SEASONAL VARIATION OF LIPID AND CAROTENOID CONTENT OF *CUCUMARIA FRONDOSA* VISCERA OIL AND THE DEVELOPMENT OF METHODS FOR ITS QUALITY CONTROL

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ABSTRACT

Little is known about the lipids and carotenoids of the lipid extracted from sea cucumber viscera (SCV) of *Cucumaria frondosa,* the species harvested from Nova Scotia, Canada. Tons of SCV are discarded as a waste stream from the processing of sea cucumber for its body wall and muscle bands, which have market value. The aim of this project was to characterize the lipid extracted from *C. frondosa's* viscera in terms of lipid class, FA and carotenoid composition and to examine the seasonal variation in these components. Lipid class analysis of sea cucumber viscera lipid (SCVL) indicated the presence of phospholipids (PL), TAG and ether lipids (diacylglyceryl ethers (DAGE) and monoacyglyceryl ethers (MAGE)). FFA, MAG, and DAG were detected in trace amounts. The proportions of lipid classes were found to vary with season with highest proportions of DAGE in winter and spring harvests (~50 % and ~40 % of total recovered lipids respectively). TAG represented ~45 % of total recovered lipids in the summer harvest. SCVL was a rich source of PUFA (29-32 % by mass FA), in particular EPA that was associated mostly with PL (> 30 %) and MAGE (> 45 %) in the winter harvest. SCVL contained 12-methyltetradecanoic acid (12-MTA), a branched fatty acid (BCFA) that exhibits anticancer activity and has been used in drug development studies. 12-MTA was associated mostly with DAGE and represented about 50 % of the total mass of FA in the winter harvest. Carotenoids in this extract occur at levels of 1.5-2.5 mg/g of the lipid extract as determined using a UV spectrophotometric method. Astaxanthin and canthaxanthin comprised 146 μg/g and 650-730 μg/g of lipid extract, respectively. The winter harvest exhibited the highest levels of lipid and carotenoid content, PUFA, EPA, 12-MTA, and BCFA when compared to spring and summer harvests. Finally, to determine the stability of the highly pigmented SCVL, a spectrophotometric method using FOX was adapted and validated to measure accurate peroxide values (PV) of SCVL. The method was linear, accurate, precise and applicable, and reduced the interference of the carotenoids in the measurement of PV of SCVL.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AOCS	American Oil Chemists Society
BCFA	Branched chain fatty acid
BHT	Butylated hydroxyltoluene
CD	Conjugated diene
CDFG	California Department of Fish and Game
CHP	Cumene hydroperoxide
DAG	Diacylglycerols
DAGE	Diacylglyceryl ether
°C	Degrees Celsius
DFA	Department of Fisheries and Agriculture
DFO	Department of Fisheries and Oceans
DHA	Docosahexaenoic acid
dwb	Dry weight basis
EI	Electronic ionization
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAME	Fatty acid methyl ester
FO	Fish oil
FFA	Free fatty acid
FOX	Ferrous Oxidation-Xylenol orange
GC	Gas chromatograph
GC-MS	Gas chromatography-mass spectrometry

GOED	The Global Organization for EPA and DHA
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography-Diode array detector
IDF	International Dairy Federation
KO	Krill oil
MAG	Monoacylglycerols
MAGE	Monoacylglyceryl ether
Min	Minute (s)
MQ	Milli-Q
12-MTA	12-Methyltetradecanoic acid, anteiso-C15:0
MTBE	Methyl *tert*- butyl ether
MUFA	Monounsaturated fatty acids
NMR	Nuclear magnetic resonance spectroscopy
n-3	Omega-3
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
R_f	Retention factor
RO	Reverse osmosis
RT	Retention time

SCV	Sea cucumber viscera
SCVL	Sea cucumber viscera lipid
SFA	Saturated fatty acid
TAG	Triacylglycerols
TBARS	Thiobarbituric acid reactive substances
TC	Total carotenoid content
TLC	Thin layer chromatography
TLC-FID	Thin layer chromatography-flame ionizationdetector
UV	Ultraviolet
WE	Wax ester
wwb	Wet weight basis
XO	Xylenol orange
β	Beta

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CHAPTER 1 INTRODUCTION

1.1 DISSERTATION OVERVIEW

Sea cucumbers are marine invertebrates that are distributed in all oceans and belong to the Holothuridea class, which is a subdivision of the *Echinodermata* phylum (Taiyeb-Ali et al., 2003). In the last three decades, sea cucumbers have rapidly become commercially important seafood throughout the world. China is considered to be the world's largest harvester of sea cucumber, followed by other Pacific regions (parts of Asia) such as the Philippines, Malaysia, Japan and Korea (Chen, 2004). Sea cucumber fisheries have been developed in non-traditional sea cucumber fishing areas, such as Canada, United States and Iceland (Hamel and Mercier, 2008). The most common species of sea cucumber in the northwest Atlantic region is *Cucumaria frondosa,* while *Parastichopus californicus* and *Parastichopus parvimensis* are the most common species found near the western Pacific coast of North America (Hamel and Mercier, 2008). The California Department of Fish and Game (CDFG) (2006) and Fisheries and Oceans Canada (DFO) (2003) have reported that thousands of metric tons of sea cucumber are processed annually.

The aboriginal people of South East Asia value the sea cucumber for its nutritional content (Taiyeb-Ali et al., 2003) as it a good source of protein (Chen, 2004). It has also been used in traditional medicine to treat asthma, hypertension, rheumatism, anemia, and sinus congestion (Fredalina et al., 1999). Chemical and bioactive extracts made from sea cucumber have been used in surgeries to heal cuts, to apply to wounds and burns, and to reduce the pain associated with arthritis (Fredalina et al., 1999; Chen et al., 2011). Sea cucumber extracts inhibit lung and galactophore cancer cells (Ma et al., 1982) and contain antioxidant components (Zhong et al., 2007). Thus, sea cucumber are a potential source of nutraceutical products.

The body wall of *C. frondosa* makes up fifty percent of its weight, which has a moisture content of ~80% (Hamel and Mercier, 2008). This species can grow to a maximum length of 50 cm, a width of 10 cm and a weight of 2-5 kg. It is typically light to dark brown in color but a few have been observed to be a pale shade of orange or a cream color. This species of sea cucumber is distributed on both sides of the North Atlantic and has been found in dense populations in the Gulf of the St. Lawrence (Hamel and Mercier, 2008). It prefers rocky or sandy substrate, and is found in depths of 30 (Newfoundland) to 300 m (New Brunswick) (Brinkhurst et al., 1975).

Harvesting of sea cucumber in Atlantic Canada takes place between May and November with *C. frondosa* being harvested as by-catch from scallop dragging fisheries. It is however highly recommended that sea cucumbers be harvested during February when their gonads are ripe and are therefore more attractive commercially because of their color, texture and taste (Mamelona et al., 2010). When sea cucumbers are handled roughly during harvest, the animal purges itself of its entire viscera, which is undesirable.

C. frondosa was expected to become one of the most important commercial sea cucumber species on the world market (DFA, 2002). To improve the market value of sea cucumber products and reduce losses, sea cucumber handling procedures during transport and/or prior to processing need to be improved (DFA, 2002). The market demand for the final product will determine how sea cucumbers are processed.

Interest in the human consumption of sea cucumber has increased in recent years because of the market demand for foods that are rich sources of protein and polyunsaturated fatty acids (PUFA) such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). The dried body wall is the most common processed product made from sea cucumbers, which is an edible part of sea cucumber that is considered to be an excellent source of protein. Dried sea cucumber is used

in soups or fried with meats and vegetables (Conan and Byrne, 1993). Sea cucumber viscera (SCV) have been eaten raw, boiled or pickled (Bruckner et al., 2003). Chen (2004) has reported that sea cucumbers contain valued nutrients (vitamin A, B1, B2, and B3) and minerals such as calcium, magnesium, iron and zinc. The nutritional facts reported for fresh and processed sea cucumber species are listed to Table 1.1. This comparison shows that proximate composition varies with sea cucumber species, whether the sea cucumber is fresh or processed, and body tissue type.

PUFAs were detected in sea cucumber extracts and represented a high percentage of its total lipid (Zhong et al., 2007). The predominant fatty acid in SCO prepared from fresh sea cucumber with or without internal organs was reported to be EPA, which represented almost 43-46% of the total fatty acid content (Zhong et al., 2007). Eicosapentaenoic acid has been reported to be involved in blood clotting (Gibson, 1983) and is thought to possess antithrombotic activity (Croft et al., 1987).

The branched fatty acid, 12-methyltetradecanoic acid (12-MTA), was first isolated from sea cucumber lipid extract of *C. frondosa* by Collin et al. in 2003. This fatty acid exhibited anti-inflammatory and anti-cancer activity and has been shown to have potent inhibitor activity against different lipoxygenases with no toxicity. As an anti-cancer agent, 12-MTA was shown to have anticancer activity on its own or in a combination with other anti-cancer compounds. Its ability to inhibit 5-lipoxygenase on its own or in combination with other co-factors, allowed it to protect the gastric mucosa against non-steroidal anti-inflammatory drugs (Collin et al., 2003).

Table 1.1 Nutritional component percentage (the range) of freshly harvested (Aydin et al., 2011), processed (Wen et al., 2010), and freeze dried tissues and body wall of sea cucumber species (Bechtel et al., 2012).

Nutritional factors	Fresh sea cucumber*	Processed sea cucumber (dried)**	Freeze dried tissues ***	Freeze dried body wall ***
	Holothuria tubulosa, *Holothuria polii,* *Holothuria mammata*	*Stichopus herrmanni,* *Thelenota ananas,* *Thelenota anax,* *Holothuria fuscogilva,* *Holothuria fuscopunctata,* *Actinopyga mauritiana,* *Actinopyga caerulea,* *Bohadschia argus*	*Parastichopus californicus*	*Parastichopus californicus*
Moisture content	81.24- 85.24	0.81-15.1	—	—
Protein	7.88- 8.82	40.7-63.3	68	47
Fat	0.09- 0.18	0.3-10.1	5	8
Ash	5.13- 7.85	15.4-39.6	12	26
Carbohydrates	—	—	9	15

*(Aydin et al., 2011), **(Wen et al., 2010), ***(Bechtel et al., 2012)

Knowledge of the lipid classes present in marine oils is important in evaluating their benefit to human health. No specific study has reported the complete lipid class content of the highly pigmented lipid obtained from *C. frondosa*. The carotenoid profile, which is responsible for the lipid's pigmentation, of the viscera of *C. frondosa* has also not been fully studied. To address these gaps in knowledge, chromatographic and spectrophotometric methods were employed to analyze the lipid class and carotenoid content of the lipid obtained from the viscera of *C. frondosa*.

The oxidative stability of lipid is an important factor to evaluate when lipid is being considered for human consumption. The determination of stability in pigmented lipids such as sea cucumber viscera lipid (SCVL) through measurement of peroxide value (PV) is challenging because pigments can obscure colored endpoints in both iodometric and spectrophotometric methods. To overcome this problem, a modified version of the ferrous oxidation-xylenol orange (FOX) method was developed so that the PV of SCVL could be determined without interference from the carotenoids.

1.1.1 Statement of the Problem and Purpose

C. frondosa is the most abundant sea cucumber species harvested in Atlantic Canada. To date most studies have been conducted on a whole body extract of sea cucumber; as a result, little is known about the lipid extract from specific body parts of *C. frondosa,* such as the viscera. This is particularly important as Atlantic sea cucumber is harvested for its muscle bands and body wall with the rest of the sea cucumber body disposed of as waste (Mamelona et al., 2010). There is local interest in utilizing this viscera waste for commercial products. If more was known about the viscera-containing waste stream, then valuable by-products, such as nutraceutical oil

products, might be developed. Sea cucumber viscera lipid is a promising extract because it contains nutritional lipids and carotenoids.

The overall objective of this study was to investigate the lipid classes present in the lipid obtained from *C. frondosa* viscera and to develop methods to evaluate its stability and carotenoid content. More specifically the objectives were:

1. To develop a method to determining the PV of the highly pigmented SCVL
2. To investigate the lipid classes present in SCVL using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and Gas chromatography-mass spectrometry (GC-MS)
3. To use HPLC to determine the carotenoid content and profile of SCVL
4. To study the lipid and total carotenoid content of SCVL extracted from the viscera of *C. frondosa* species harvested from Atlantic Canada of three different seasonal harvests

CHAPTER 2 MODIFICATION OF THE FERROUS OXIDATION-XYLENOL ORANGE METHOD FOR DETERMINATION OF PEROXIDE VALUE IN HIGHLY PIGMENTED SEA CUCUMBER VISCERA LIPID

2.1 ABSTRACT

A ferrous oxidation-xylenol orange method (FOX) was modified to measure the levels of hydroperoxides in highly pigmented sea cucumber viscera lipid (SCVL). With the FOX method, oil hydroperoxides oxidize Fe (II) to Fe (III) ions, which in turn bind to xylenol orange (XO) to form a complex that has a maximum absorbance at 560 nm. When applied to lipid extracts containing high levels of carotenoid pigments, the carotenoids interfere in the absorbance measurement because the XO-complex and carotenoids absorb at the same wavelength. To avoid this, an approach was developed where carotenoids were removed from the XO-complex containing solution before absorbance measurements were made. This was accomplished by aqueous extraction of the reaction solution, leaving an organic layer that was highly pigmented. The absorbance of the aqueous layer was measured at 560 nm and the lipid hydroperoxide content was determined (mmol/kg) using a cumene hydroperoxide calibration curve. The method was validated using oxidized, un-pigmented fish oils. The modified FOX method described herein was linear, accurate and precise. The method was also shown to be applicable to pigmented oils with hydroperoxide concentrations ranging from 5-35 meq/kg lipid. This modified FOX method for hydroperoxide determination of highly pigmented lipids, such as SCVL, is a valuable alternative to the methods presently available for determination of peroxide values.

2.2 INTRODUCTION

Lipid oxidation is a chemical change that may negatively affect the flavor, aroma, and nutritional quality of oils and food, and may lead to the production of toxic compounds (Chow and Gupta, 1994). It involves the continuous formation of hydroperoxides as primary oxidation products that may break down into a range of non-volatile and volatile secondary products (Dobarganes and Velasco, 2002). Concentrations of peroxides and conjugated dienes (CD) are frequently used to assess the extent of primary oxidation, while thiobarbituric acid reactive substances (TBARS) and headspace volatiles are commonly employed to monitor secondary products of oxidation (Wong, 1989; Shahidi and Wanasundara, 1998).

Among the various methods of oxidation assessment, much focus has been placed on determining the concentrations of hydroperoxides in edible oils. The peroxide value (PV) is a measure of the total hydroperoxide content of an oil (expressed as meq/kg). It is one of the most commonly used quality indicators to monitor fats and oils during production and storage (Antolovich et al., 2002). Accurate measurement of PV can be difficult as hydroperoxides are very unstable and can be rapidly decomposed into secondary products (Frankel, 2005). Both iodometric titration and ferric ion complexation approaches, which rely on color formation for endpoint detection or absorbance determination, respectively, have been used to measure PV (Dobarganes and Velasco, 2002).

Measurement of PV using an iodometric titration is the standard method described by the American Oil Chemists Society (AOCS); hydroperoxides (ROOH) are reduced with iodide (AOCS Method Cd 8-53, 1997) and the resultant iodine is titrated against a standardized solution of sodium thiosulfate in the presence of startch (Gray and Monahan, 1992). When a non-pigmented lipid is titrated, the endpoint is reached when the solution being titrated becomes

colorless. The presence of carotenoid pigments in lipids makes it difficult to detect the titration endpoint, reducing the accuracy of measurement. Ali-Nehari et al. (2012) noted that a potential solution to PV determination in pigmented lipids is to simply use less than the recommended 5 g of lipid so there is less masking of the end point by the pigments.

PV of lipids may also be determined using spectrophotometric methods that involve the determination of an Fe (III)-thiocyanate complex; Fe (III) is produced from the oxidation of Fe (II) by peroxides in an acidic environment and then reacts with thiocyanate. The resulting Fe (III) - thiocyanate complex has a red-violet color with a maximum absorption at 500-510 nm (IDF 74A, 1991). Application of this method to pigmented lipids generates inaccurate PV measurements because carotenoids also absorb at 500-510 nm. To address this interference, Hornero-Méndez et al. (2001) removed the carotenoids from paprika and palm oil by extracting the oil-containing assay mixture with diethyl ether after oxidation to Fe (III). This allowed for the measurement of the Fe (III)- thiocyanate complex without carotenoid interference. This method was developed for the measurement of PV in plant oils and has not been shown to be applicable to marine derived oils.

A similar iron-based spectrophotometric method, known as the ferrous oxidation-xylenol orange (FOX) method, is also based on the oxidation of Fe (II) to Fe (III) by hydroperoxides in an acidic medium (Eq 1). The Fe (III) immediately reacts with xylenol orange (XO) to produce a blue-purple Fe (III)-(XO) complex (Eq 2) which is measured spectrophotometrically between 550-600 nm (Dobarganes and Velasco, 2002; Hornero-Méndez et al., 2001; Shantha and Decker, 1993). This method also suffers from the drawback that pigmented oils interfere with the measurement of an accurate PV. In a literature search, no modifications were reported for this method that would make it suitable for the PV analysis of highly pigmented lipids.

$$ROOH + Fe^{2+} \longrightarrow Fe^{3+} + RO^{\bullet} + OH^{-} \quad \text{(Eq 1)}$$

$$Fe^{3+} + XO \longrightarrow \text{Fe-XO complex} \quad \text{(Eq 2)}$$

Recently the lipid obtained from sea cucumber viscera (SCV) and other sea cucumber body parts has attracted interest as it contains 43-57 % w/w eicosapentaenoic acid (EPA), 2.0-5.8 % w/w docosahexaenoic acid (DHA) and 2-9 % w/w of 12-methyltetradecanoic acid (12-MTA) (Zhong et al., 2007) which has been shown to inhibit proliferation of cancer cells and the extracellular production of surfactant (Fredalina et al., 1999; Yang et al., 2003; Inoue et al., 2012). Carotenoids that make PV determination difficult in pigmented oils are also found in SCV lipid (SCVL) and are reported to possess antioxidant activity (Zhong et al., 2007).

The present study describes a modification of the FOX spectrophotometric method to make it suitable for the determination of PV in highly pigmented lipids such as SCVL. The resulting modified FOX method was validated using fish oil, an unpigmented oil, by testing for linearity, accuracy and precision. The repeatability of the modified FOX method was demonstrated by applying the method to pigmented SCVL samples spiked with different proportions of oxidized fish oil to generate different concentrations of hydroperoxides.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Materials and Methods

Xylenol orange (tetrasodium salt) and cumene hydroperoxide were purchased from Sigma-Aldrich (Canada). Ammonium iron (II) sulfate hexahydrate was purchased from Fluka (Sigma-Aldrich Co, St. Louis, US) and starch indicator (1 % w/v) aqueous solution for iodometric titrations was obtained from Ricca Chemical company (LLC). All solvents (Optima grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). The 95% ethanol used in this work was obtained from Commercial Alcohols (Chatham, ON). Reverse osmosis (RO) water was used for all reactions. Fish oils (FO) were provided by Ascenta Health (Dartmouth, NS). SCVL was extracted from SCV of *Cucumaria frondosa* obtained from Ocean Pride Fisheries Limited (Lower Wedgeport, NS). All data were collected using a UV spectrophotometer (Novaspec II, LKB Biochrom, Holliston, MA, USA) set at 560 nm for both FOX and modified FOX methods. Absorbance was measured using a 1-cm path amber glass cuvette.

2.3.2 Extraction of SCVL

Lipid for these studies was prepared from frozen SCV using the Bligh and Dyer (1959) method with slight modification (Christie, 1982). Frozen SCV samples were obtained from three randomly selected sites in the sample box and were homogenized using a food processor. Then 200 g of ground SCV was blended with 200 mL of chloroform and 400 mL of methanol. The mixture was filtered using a Buchner funnel equipped with a Whatman # 1, 12.5 cm filter. The residual SCV was placed in a blender, re-extracted with 200 mL chloroform and filtered using a Buchner funnel. The combined chloroform/methanol extracts were then mixed with 200 mL of aqueous 0.88% potassium chloride, transferred to a separatory funnel covered in aluminum foil,

flushed with nitrogen and allowed to sit overnight in a dark fume hood at room temperature. The ratio of chloroform: methanol: water in the final mixture was 2:2:1.8, as the moisture content for SCV was ~ 80% (see Chapter 4). The lower organic layer was collected and dried over anhydrous sodium sulfate for 2 h at 4^{o}C. The cloudy organic layer was then filtered through a bed of anhydrous sodium sulfate to remove the residual water in the lipid. The solvent was removed from the resulting filtrate by evaporation under reduced pressure using a rotator evaporator at 40 °C. Samples were flushed with nitrogen and stored at -20 °C in preparation for analysis.

2.3.3 Iodometric AOCS Method

The iodometric AOCS method (Cd 8-53, 1997) was used as a reference to determine PV. Aliquots of lipid of known mass (normally 5 g) were dissolved in 30 mL of 3:2 (v/v) glacial acetic acid: chloroform in a 250 mL stoppered Erlenmeyer flask and mixed well with 500 µL of saturated potassium iodide. After exactly 1 min, the reaction was stopped by adding 30 mL of water. The mixture was then titrated using a 0.01 N standardized sodium thiosulfate solution in the presence of 1% starch indicator. The PV (meq/kg) was calculated using the following equation:

$$PV = ((S - B) \times N \times 1000) / W$$

where S is the volume of sodium thiosulfate solution used in titration of the lipid sample in mL, B is the volume of titration of the blank in mL, N is the normality of the titrant (sodium thiosulfate solution) and W is the weight of the lipid sample in grams. This method was also applied to lipid masses of 3, 2, 1 and 0.5 g (Ali-Nehari et al., 2012).

2.3.4 FOX Method

The FOX method was adapted from Navas et al. (2004). The lipid extract (up to 25 mg/mL of reaction medium) was placed in a 10 mL screw-capped test tube and dissolved in 200 µL dichloromethane/ ethanol 3:2 (v/v). The following solutions were then added to the tube: 100 µL of 5 mM aqueous ferrous ammonium sulfate, 200 µL of 0.25 M methanolic H_2SO_4, and 200 µL of 1 M methanolic xylenol orange. The final volume was adjusted to 2 mL by adding 1300 µL dichloromethane/ ethanol 3:2 (v/v). The reaction was incubated for 30 min in the dark at room temperature and the absorbance of the mixture was read at 560 nm against its blank (i.e., reaction mixture without lipid). The PV was determined using a cumene hydroperoxide (CHP) calibration curve that was constructed following the above procedure where five different concentrations (5-15 CHP nmol/mL reaction medium) were prepared in triplicate (n=3). PV obtained were expressed as mmol CHP/kg oil sample.

2.3.5 Trials to Modify FOX Method

Efforts here were focused on modifying the FOX method so that the carotenoids were separated from the XO-complex containing solution before an absorbance reading at 560 nm was obtained. Initially, I tested the reproducibility of the FOX method described above, followed by testing modifications to the method using unpigmented FOs; this allowed for the comparison of the PV results to those obtained from a standard method (the AOCS titration method). Three approaches to the modification of the FOX method were investigated.

In the first attempt, the aim was to produce Fe (III) first and to then remove the pigments, before the XO indicator was added. To determine if the Fe (III)-XO complex would form if XO was added after generating Fe (III), Fe (II) was incubated with FO for 30 min. XO indicator was then added and the mixture was again incubated for 30 min to allow the formation of the XO-

complex. A second approach attempted to remove carotenoid interference by solubilisation of the XO-complex in methanol; since carotenoids are insoluble in methanol, this approach should effectively remove them from the solution. This was accomplished by evaporation of the solvent mixture (dichloromethane, ethanol, water and methanol) after a 30 min reaction time, followed by solubilisation of the resulting dark red solid containing the XO-complex in 2 mL methanol. In the final attempt, water was used to recover the XO-complex from the reaction mixture. FO was again incubated according to the original FOX method described above but once the XO-complex had formed, 2 mL of water was added and the mixture was vortexed. Centrifugation at ~160 X g (for 3 min) afforded two layers. Absorbance of the aqueous purple XO-complex layer (top layer) at 560 nm was measured against a blank. For comparison, the absorbance at 560 nm of the lower organic layer was also measured and PV was determined using a cumene hydroperoxide (CHP) standard calibration curve as described above.

The modified FOX method was validated using FO, by testing for linearity, accuracy, precision and repeatability, following the guidelines set out by the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002). A calibration curve to relate PV determined by the modified FOX and AOCS methods was established by plotting PV (mmol/kg) determined by the modified FOX method versus PV (meq/kg) determined by the AOCS method using oxidized unpigmented FOs (15 samples; each sample represented 5 replicate measurements of the same oxidized lipid at five different points of oxidation). Three different types of FO were used for the calibration curve. Each type contained mixtures of fatty acids with different degrees of unsaturation. Fish oil samples (300 mL) were oxidized for 10 days in an oven at 40 °C. Samples were withdrawn daily and placed in amber vials, flushed with nitrogen and stored at -20 °C in preparation for analysis.

The residuals of the calibration curve were plotted and tested for their normality using the Anderson-Darling test (Anderson and Darling, 1954). To test for accuracy, PV measurements were made of samples derived from two other batches of oxidized FO not used to construct the calibration curve (5 samples with each sample consisting of 5 replicate measurements of the same lipid). PV (mmol/kg) was determined using the modified FOX method and the calibration curve to yield a PV (meq/kg) equivalent to that determined by the AOCS method; this is referred to as 'derived PV'. This derived PV was then compared to that determined when the AOCS method was followed (called the "AOCS PV" and considered the true value). Accuracy was assessed by comparing the 'AOCS PV' and the 'derived PV' (meq/kg) using a two-sample *t* test ($p > 0.05$).

To test for precision, the percent relative standard deviation (%RSD) for both values ('AOCS PV' versus 'derived PV') were determined and evaluated using the Horwitz equation. The acceptable Horwitz %RSD was calculated using the following equation:

$$\%RSD = \tfrac{2}{3} \times 2^{(1-0.5\log C)}$$

where C is the mass concentration of EPA-hydroperoxide in decimal fraction. Sufficient precision was obtained when the %RSD of the derived PV fell below the acceptable Horwitz %RSD (Boyer et al., 1985). Precision was also evaluated by measuring the repeatability of the method applied to SCVL. However, SCVL was observed to be very resistant to oxidation under different conditions. In order to establish measureable PV in a mixture containing SCVL, oxidized FO was added at different concentrations to generate a series of samples containing 0 - 90% SCVL in fish oil by mass. The FO sample used in this test had been oxidized for 3 days in an oven at 40 °C. The repeatability of the method was then accessed by comparing %RSD.

In all the attempts described, a 50 or 100 μL glass syringe was used when measuring lipid volumes to minimize error. Similarly, the same source of reverse osmosis (RO) water was used for sample analysis and the development of the cumene hydroperoxide calibration curve, and the xylenol-orange indicator was obtained from the same supplier and had the same batch number.

2.3.6 Statistical Analysis

All statistical tests were conducted using Minitab 17. Normality of the residuals of the calibration curves was tested using the Anderson-Darling test. A two-sample *t* test was used to evaluate accuracy by determining differences between the AOCS PV and the derived PV.

2.4 RESULTS

When the iodometric AOCS (AOCS Method Cd 8-53; 1997) method and its modified version (Ali-Nehari et al., 2012) were tested using 5, 3, 2, 1 and 0.5 g of SCVL, the titration endpoint could not be determined due to the dark red color of the lipid.

In the first attempt to modify the FOX method, XO indicator was added after the 30 min incubation period but no color development was observed. In the second attempt, when the reaction mixture was evaporated to dryness after a 30 min incubation period, a dark red film formed on the test tube that yielded a pale yellow solution when it was suspended in methanol. This likely indicated the breakdown of the XO-complex. In the last attempt, addition of water to the reaction mixture after a 30 min incubation period resulted in presence of a purple color in the water layer that was indicative of the presence of the XO-complex. Measurement of the absorbance of the water layer at 560 nm gave a reading of 0.54 ± 0.02 while that of the organic layer had a low absorbance (0.013 <0.00). This water extraction step was then adopted for all other measurements using the FOX method.

The residuals of the calibration curve (Fig 2.1) were found to be normally distributed ($p > 0.05$, Anderson-Darling test). The test of accuracy using the two-sample *t* test (Table 2.1) did not show a significant difference ($p > 0.05$) between the 'derived PV' values and the 'AOCS P' for any sample. The acceptable Horwitz %RSD values were greater than those for the derived PV, indicating that the precision of the modified FOX method was acceptable (Table 2.1). When this calibration curve was then used to determine a PV that was equivalent to the AOCS PV in oxidized SCVL, unoxidized SCVL samples exhibited a higher PV (0.57 ± 0.02 meq/kg) than the SCVL samples that were exposed to oxidation conditions (Table 2.2). When applied to SVCO samples spiked with oxidized FO, relative standard deviations (%RSD) of the triplicate results were low, indicating high precision of the modified FOX method (Table 2.3). The 'derived PV' determined for the six samples ranged from 5.84-35.17 meq/kg. The PV increased with increasing amounts of oxidized FO percentage in the samples.

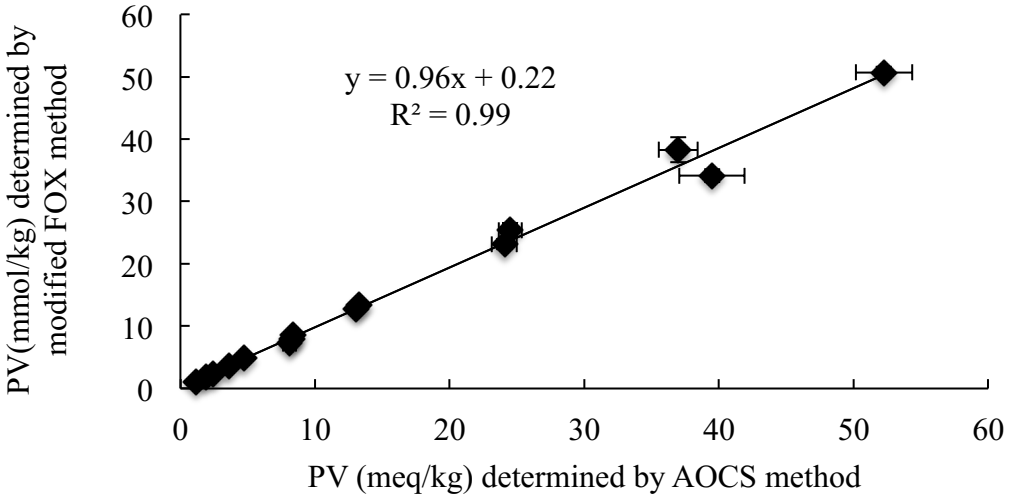


Figure 2.1 Calibration curve relating PV (meq/kg) determined by AOCS method to PV (mmol/kg) determined by the modified FOX method for three different unpigmented oxidized FO (mean +/- SD; 15 samples were plotted on each axis with n=5 for each sample).

Table 2.1 Tests of accuracy and precision of the modified FOX method (mean +/- SD, n=5 for each sample) applied to unpigmented FO evaluated by the two-sample *t* test and the acceptable Horwitz %RSDs, respectively.

AOCS determined PV (meq/kg)	%RSD	Derived PV (meq/kg)	%RSD	Two-Sample *t* test *p* Value	Acceptable Horwitz %RSD
6.89 ± 0.11	1.60	6.81 ± 0.16	2.35	0.39	3.32
7.52 ± 0.16	2.13	7.63 ± 0.11	1.44	0.71	3.28
7.75 ± 0.25	3.22	7.77 ± 0.20	2.57	0.89	3.27
10.32 ± 0.24	2.32	10.42 ± 0.15	1.44	0.46	3.13
33.68 ± 1.37	4.07	33.74 ± 0.20	0.60	0.93	2.62

Table 2.2 Derived peroxide value (PV) (mean +/- SD, n=3) determined for SCVL oxidized in an oven at 40 °C to test for repeatability expressed by % RSD.

Oxidation time (Days)	Derived PV (meq/kg)	%RSD
0	0.57 ± 0.02	2.79
5	0.34 ± 0.00	0.46
10	0.39 ± 0.00	1.17
15	0.37 ± 0.00	0.31

Table 2.3 Derived peroxide value (PV) (mean +/- SD, n=3) determined for samples prepared by mixing SCVL with oxidized FO in different proportions for the test of repeatability expressed by % RSD.

Sample	Derived PV (meq/kg)	%RSD
1	5.84 ± 0.17	2.89
2	12.05 ± 0.52	4.34
3	24.32 ± 0.66	2.70
4	25.62 ± 0.07	0.29
5	31.62 ± 0.88	2.78
6	35.17 ± 0.47	1.32

2.5 DISCUSSION

When the original AOCS method and its modified version (Ali-Nehari et al. 2012) were used to determine the PV of SCVL, the color change could not be detected due to the color masquing effect of the carotenoids present in the SCVL even when the amount of oil used was much less than that recommended. Thus, no data could be reported for these analyses. These observations urged us to develop an alternative method to determine the PV in the SCVL samples. Both the FOX and IDF method were reported as rapid and sensitive methods which exhibit good precision (Nielsen et al., 2003); however, the FOX method was selected over the IDF method in this study because it can be applied to a wider variety of oil types (Navas et al., 2004; Grau et al., 2000).

The FOX spectrophotometric method by Navas et al. (2004) was chosen as the starting point for this work. It was reported to have high sensitivity and was useful for samples with low lipid hydroperoxide content; however, it had only been used to date for non-pigmented edible lipids. In this method, dichloromethane/ethanol 3:2 (*v,v*) was used as the main solvent in the reaction medium, where lipid precipitation did not occur even at high concentrations (up to 25 mg SCVL/mL). The procedure required the lipid to be dissolved under acidic conditions to prevent the rapid conversion of Fe (II) to Fe (III) which occurs under basic conditions. This makes the Fe (II) more stable and specific for the hydroperoxide reaction (Bou et al., 2008). Acidic conditions also prevent the formation of $Fe(OH)_2$ and $Fe(OH)_3$ precipitates, allowing Fe (II) to stay in solution and be available for oxidation by hydroperoxides. Importantly, uncomplexed XO is not detected at 560 nm so does not interfere with the absorbance reading (Gay et al., 1999a).

Initially, unpigmented FO was used to test the conditions of the FOX method and modifications to it. This allowed for the comparison of the PV results to those obtained from the standard AOCS method. The purple color observed when applying the original FOX method indicated that the formation of the XO-complex had occurred. With the FOX method established, modification steps were then introduced. Efforts were then focused on modifying the FOX method described by Navas et al. (2004) so that the carotenoids were separated from the solution containing the XO-complex before an absorbance measurement was obtained.

The first attempt evaluated the feasibility of oxidizing the ferrous ions, before adding the XO indicator, similar to the modified version of the IDF method used for PV determination of pigmented plant samples (Hornero-Méndez et al., 2001). Addition of XO after the 30 min reaction time did not result in formation of the colored XO-complex. This indicated that ferric ions were not being produced under these conditions, and/or the reaction conditions were not suitable for the formation of the Fe (III)-XO complex. Hermes-Lima et al. (1995) suggested that when XO is added to an acidic reaction mixture containing hydroperoxides, it does not catalyze the oxidation of Fe (II). This would suggest that adding XO after Fe (III) had been formed should have no effect on the formation of the XO complex. It is therefore postulated that the Fe (III) is not stable in the reaction mixture for 30 min.

In the second approach, methanol was used to selectively dissolve the dried purple XO-complex from the film deposited on the tube when the reaction solution was evaporated. The methanol-insoluble carotenoids were expected to remain on the tube surface. Unfortunately a pale yellow methanol solution was obtained which indicated that the metal-xylenol orange complex had broken down and was therefore not stable in methanol. The reason for the instability of the complex in a 100 % methanol solution is unknown.

The last modification attempt, which used aqueous extraction of the XO-complex to isolate it from the pigments, successfully separated the XO-complex from the reaction medium. Trials using FO revealed that the XO-complex was easily extracted into the aqueous layer due to its polar nature. The negligible absorbance reading obtained for the organic layer after water extraction indicated that the XO-complex was extracted into the aqueous layer.

To validate the modified FOX method and report a PV that would be equivalent to that from the iodometric titration, a calibration curve (Fig 2.1) was constructed. PV at low ranges (2-50 meq/kg) were selected to establish this calibration curve as the Global Organization for EPA and DHA Omega-3s (GOED) Voluntary Monograph (2012) sets a maximum PV at 5 meq/kg; validation in this range would ensure that this method is reliable for low PV. The higher relative standard deviation we found at the highest PV using the AOCS method may have occurred because a longer time (more than 1 min reaction time recommended for the AOCS reference method) is required to complete the titration to reach the endpoint, during which new hydroperoxides might be rapidly formed in marine oils which in turn would affect the method's precision (Shahidi and Zhong, 2005).

In practical applications, the calibration curve is necessary to relate the results of the modified FOX method to a standard method. The stoichiometry between the molar amounts of hydroperoxide and iodine in the reaction is known (1:1) in the AOCS method (Gay et al., 1999b). However, the exact stoichiometry between the amounts of XO-Fe (III) formed per mole of hydroperoxide in the FOX method is not known but has been reported to be between 2 and 3 for hydrogen peroxide (Gay et al., 1999b). In one study, the XO-Fe (III) was reported to have 1:1 stoichiometry (Gay et al., 1999a), but, Mizuguchi and Yotsuyanagi (2001) reported that in the presence of excess Fe (III), a 2:1 stoichiometry existed. A 1:1 ratio is thought to exist when

excess XO is present in the reaction mixture (Pribil, 1982). Good agreement was observed between the PV determined using this modified FOX method and those determined by AOCS method; a strong correlation has been previously observed between titration methods and the FOX method (Yildiz et al., 2003).

The acceptable Horwitz % RSD values were greater than the RSD determined for the derived PV, indicating acceptable precision of the modified method. The range of %RSD (0.60-2.57) for the modified FOX method was slightly lower than the %RSD range for the AOCS (1.6-4.07) method, which suggested that the modified method exhibited a higher level of precision than the AOCS method. The routinely used iodometric titration method to determine PV has been reported to be less sensitive than spectrophotometric methods (Dobarganes and Velasco, 2002). For instance, external conditions such as the presence of oxygen are known to affect the sensitivity of iodometric method (Jessup et al., 1994). Also, the large amount of lipid (5 g) used in the iodometric titration method, especially when it is highly oxidized, might be involved in lowering its precision due to the subjective nature of determining the titration endpoint.

The main objective of this study was to develop a method which could be used to determine the PV in pigmented lipids. Therefore, it was necessary to test the repeatability of the modified FOX method on a pigmented lipid such as SCVL (Table 2.3). SCVL was mixed with different percentages of oxidized FO, ensuring that a range of different concentrations of hydroperoxides could be evaluated. When SCVL containing samples were subjected to the modified FOX method the pigments originating from the lipid remained in the organic layer thus enabling the measurement of the XO-complex in the aqueous layer without interference from the carotenoids. For this analysis %RSD between 0.29-4.34 (Table 2.3) were observed. The %RSD

did not consistently vary with PV, therefore indicating that pigment concentration did not influence reproducibility.

In this study, all attempts to oxidize pure SCVL failed to show increases in PV in lipids exposed to a variety of oxidizing conditions (Table 2.3). This was surprising as Takeungwongtrakul et al. (2012) suggested that carotenoids are very unstable to heat and/or oxygen due to their unsaturated structure. Additionally, SCVL extracted from the whole body of *C. frondosa* is a typical marine oil in that it contains unsaturated FA including palmitoleic acid (16:1n-7; 20 %) and EPA (40 %) (Zhong et al., 2007) which make the lipid susceptible to oxidation under the conditions used here. Similar behavior has recently been observed for krill oil (Thomsen et al., 2013) in which only very low levels of primary and secondary oxidation products were detected in oil incubated at 40 °C for 20 days. Thomsen suggested that methods such as PV determination are not the best approaches to be used in determining the stability of highly pigmented oils such as krill oil.

2.6 CONCLUSIONS

A modified FOX method was developed to determine the PV of pigmented lipids such as SCVL. The strong color in pigmented lipids rich in carotenoids such as SCVL complicates color-dependence analyses such as the iodometric AOCS official method, as well as spectroscopy methods. The method developed here incorporated a water extraction step after the formation of the XO-complex to remove the carotenoids from the aqueous phase containing the Fe (III)-XO complex. Linearity, accuracy, precision and repeatability parameters were tested for the modified FOX method and data indicated an acceptable validity. This method can therefore be successfully used to determine the PV of pigmented oils including solvent extracted SCVL. It is

a rapid and simple method that requires low levels of organic solvents, and no derivatization or sample preparation steps. All of these characteristics make the modified FOX method a favourable approach for determination of PV in pigmented oils. In this study, I was not able to track changes in hydroperoxides in oxidized SCVL samples under different oxidation conditions. That suggested either hydroperoxides were not produced in such lipid and/or some other degradation products were existed. Further investigation into the behavior of SCVL upon exposure to different oxidation conditions is recommended.

CHAPTER 3. LIPID CLASS IDENTIFICATION OF VISCERA OF *CUCUMARIA FRONDOSA* HARVESTED FROM THE ATLANTIC CANADA

3.1 ABSTRACT

Lipid classes isolated from lipids extracted from the viscera of *Cucumaria frondosa* species were investigated. The presence of triacyglycerols (TAG), free fatty acids (FFA), phospholipids (PL), and non-polar diacylglycerol ethers (DAGE) were confirmed using HPLC analysis. FFA were present in trace amounts. Monoacylglycerols (MAG) and diacylglycerols (DAG) were identified using HPLC analysis but neither class was detected using TLC. The alkyl and acyl structures of both DAGE and monoacylglyceryl ether (MAGE) were determined using gas chromatography-mass spectrometry (GC-MS) analyses of isolated DAGE and MAGE fractions that had been saponified and then acetylated. Alkyl chains associated with the ether bond in both DAGE and MAGE contained a series of saturated and monounsaturated structures ranging from C_{15}-C_{21}. The main alkyl structures present in DAGE and MAGE were 16:0 and 18:0 alkyl moieties, respectively but branched alkyl chains were identified in both ether lipid classes. For acyl structures, MAGE contained > 50% mass total FA of EPA, while the branched chain fatty acid (BCFA) 12-methyltetradecanoic acid (12-MTA) comprised ~ 40% of total FA in DAGE. Multimethyl BCFA were also identified using GC-MS of picolinyl esters prepared from recovered DAGE and MAGE lipid classes. This represents the first in depth study of the lipid classes present in sea cucumber and more specifically in *C. frondosa*.

3.2 INTRODUCTION

Cucumaria frondosa is a species of sea cucumber that is widely distributed in the cold-waters off the coast of the United States and Canada. The lipid extracted from the viscera, which is a processing by-product, has recently been identified as a potential source of marine lipids for nutraceutical applications (Vaidya and Cheema, 2014). Little, however, is known about the lipid classes present in sea cucumber viscera lipid extracts. The lipid class composition of methanol extracts of freeze dried powders of *C. frondosa* harvested off the coast of Newfoundland (Vaidya and Cheema, 2014) have been reported to contain up to nine lipid classes including hydrocarbons, steryl esters, ethyl ketones, TAG, FFA, alcohols, sterols, and phospholipids (PL).

Lipid studies of members of the Holothurian class of sea cucumber including *C. japonica*, *C. okhotensis*, *C. fraudatrix* and *Stichopus japonicas* have revealed the presence of the ether lipid, 1-*O*-alkylglyceryl ether (Rybin et al., 2007; Isay et al., 1976), in these species. Diacylglyceryl ethers (DAGE) and monoacylglyceryl ethers (MAGE) are reported to be constituents in the membranes of marine animals such as in the livers of dogfish and shark (Haraldsson and Kristinsson, 1998) and deep-sea squids (*Berryteuthis magister*) (Hayashi and Kishimura, 2002). In the last two decades, ether lipids have attracted the interest of researchers due to their health promoting effects in humans. Andreesen (1988), Berdel (1991) and Diomede et al. (1993) used ether lipids in cancer therapy and more recently, Torres et al. (2009) and Martin et al. (2011) showed that ether lipids improved the bioavailability of other lipid molecules such as butyric or omega-3 fatty acids.

The major PL found in the organs and tissues of *C. frondosa japonica*, *Eupentacta fraudatrix* and *Apostichopus japonicus* sea cucumber species were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) which ranged between 40.4-75.4,

10.7-34.2 and 3.6-18.3 % w/w of the total PL respectively (Kostetsky et al., 2012). Lower levels of phosphatidylinositol, lysophosphatidylcholine, lysophosphatidylethanolamine, diphosphatidyl -glycerol, phosphatidic acid, phosphatidylglycerol and phosphatidylinositol-4-phosphate were also found. Ceramide aminoethylphosphonate (0.1-0.7% of total PL) was only found in the intestine and respiratory trees suggesting its presence was due to an organism outside of the sea cucumber. Sphingomyelin and sphingomyelin hydroxy acids were not found in these species. Very little variation was observed in the PL composition between individual organs and tissues and the whole sea cucumber. Unfortunately, the total PL content of sea cucumber was not reported in that study (Kostetsky et al., 2012). Information on the PL content of sea cucumber species especially that of *C. frondosa* is therefore scarce.

Alkyl structures including saturated (branched and unbranched) and monounsaturated hydrocarbon chains associated with the ether bond in glycerol ethers have been previously identified in some Holothurian species (Rybin et al., 2007; Santos et al., 2002). The identification of branched chain fatty acids (BCFA) is normally accomplished using electron impact mass spectroscopy (EIMS) analysis (Ran-Ressler et al., 2012) of dimethyloxazoline (DMOX) or picolinyl esters derivatives of fatty acid methyl esters (FAME) (Harvey 1982; Yu et al., 1988). The BCFA called 12-MTA, which has been identified in *C. frondosa*, is known for its beneficial health effects (Collin et al., 2003) but no information regarding its presence in specific lipid classes has been reported to date.

The main objective of this study was to identify the lipid classes and characterize the fatty acid composition of MAGE and DAGE lipid classes present in the viscera of *C. frondosa* harvested off the coast of Nova Scotia, Canada. While the presence of ether lipids in both the neutral and polar fractions was expected, our initial focus was on confirming and identifying

their structures in simple lipids in SCVL. This information will be very important in evaluating the nutritional value of SCVL and identifying its potential niche in the marine oil supplement market.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Materials and Methods

Harvests of sea cucumber viscera from *C. frondosa* were conducted in January 2015 (winter; latitude 4439/ longitude 6041) and March 2015 (spring; latitude 4439/ longitude 6040) from the Sable Island Banks off Nova Scotia, Canada by Ocean Pride Fisheries Limited (Lower Wedgeport, NS). The viscera sample obtained from each harvest timepoint was a pooled sample. The samples were stored in two boxes (each with a dimension of 24, 18, 15 cm), weighing 1.5 kg each, at -30 °C prior to evaluation.

Butylated hydroxyltoluene (BHT) and all standards used in TLC and HPLC studies were purchased from Sigma-Aldrich (Oakville, ON, Canada). Standards used for TLC study were: arachidic acid stearyl ester (wax ester; WE), 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol (diacylglyceryl ether (DAGE)), tristearin (triacylglycerol (TAG)); 1,2-distearine (diacylglycerol (DAG)), 1-monostearin (monoacylglycerol (MAG)), stearic acid (18:0) (free fatty acid (FFA)), phosphatidyl choline dipalmitoyl (PC), cholesterol (sterol). Standards used for HPLC work included free fatty acids (oleic acid (18:1 cis-9), elaidic acid (18:1 trans-9) and 9-cis,12-cis-linoleic acid (18:2n-6 cis)), 1-oleoyl-*rac*-glycerol MAG (18:1), olive oil as a TAG standard, 1,2-dipalmitoyl-*sn*-glycerol DAG (16:0) standard, 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol (diacylglyceryl ether; DAGE)), and a PL mixture containing L-α-lysophosphatidylcholine from *Glycine max* (soybean), L-α-phosphatidylcholine, L-α-phosphatidylethanolamine

from *Glycine max* (soybean), and L-α-phosphatidylinositol sodium salt from *Glycine max* (soybean). TLC plates (20 X 20 cm, DC-Fertigplatten Kieselgel 60 F-254, layer thickness 0.25 mm) were purchased from Merck (Canada). All solvents (Optima grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). The 95% ethanol used in this work was obtained from Commercial Alcohols (Chatham, ON). Reverse osmosis (RO) water was used in SCVL extraction.

3.3.2 Extraction of SCVL

SCVL was extracted using the Bligh and Dyer (1959) method described in Chapter 2; section 2.3.2 of this thesis.

3.3.3 HPLC Analysis

Samples for lipid class profiling using HPLC were prepared by dissolving 30-35 mg of the SCVL in 1.0 mL of dichloromethane. A 1.0 µL aliquot of the solution was injected onto an Agilent 1100 HPLC equipped with an YMC PAK-PVA-SIL-NP column (250 X 4.6 mm I.D.; 5 µm) and an ESA Corona Charged Aerosol Detector (CAD). The column was eluted with a gradient (Table 3.1) containing 0.2 % v/v ethyl acetate in isooctane (solvent A), 0.02 % v/v acetic acid in 2:1 acetone: ethyl acetate (solvent B) and 0.1 % acetic acid in 3:3:1 v/v/v isopropyl alcohol: methanol: water (RO-water from a milli-Q (MQ) system) (solvent C), at a flow rate of 1.50 mL/min over a run time of 77 min with a post-run time of 12 min. The eluent was monitored with Charged Aerosol Detector (CAD) through an Agilent Analog Digital Convertor 35900 which was set to 2000.000 mAu/Volt and a peak width of 0.027 min (10.00 Hz). Lipid standards (described in section 3.3.1) prepared at a concentration 1 mg/mL in dichloromethane were injected to identify the retention time (RT) of each lipid class component.

Table 3.1 Gradient elution system for SCVL lipid class profiling.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
12	99	1	0
16	98	2	0
25	94	6	0
40	92	8	0
44	75	25	0
48	20	80	0
52	0	100	0
62	0	50	50
68	0	15	85
70	0	0	100
72	0	0	100
74	0	100	0
77	90	10	0

Solvent A = 0.2 % v/v ethyl acetate in isooctane, Solvent B = 0.02 % v/v acetic acid in 2:1 v/v acetone: ethyl acetate and Solvent C = 0.1 % v/v acetic acid in 3:3:1 v/v/v isopropyl alcohol: methanol: water.

3.3.4 Isolation of Lipid Classes by TLC

Lipid samples were dissolved in chloroform at a concentration of 250 mg/mL. A hundred microliters of this concentrate was streaked onto a pre-coated silica gel TLC plate which had been previously developed in ethyl acetate and activated in an oven for one hour at 100°C. The streaked plate was developed in hexane: diethyl ether: acetic acid (80:20:1 by volume) and sprayed with 0.2% methanolic 2,7-dichloroflourescein for visualization of the bands upon exposure to ultraviolet light (360 nm).

Lipid standards including WE, DAGE, TAG, DAG, MAG, FFA, PC and cholesterol were used to identify the five lipid classes present in SCVL by comparison of (Retention factor) R_f

values. Non-polar lipid (DAGE, TAG, FFA) bands were scraped off the plate and extracted three times with 3 mL of 1:1 (*v,v*) hexane: chloroform. Polar lipids (MAGE, PL) were recovered similarly using 2:1 methanol: chloroform (*v,v*). The solvent of the three combined extracts for each lipid class was evaporated using a stream of nitrogen and the weight of each lipid class determined. Recovered simple lipids (MAGE, DAGE, TAG and FFA) were dissolved in hexane, and PL was dissolved in methanol, flushed with nitrogen and stored at -20°C in preparation for analysis.

3.3.5 ^{1}H-NMR and ^{13}C -NMR Analysis

The NMR spectra of lipid classes isolated using TLC (section 3.3.4) were recorded on a Bruker Avance 500 MHz spectrometer. The recovered bands were dissolved in deuterated chloroform ($CDCl_3$) at 16 mg/mL for ^{1}H NMR, and 73 mg/mL for ^{13}C NMR analysis before being transferred to NMR tubes (5 mm diameter, 8 in. in length, Wilmad-LabGlass, Vineland, NJ, USA). The solvent residual signal in the $CDCl_3$ was used as a reference for chemical shift assignments. The ^{1}H NMR acquisition parameters were modified from a previous study (Guillen and Ruiz, 2004): spectral width, 10080 Hz; relaxation delay, 3 s; number of scans, 32; acquisition time, 3.25 s; total acquisition time of 6.9 min. The ^{13}C NMR acquisition parameters were as follows: spectral width, 33333 Hz; number of scans, 512; acquisition time, 0.81 s; with a total acquisition time of 16.07 min.

3.3.6 Ether Lipid Identification Using GC-MS

The detection of ether lipids required some preparative steps before chromatographic analysis could be conducted. Similar to other compounds that have hydroxyl groups, such as long chain alcohol, sterols, mono- and diacylglycerols, ether lipids must be converted to non-polar volatile derivatives. For such analysis, the hydroxyl group of the ether lipids must be free

which can be achieved by enzymatic hydrolysis or saponification. In this study, saponification was chosen to convert DAGE and MAGE (Fig 3.1) into glyceryl ether diols, retaining the *O*-alkyl moiety at the 1-position in glycerol backbone.

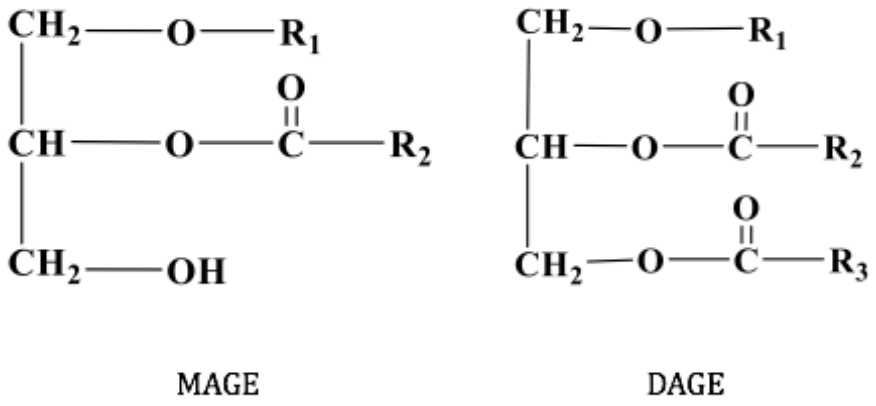


Figure 3.1 Chemical structure of monoacylglyceryl ether (MAGE) and diacylglyceryl ether (DAGE) (R_1, R_2 and R_3= hydrocarbon chains).

DAGE and MAGE were saponified according to Christie (1989) with slight modifications. The lipid was suspended in 5 mL of 2 M ethanolic potassium hydroxide and was placed in a 10 mL capped heavy-duty test tube. The mixture was flushed with nitrogen, sealed and heated at 100 °C for one hour. The mixture was then cooled at room temperature and transferred into a 50 mL test tube. Sixteen milliliters of RO water and 8 mL of a mixture of organic solvents (hexane: diethyl ether, 1:1, *v,v*) were added to the test tube. The top organic layer containing unsaponified material was transferred into a second 50 mL test tube after the sample had been mixed well. The isolated organic layer was washed with 8 mL RO water and evaporated under a stream of nitrogen.

The resulting diols present in the unsaponifiable material were converted into volatile

derivatives so that they were amenable to GC analysis. Several derivatives have been suggested such as isopropylidene (Kang et al., 1998; Malins et al., 1965), diacetylated (Sargent et al., 1973), disilylated (TMS) (Kang et al., 1998) or dimethoxylated (Hallgren and Larsson, 1962) derivatives. In these studies, acetylation was used as the resulting product is stable and volatile enough to be detected by GC analysis (Renkonen, 1966). The unsaponifiable material was acetylated by adding 0.5 ml of a mixture of acetic anhydride and pyridine (5:1, *v,v*) and allowing the mixture to incubate overnight at room temperature (Renkonen, 1966). The solvent was then evaporated, the remaining acetylated material was dissolved in hexane and the sample was transferred to a GC vial in preparation for GC-MS analysis. For this work, the DAGE standard was similarly saponified and acetylated.

Ether lipids were analyzed using a GC Ultra gas chromatograph coupled with a PolarisQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Saponified and acetylated recovered MAGE and DAGE were dissolved in hexane for GC-MS analysis. The analysis was performed using electron ionization (EI) and chemical ionization (CI) modes with split-injection at 250 °C. The separation was performed on a ZB-35 capillary column (30 m × 0.25 mm i.d.) with helium as the carrier gas at 1.2 mL/min. The initial temperature for the program was held at 180 °C for 1 min, then increased to 350 °C at 5 °C/min and held for 3 min. The ionization energy used was 70 eV, with a multiplier voltage of 1643 V, source temperature at 200 °C, and transfer line temperature of 350 °C. Spectral data were acquired over a mass range of m/z 60–600 in both modes and the emission current was 250 μA. In CI mode, methane was used as the reagent gas at a flow rate of 1.5 mL/min.

Alkyl groups associated with an ether bond in both DAGE and MAGE lipid classes were identified by ion spectra obtained for peaks occurring at different RT after subtracting 59 (acetoxy group) from the total molecular mass of DAGE and MAGE.

3.3.7 GC-FID Analysis of FAME

Acid catalyzed transesterification with 0.5 N H_2SO_4 in methanol was used to produce FAME of recovered lipid classes following the procedure described by Budge et al. (2006). FAME were determined using a GC (Bruker, SCION 436-GC) fitted with a DB-23 column ((50%-cyanopropyl)-methylpolysiloxane) (30 m X 0.25 m X 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector (FID). Splitless injection was used with an injector temperature of 250 °C. FAME samples were separated using the following temperature program: The initial temperature was held at 60 °C for 1 min, then increased to 153 °C at 45 ^{o}C/min, held for 2 min, then increased to 174 ^{o}C at 2.3°C/ min and held for 0.2 min, increased to 205°C at 2.5 °C /min and held for 2.5 min for a total run time of 41 min.

3.3.8 Identification of BCFA Using GC-MS

The identification of BCFA was accomplished by preparing FAME and picolinyl esters derivatives that were subjected to GC-MS analysis. Specifically, FAME was analyzed using GC-MS and GC-MS/MS, while picolinyl esters were analyzed with GC-MS only. FAME analysis, using GC-MS in EI mode, was completed first so that the molecular ions of the analytes could be used as precursor ions for MS/MS. Previously, MS/MS has been shown to be useful in distinguishing between straight chain and branched chains as well as between *iso*- and *anteiso*-fatty acids (Ran-Ressler et al., 2012). Picolinyl esters were used to confirm the identities of FA determined by MS/MS and to establish the identities of multimethyl branched FA. Both derivatives were analyzed using GC Ultra GC coupled with a PolarisQ mass spectrometer

(Thermo Fisher Scientific Inc., Waltham, USA).

FAME samples were made up to a concentration of 0.4 mg/mL and injected in splitless mode at 250 ^{o}C. The separation of FAME was performed on a DB-23 capillary column ((50%-cyanopropyl)-methylpolysiloxane, 30 m × 0.25 mm i.d.) with helium as the carrier gas at a flow rate of 1.0 mL/min. The initial temperature was held at 60 °C for 2 min, then increased to 153 °C at 40 ^{o}C/min, increased to 220 ^{o}C at 2.5 min and held for 6.9 min. The ionization energy was 70 eV, with multiplier voltage of 1643 V, source temperature at 200 °C, and transfer line was kept at 250 °C. Spectral data were acquired over a mass range of m/z 60–450. In MS/MS mode, the molecular ion was used as the precursor ion and the isolation width was 3. The excitation voltage was 0.75 V and maximum excitation energy (q) was 0.225.

The structures of multimethyl branched FA were determined by MS analysis of the picolinyl esters derivatives. 3-Pyridylcarbinol (picolinyl) ester derivatives were prepared according to the method described by Harvey (1982). Briefly, 10 mg of lipid was weighed into a tube and dissolved in 1 mL dry dichloromethane. Potassium *tert*-butoxide in tetrahydrofuran (0.1 mL, 1.0 M) was mixed with 3-pyridylcarbinol (0.2 mL) and then added to the tube. The tube was sealed and the mixture was allowed to incubate at 40 °C for 30 min. After the solution had cooled to room temperature, water (2 mL) and hexane (4 mL) were added and the contents of the tube were mixed. The organic layer was collected, dried over anhydrous sodium sulfate, and evaporated to dryness using a flow of nitrogen. The resulting picolinyl esters were dissolved in hexane in preparation for GC-MS analysis.

GC-MS analysis of the picolinyl esters was conducted using a DB-1ms capillary column (100% dimethylpolysiloxane, 30 m × 0.25 mm i.d.), with helium as the carrier gas at a flow rate of 1 mL/min. Samples were prepared at a concentration of 0.4 mg/mL and injected in splitless

mode at 280 °C. The initial oven temperature was held at 60 °C for 2 min, then increased to 235 °C at 20 °C/min, followed by increasing to 280 °C at 2 °C/min and holding for 10 min. The ionization energy was 70 eV, with multiplier voltage of 1643 V, source temperature at 200 °C, and transfer line at 280 °C. Spectral data were acquired over a mass range of m/z 60–500.

The molecular weight of a BCFA picolinyl ester was determined by the molecular ion, while a 28 amu gap in the EI spectra indicated the location of the branched methyl group (-CH (CH_3)-).

3.4 RESULTS

3.4.1. Determination of Lipid Classes by HPLC

HPLC analysis of the lipid classes in SCVL showed chromatographic peaks between ~10-67 min (Fig 3.2). Injection of lipid class standards allowed for the identification of DAGE, TAG, and FFA at 13, 14, and 26 min respectively in SCVL (Fig. 3.3) by comparison of RT. Injection of a mixture of PL, which was a composite of 4 types of PL but multiple PL species, resulted in a cluster of broad peaks between 58-66 min. DAG and MAG lipid classes in SCVL sample were detected at 28 and 48 min respectively (Fig 3.2). A distinct cholesterol peak was not observed at 25 min, which suggested that the lipid extract may not contain cholesterol or that the cholesterol co-eluted with the FFA lipid class. The elution time for MAGE lipid class remained unknown, as we did not run a MAGE standard. Based on similar RT of TAG and DAGE, MAGE may co-elute with DAG lipid class.

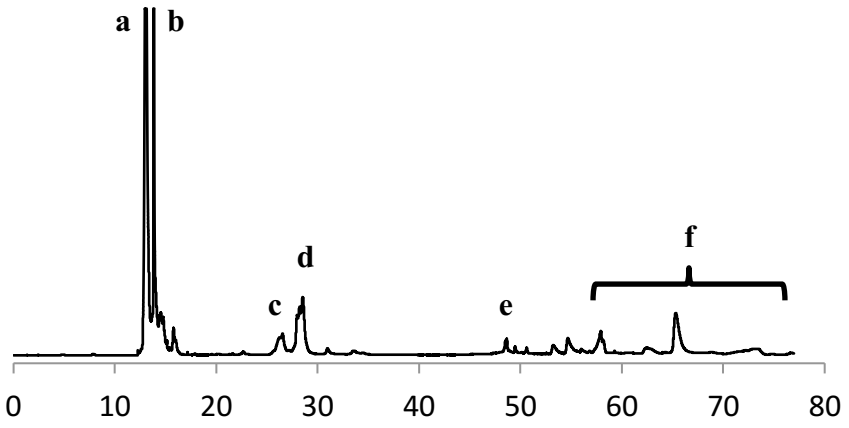


Figure 3.2 Lipid class profiling of SCVL using HPLC: a) DAGE, b) TAG, c) FFA, d) DAG, e) MAG and f) PL.

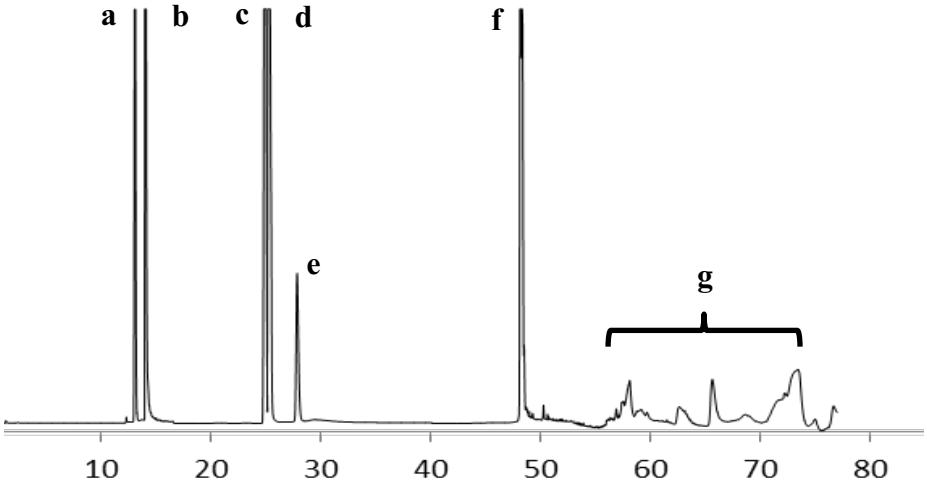


Figure 3.3 HPLC chromatogram of lipid class standards: a) DAGE, b) TAG, c) cholesterol, d) FFA, e) DAG, f) MAG and g) PL (wide peaks in (g) region are different molecular species of each type of PL injected).

3.4.2 Isolation of Lipid Classes by TLC

Lipid classes that were detected and isolated in SCVL using TLC were identified as PL, FFA, TAG and DAGE by co-spotting SCVL with lipid standards (Fig 3.4). The R_f value for a cholesterol standard was found to be slightly lower than the R_f value of band 2 suggesting that cholesterol was present in levels below the limits of detection by TLC or was not present in SCVL, which was in agreement with the HPLC results. While DAG and MAG lipid classes were not detected by TLC analysis, the DAG standard was found to exhibit an R_f that was close to the R_f of band 2. Band 2 was isolated as it was suspected to contain MAGE but we were unable to confirm this by TLC as we did not have access to a MAGE standard.

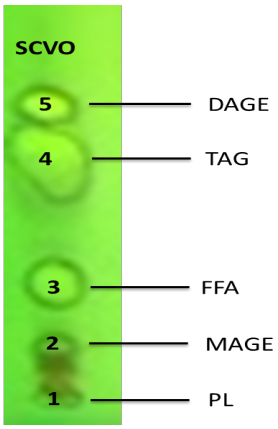


Figure 3.4 Lipid classes in TLC of SCVL.

3.4.3 Characterization of DAGE and MAGE

Analysis of recovered bands 2 and 5 (Fig 3.4), that were thought to contain MAGE and DAGE respectively, contained peaks between 70-75 ppm in ^{13}C NMR spectra (Fig 3.5A) and at ~3.5 ppm in the ^{1}H NMR (Fig 3.5B) spectra which supported the presence of compounds containing ether bonds (Lambert et al., 1987). The presence of glyceryl ethers was confirmed using GC-MS analysis of saponified and acetylated isolated bands using EI (Fig 3.6). Inspection of the mass spectra of peaks eluting at 18.05 and 18.77 min in band 5 (Fig 3.7A and Fig 3.7B

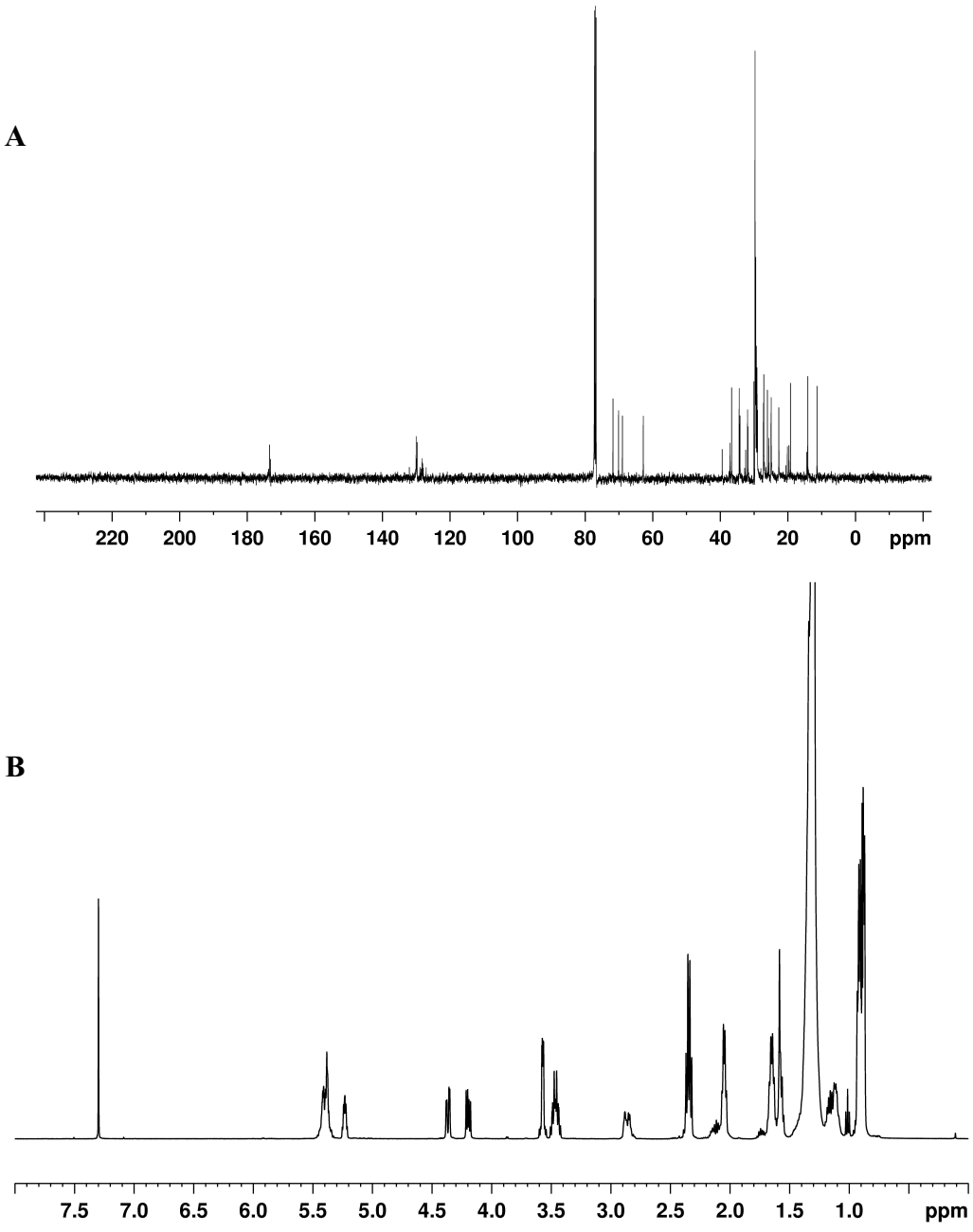


Figure 3.5 ^{13}C-NMR (A) and ^{1}H NMR (B) of recovered band 5 from SCVL in $CDCl_3$.

respectively) did not indicate the presence of a molecular ion. The peak eluting at 18.05 min showed a good MS library match to a molecule containing a branched alkyl structure (16:0). The peak at 18.77 min was identified as a molecule containing an unbranched 16:0 alkyl structure that had the same retention time of the saponified, acetylated 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol standard.

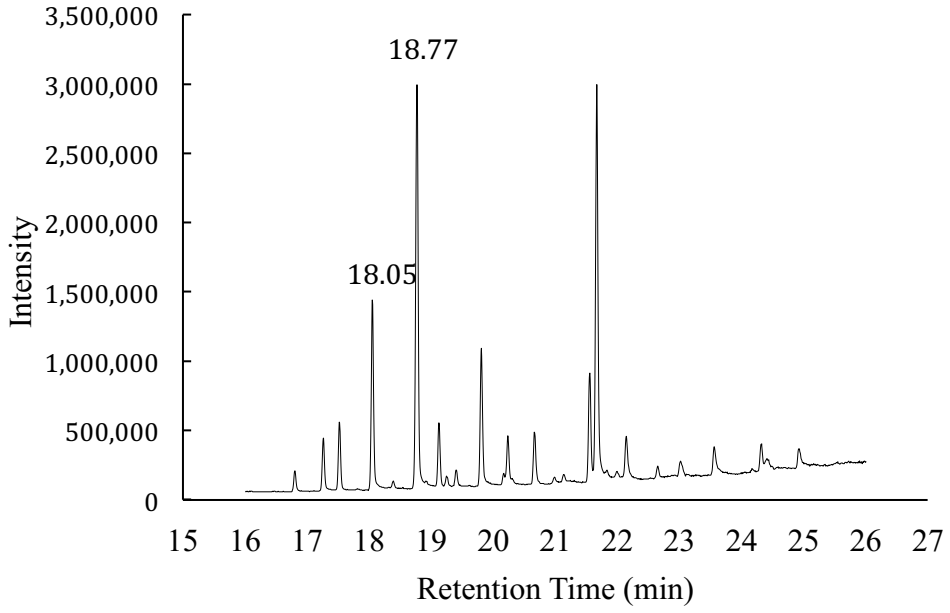


Figure 3.6 GC-MS analysis of saponified, acetylated band 5 in EI mode. The peak at 18.05 min was identified to be a molecule containing a branched alkyl structure (16:0) by MS library matching. The peak at 18.77 min was identified as a molecule containing an unbranched 16:0 alkyl structure that had the same RT of the saponified, acetylated 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol standard.

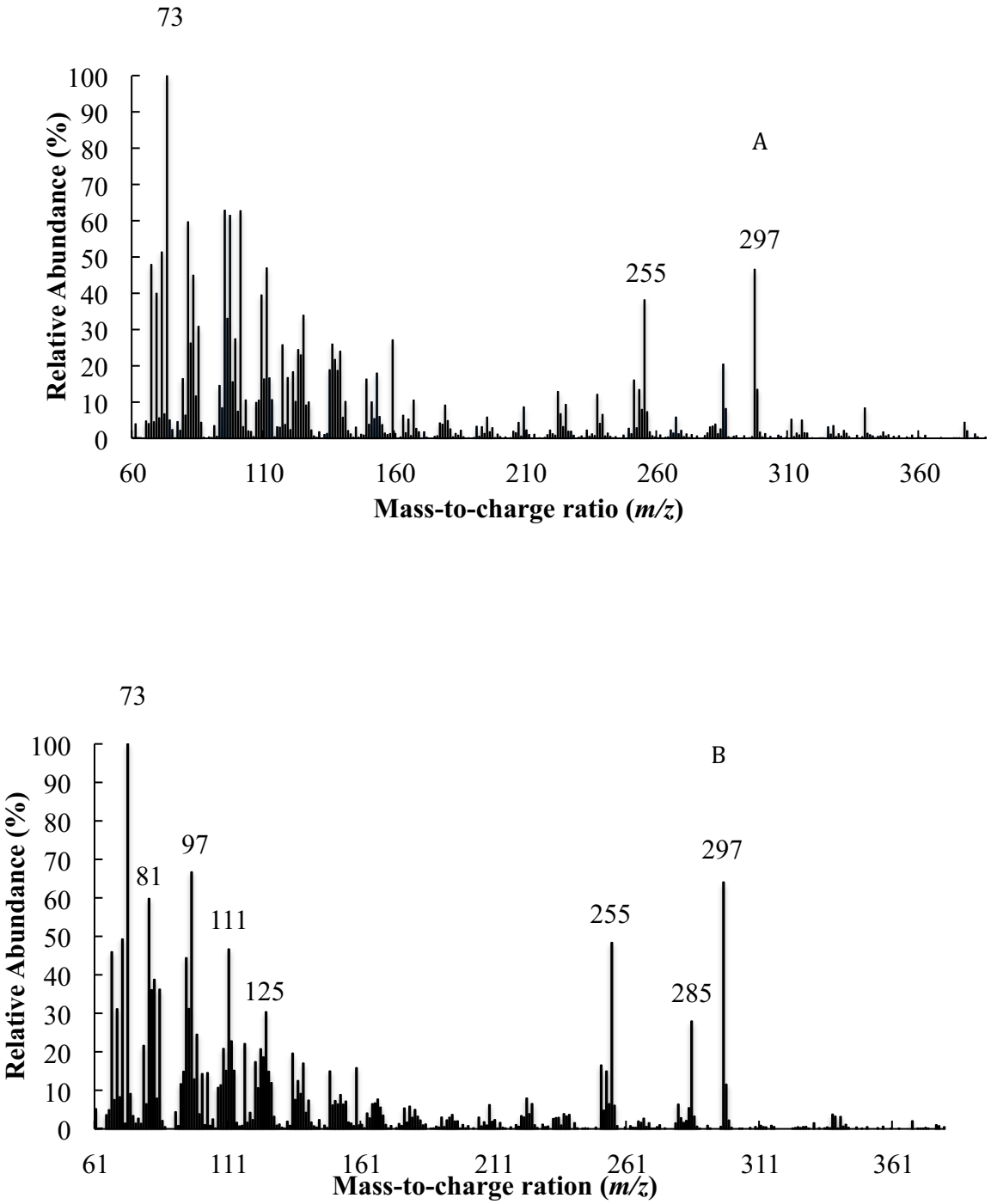


Figure 3.7 Electron ionization mass spectra (EIMS) for structures eluted at 18.05 (A) and 18.77 (B) min.

GC-MS analysis using CI of saponified, acetylated band 5 yielded a chromatogram similar to that obtained by EI detection (Fig 3.8). The CI mass spectra (CIMS) for peaks occurring at 18.09 (Fig 3.9A) and 18.87 min (Fig 3.9B) both showed base peaks at *m/z* 341 which represented a $[M-59]^+$ fragment for a sixteen carbon containing alkyl group of DAGE that had been saponified and then acetylated. This fragment was associated with the loss of an acetoxy group from a molecular ion at *m/z* of 400 (Fig 3.10 A and B). GC-MS analysis of recovered band 2 of SCVL (Fig 3.4) gave results similar to those observed for band 5 suggesting both bands 2 and 5 belonged to a glycerol acyl ether lipid class.

DAGE was composed primarily of saturated alkyl structures (85 % of total by mass) with 16:0 and 18:0 dominating at ~27 % and 26 % respectively (Table 3.2). Saturated branched alkyl chains (15:0, 16:0, 17:0) were also identified in DAGE with branched 16:0 (11.5 %) being the major structure present followed by branched 17:0 (8.7 %) eluting at 19.84 min. Monoenoic alkyl chains were rarer but detected at a total of 14 % where 18:1 (7 %) was the major structure present.

Saturated alkyl structures in MAGE (91 %) were slightly higher than that detected in DAGE (85 %) (Table 3.2). The predominant alkyl chains in MAGE were 18:0 (50 %) and 16:0 (15 %). The branched 16:0 and 17:0 alkyl chains occurred at 5.6 and 5.4 % while the monounsaturated 18:1 occurred at ~5 %. The saturated 21:0 alkyl structure (~11 %) was present in MAGE at a level 10 times higher than in DAGE. The only alkyl polyenoic alkyl chain detected in both DAGE and MAGE contained an 18:2 alkyl structure but was only present at levels less than 1%.

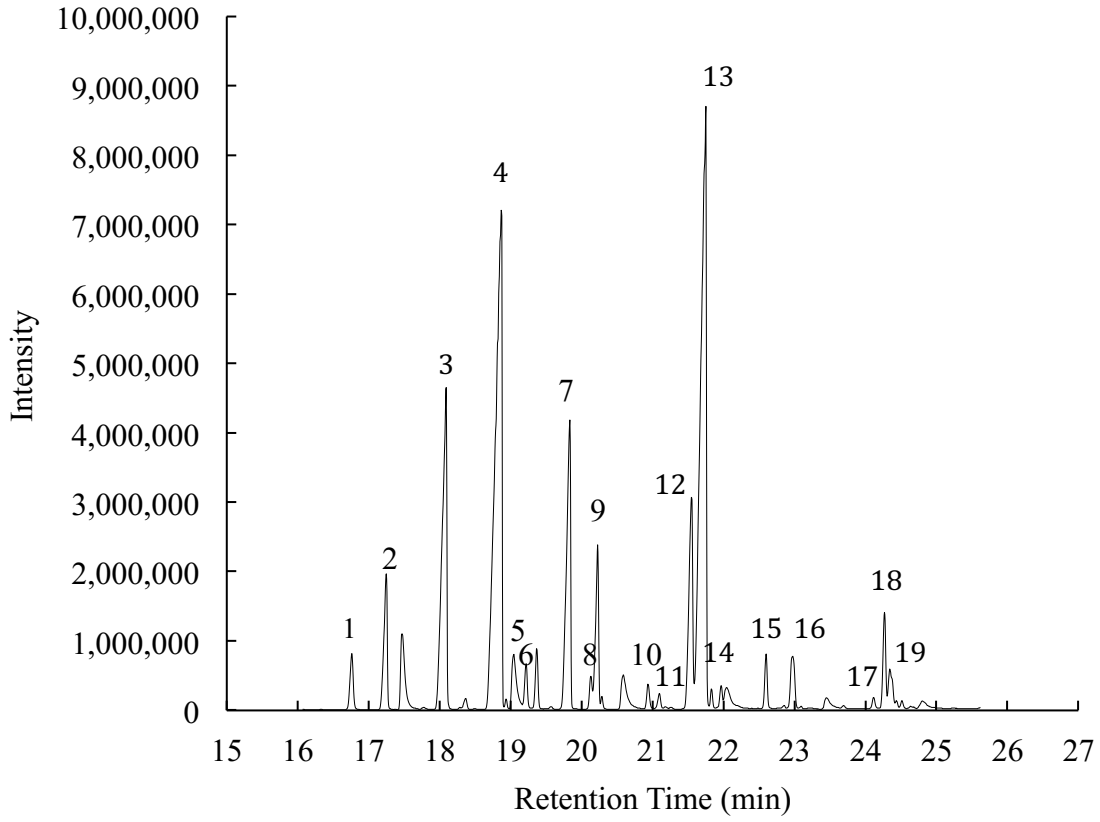


Figure 3.8 GC-MS analysis of saponified, acetylated band 5 using CI mode. Peak 3 at 18.09 min was identified to be a molecule containing a 16:0 branched alkyl structure using an MS library evaluation. Peak 4 at 18.87 min was identified as a molecule containing an 16:0 unbranched alkyl structure that had the same RT of the saponified and acetylated 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol standard.

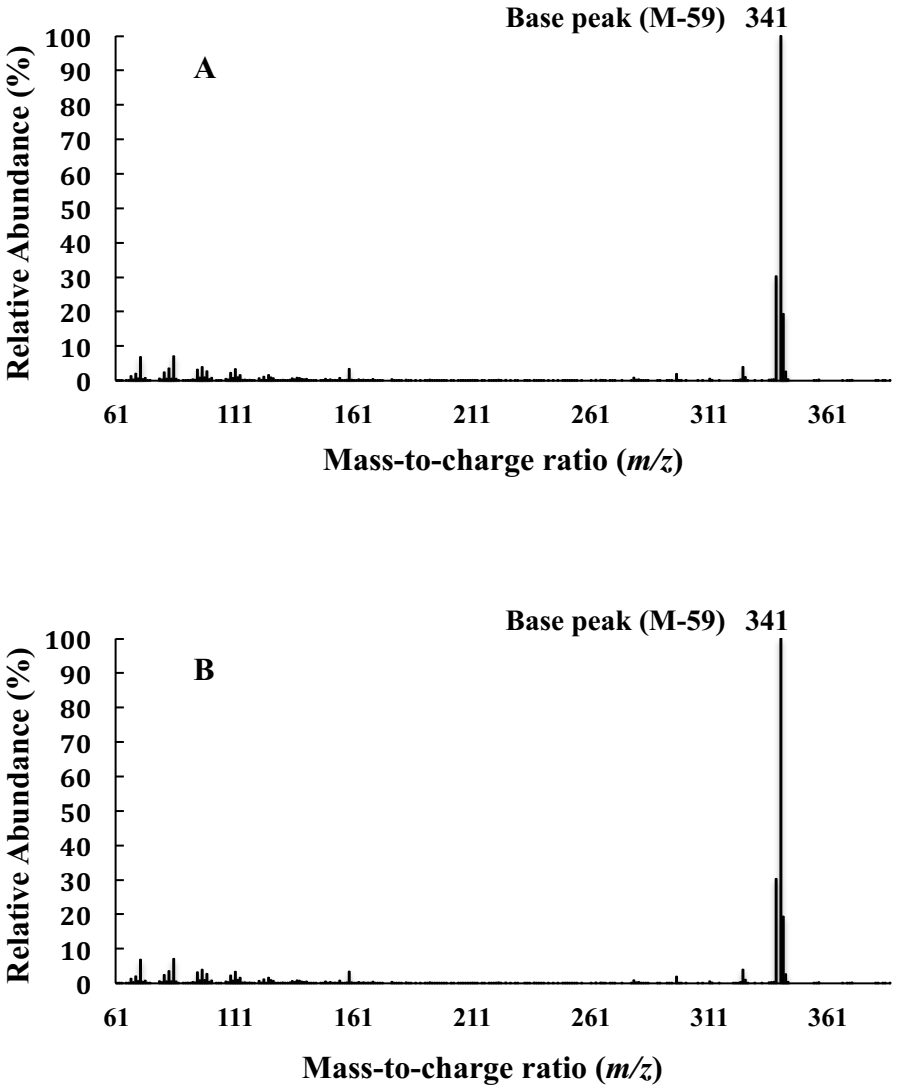


Figure 3.9 Chemical ionization mass spectra (CIMS) for structures eluting at 18.09 (A) and 18.87 (B) min.

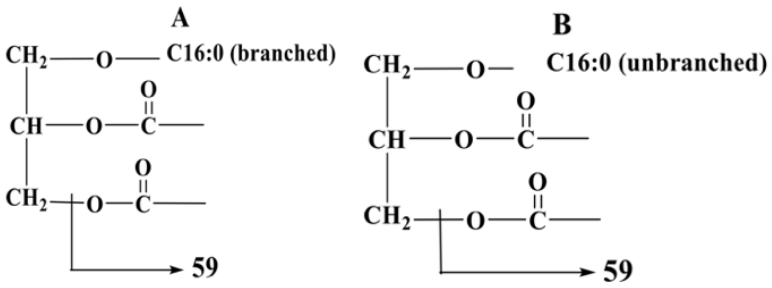


Figure 3.10 The loss of an acetoxy group from position n-3 from the glycerol backbone for structures eluting at 18.09 (peak 3) (A) and 18.87 min (peak 4) (B) of saponified, acetylated DAGE.

Table 3.2 Area % of alkyl chains identified in DAGE and MAGE lipid classes associated with the ether linkage identified by GC-MS in CI mode.

RT (min)	Peak number (DAGE band)	Molecular Mass	Alkyl chain ether bond	DAGE (Area %)	MAGE (Area %)
16.76	1	386	15:0 (branched)	1.3	0.6
17.25	2	386	15:0	3.3	1.3
18.09	3	400	16:0 (branched)	11.5	5.6
18.87	4	400	16:0	27.3	15.0
19.22	5	414	17:0 (branched)	0.7	0.1
19.36	6	414	17:0 (branched)	0.9	0.1
19.84	7	414	17:0 (branched)	8.7	5.2
20.23	8	414	17:0	4.1	2.2
20.29	9	412	17:1	3.3	0.0
20.93	10	*426/428	18:1/18:0	0.4	0.1
21.08	11	426	18:1	0.5	0.3
21.53	12	426	18:1	6.9	5.0
21.75	13	428	18:0	25.6	50.0
21.97	14	424	18:2	0.4	0.9
22.60	15	442	20:0	0.7	0.0
22.97	16	440	20:1	1.4	2.4
24.10	17	454	21:1	0.2	0.2
24.27	18	454	21:1	1.8	0.4
24.34	19	456	21:0	1.1	10.6
Saturated, straight and branched				85.2	90.8
Monoenoic				14.4	8.0
Polyenoic				0.4	0.9

* Two compounds were co-eluting at 20.93 min. One of the compounds was 18:1 (MW=426), the other is 18:0 (MW=428)

The major FA identified in DAGE were 12-MTA and 16:1n-7 at ~ 42 and 20 % respectively (Table 3.3). A number of interesting branched chain FA were identified (17 %), including several with multimethyl branches (such as 4,8,12-Me-13:0; 8,12-Me-14:0; 12-Me-15:0 and 8,12-Me-15:0). In contrast, MAGE contained relatively little 12-MTA (~ 4 %) and much higher levels of EPA (58 %) than DAGE, where EPA represented most of the MAGE PUFA content. The total BCFA content of MAGE was detected at ~ 8 % which is much lower than that observed for DAGE.

3.4.4 Identification of BCFA Using GC-MS

In this work, straight chain FA, analyzed as FAME, had EI spectra similar to *iso* branched FAME, where a homologous series of ions between $[M-29]^+$ and the McLafferty rearrangement ion *m/z* 74 were present, representing the elimination of methylene groups from the chain. GC MS/MS spectra of straight chain FAME (14:0, 15:0 and 16:0) characteristically exhibit ions that are regularly spaced 14 amu apart. In the case of monomethyl branched FAME, only 12-methyl-14:0 (12-MTA) and 12-methyl-15:0 were identifiable using MS/MS because the intensities of molecular ions were too low for all the other monobranched chains. In the MS/MS spectrum of 12-methyl-14:0 FAME, the two major ions at *m/z* 199 and 227 indicated the presence of a 12-methyl group. A similar approach was used to identify branch positions in other singly and multi branched FAME.

Table 3.3 The FA profile (weight percentage of total FAME identified; mean +/- SD, n=3) of recovered MAGE and DAGE lipid classes from the lipid of SCV of *C. frondosa.* BCFA were identified using GC-MS; the rest of the FA data were identified by GC-FID.

Fatty acid	**Weight percentage (%) in DAGE**	**Weight percentage (%) in MAGE**
14:0	0.52 ± 0.03	0.15 ± 0.01
4,8,12-Me-13:0	3.61 ± 0.05	0.39 ± 0.00
*Me-14:0(a)	0.84 ± 0.03	0.09 ± 0.00
Me-14:0(b)	0.74 ± 0.01	0.16 ± 0.03
i-15:0	0.07 ± 0.02	0.02 ± 0.00
12-MTA	41.75 ± 0.61	4.48 ± 0.18
8,12-Me-14:0	3.29 ± 0.44	0.41 ± 0.03
15:0	0.23 ± 0.02	0.03 ± 0.01
Me-14:0(c)	0.66 ± 0.03	0.07 ± 0.00
12-Me-15:0	6.98 ± 0.22	1.95 ± 0.35
8,12-Me-15:0	0.47 ± 0.01	0.12 ± 0.04
16:0	0.67 ± 0.05	0.71 ± 0.05
16:1n-9	0.15 ± 0.03	1.11 ± 0.42
16:1n-7	20.40 ± 0.56	10.49 ± 0.38
**17:1(a)	0.04 ± 0.01	0.05 ± 0.01
i-17:0	0.04 ± 0.02	0.20 ± 0.05
ai-17:0	0.55 ± 0.02	0.10 ± 0.01
17:1(b)	0.03 ± 0.01	0.03 ± 0.00
16:2n-4	0.72 ± 0.01	0.31 ± 0.03
16:3n-4	0.23 ± 0.02	0.22 ± 0.01
17:1(c)	0.44 ± 0.01	0.20 ± 0.01
17:1(d)	0.29 ± 0.05	4.72 ± 0.14
16:4n-1	0.17 ± 0.03	0.13 ± 0.02

Fatty acid	Weight percentage (%) in DAGE	Weight percentage (%) in MAGE
18:0	0.49 ± 0.07	0.92 ± 0.02
18:1n-9	0.85 ± 0.04	0.45 ± 0.03
18:1n-7	2.71 ± 0.06	2.33 ± 0.13
18:2n-7	0.02 ± 0.00	0.02 ± 0.00
18:4n-3	0.28 ± 0.02	0.28 ± 0.06
20:0	0.04 ± 0.01	0.03 ± 0.01
20:1n-11	0.20 ± 0.01	0.20 ± 0.02
20:1n-9	0.29 ±0.01	0.41 ± 0.01
20:1n-7	0.07 ± 0.01	0.07 ± 0.01
20:4n-6	0.13 ± 0.03	1.39 ± 0.07
EPA	5.21 ± 0.28	57.67 ± 0.31
22:0	0.10 ± 0.04	0.08 ± 0.06
22:1n-9	0.09 ± 0.02	0.13 ± 0.05
22:1n-7	0.13 ± 0.02	0.10 ± 0.04
22:4n-6	0.29 ± 0.03	0.30 ± 0.01
DHA	0.30 ± 0.02	0.99 ± 0.02
24:1	0.61 ± 0.04	1.20 ± 0.07
Others	5.30 ± 0.15	7.28 ± 0.40
Σ Saturated FA	2.05 ± 0.09	1.92 ± 0.07
Σ MUFA	26.31 ± 0.51	21.51 ± 0.54
Σ PUFA	7.35 ± 0.27	61.31 ± 0.36
Σ BCFA	58.99 ± 0.72	7.98 ± 0.38
Σ BCFA without 12-MTA	17.24 ± 0.45	3.50 ± 0.34

*Me-14:0 (a, b and c), and ** 17:1 (a, b, c and d) are isomers

3.5 DISCUSSION

The presence of ether lipids in SCVL was expected as alkyl structures including saturated (branched and unbranched) and monounsaturated alkyl chains associated with an ether bond in glycerol ethers have been previously identified in some Holothurians (Rybin et al., 2007; Santos et al., 2002; Isay et al., 1976), the class under which sea cucumber are classified. High levels of α-glyceryl ethers have been reported in lipid extracts of *Stichopus japonicas* (18 %), *C. fraudatrix* (9.3 %), and *C. japonica* and *C. okhotensis* (25-27 %) (Isay et al., 1976; Rybin et al., 2007). The presence of DAGE ether lipids in dogfish, shark, deep-sea squids, elasmobranch fish, oysters, sponges, pteropoda and corals (Haraldsson and Kristinsson, 1998; Hayashi and Kishimura, 2002; Hallgren and Larson, 1962; Guella et al., 1986; Myers and Crews, 1983; Smith and Djerassi, 1987; Boer et al., 2005; Imbs et al., 2006) suggests that they are ubiquitous in marine animals and therefore their presence in sea cucumber species such as *C. frondosa* was expected.

The CIMS spectra obtained from the saponified/acetylated DAGE band of SCVL showed that the same ion spectra were obtained for peaks occurring at 18.09 (Fig 3.9A) and 18.87 min (Fig 3.9B). This suggested that the two ether lipid derivatives contained the same number of carbons. The difference in RT of the two peaks indicated that the two were structurally different such that one contained a branched alkyl chain while the other was unbranched. The branched structure was predicted to elute at 18.09 min (Fig 3.8; peak 3) which was before the non-branched structure at 18.87 min (Fig 3.8; peak 4) (Ran-Ressler et al., 2012). Both peaks had base peaks at *m/z* 341 which was a result of the loss of an acetoxy fragment $[M-59]^+$ (Fig 3.10; A and B). Using this observation (Fig 3.10; A and B) a branched 16:0 alkyl structure was assigned

to the ether bond in peak 3 that eluted at 18.09 min and an unbranched 16:0 alkyl structure to peak 4 at 18.87 min (Table 3.2).

Following this procedure, several alkyl chains associated with the ether bond in the DAGE lipid class (Table 3.2) were assigned. For instance, peaks 1 and 2 in Fig 3.8 were determined to contain a molecular weight of 386 which indicated the presence of a branched 15:0 alkyl chain at 16.76 min and an unbranched 15:0 alkyl chain at 17.25 min. Similarly, peaks 7 and 8 which had the same molecular weight (414) were determined to contain a branched and unbranched 17:0 alkyl chain ether lipid at 19.84 and 20.23 min respectively (Fig 3.8; Table 3.2).

Analysis of the components of glyceryl ethers derived from both DAGE and MAGE revealed unique alkyl chain structures that were characteristically rich in 16:0 and 18:0 (Table 3.2). Rybin et al. (2007) and Santos et al. (2002) also found a similar dominance of 16 and 18 carbon *O*-alkyl chains in DAGE and MAGE of *C. japonica, C. okhotensis* and *Oneirophanta mutabilis*. This narrow range of alkyl structures indicates a high specificity for particular substrates in the pathways responsible for the biosynthesis of alkylglycerols (Reichwald-Hacker, 1983). Rybin et al. (2007) and Santos et al. (2002) had suggested that the specificity might be due to two acyl-CoA reductase isoenzymes (FAR1 and FAR2) (Cheng and Russel, 2004; Hartvigsen et al., 2006). Both reductase isoenzymes select fatty acyl-CoA of 16 and 18 carbon chains; FAR2 is specific for palmityl-and stearyl-CoA substrates, while FAR1 targets monounsaturated palmitoleyl- and oleyl-CoA and polyunsaturated linoleyl-CoA (Magnusson and Haraldsson, 2011). The effect of this specificity was suggested in mammalian cells, and might exist in sea cucumbers and other marine organisms.

The FA profile for DAGE and MAGE were characterized by the presence of high concentrations of 12-MTA and EPA (Table 3.3). The patterns between the alkyl groups and FA

associated with DAGE and MAGE were therefore quite different; the FA present in the highest proportions are not the same as the alkyl groups present in the highest amounts. In the biosynthesis of ether lipids, 1-*O*-alkyl-*sn*-glycero-3-phosphate has been reported to be a glycerol based intermediate found mostly in mammalian cells (Brites et al., 2004). Esterification of this intermediate with acyl-CoA at the *sn*-2 position in the presence of alkyl-acyl-glycero-3-phosphate acyltransferase results in the formation of 1-*O*-alkyl, 2-acyl-*sn*-glycero-3-phosphate. Removal of the phosphate group from the sn-3 position of that structure by phosphohydrolase results in the formation of 1-*O*-alkyl-2-acyl-*sn*-glycerol (MAGE). DAGE is then synthesized when 1-*O*-alkyl-2-acyl-*sn*-glycerol (MAGE) is esterified with a long-chain acyl-CoA ester by an acyltransferase. Thus, the high concentration of EPA in MAGE is likely due to a high specificity of alkyl-acyl-glycero-3-phosphate acyltransferase in positioning long chain FA (EPA) at *sn*-2 position. An elevated level of EPA is not observed in DAGE. If DAGE are derived from MAGE then some other mechanisms must be involved and responsible for the hydrolysis of EPA in MAGE and replacement of 12-MTA in DAGE. Alternatively, co-elution of DAG, found in the HPLC analysis, with MAGE, would also lead to an elevated EPA proportion if EPA was present in a high proportion in DAG.

Different species of BCFA were identified in both DAGE and MAGE lipid classes. *Iso*- and *anteiso* BCFA are the most abundant monomethyl BCFA. Zhong et al. (2007) reported the presence of *ai*-15:0 in fresh and dried *C. frondosa* harvested near Newfoundland. *Iso*-14:0, *iso*- and *anteiso*-15:0, *anteiso*-16:0 and *anteiso*-17:0 were identified in oil extracts of many marine organisms including Atlantic and Pacific dogfish in trace amounts (Kang et al., 1998). The BCFA (including 12-MTA) in the DAGE of SCVL represented 59 % of its FA weight

percentage. Multimethyl branched FA, such as 4,8,12-Me-13:0, 8,12-Me-14:0, and 8,12-Me-15:0, have not been previously reported in lipid extracts of sea cucumber species.

Such BCFA can be synthesized in animal tissues *per se.* In bacteria and in animal tissues, *iso*- and *anteiso*-BCFA can be produced biosynthetically through regular mechanisms for the synthesis of saturated FA (including involvement of acyl carrier protein) except the primer molecule differs (2-methylpropanyl-CoA, 3-methylbutyryl-CoA, and 2-methylbutyryl-CoA) and is activated by a similar enzyme (FA synthase) (Fig 3.11) (Kaneda, 1991). For example, the initial step for the syntheses of 4,8,12-Me-13:0 BCFA (an *iso*-BCFA) would involve the use of 2-methylpropanyl-CoA as a primer molecule followed by a combination of two successive 2-methylbutyryl-CoA and one acetyl-CoA additions. Similarly, the synthesis of 12-MTA (*anteiso*-BCFA) can be envisioned by using one 2-methylbutyryl-CoA and four malonyl-CoA as chain extenders, followed by one acetyl-CoA addition (Fig 3.12).

It has been reported that BCFA are involved in membrane fluidity in animal tissue (Annous et al., 1997) and are well known for their low melting points (Kaneda, 1983). An increase in the concentration of 12-MTA in cell membranes might be expected when an animal is exposed to low temperatures especially when there is a deficiency or no cholesterol, such as observed in bacteria (de Mendoza and Cronan, 1983; Suutari and Laakso, 1994) and in SCVL. As a result this BCFA could be important in maintaining cell membrane fluidity (Annous et al., 1997) in sea cucumbers as they live in the cool waters of the ocean floor.

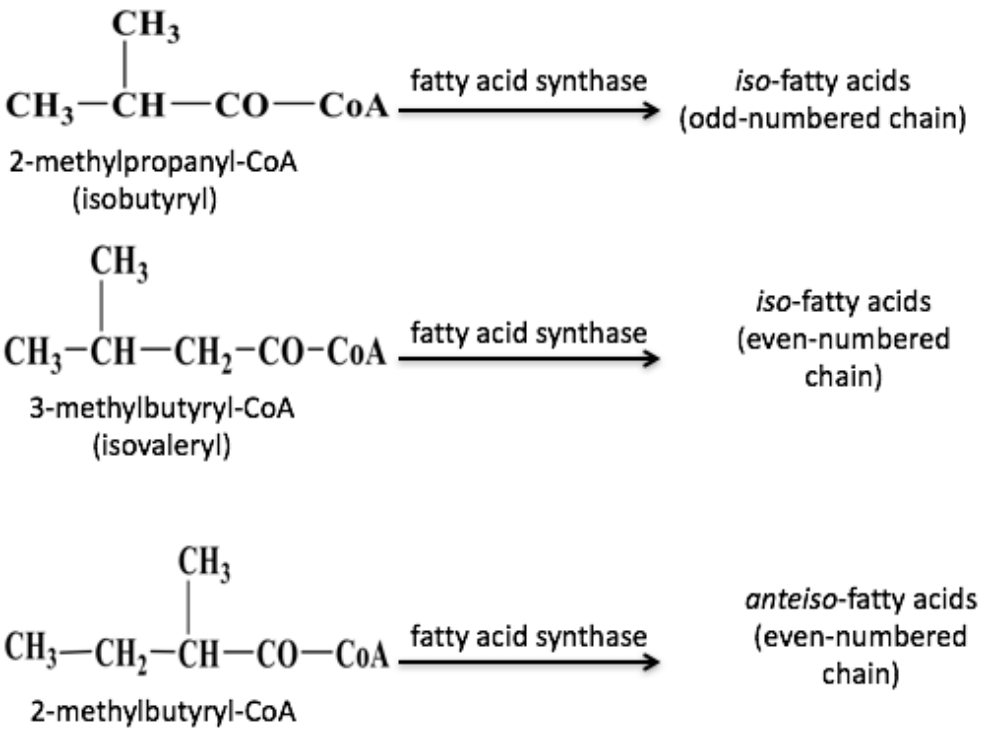


Figure 3.11 Mechanisms for the synthesis of BCFA in bacteria (Adopted from Kaneda, 1991).

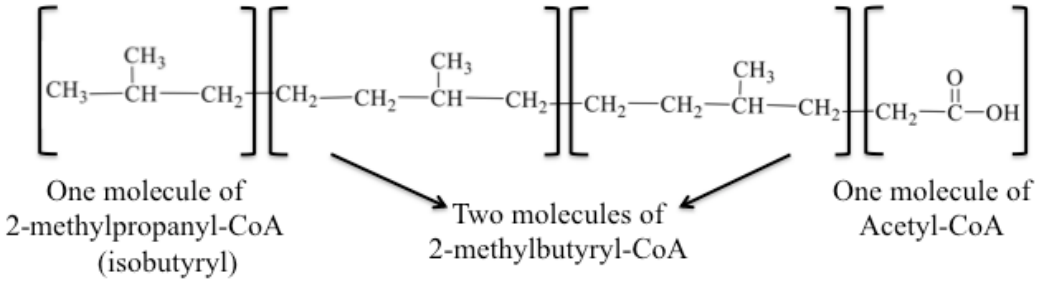


Figure 3.12 Suggested mechanism for the synthesis of 4,8,12 Me-13:0 BCFA.

The BCFA and specifically 12-MTA have been shown to exhibit promising health benefits including anti-inflammatory, anti-cancer activity and wound healing activity (Collin et al., 2003). SCVL in this study is concentrated with this particular BCFA, which might be seen as a natural source of 12-MTA for medical applications.

3.6 CONCLUSIONS

Five lipid classes were identified in SCV lipid extract of *C. frondosa* which included non-polar ether lipids (diacylglyceryl ether; DAGE), TAG, polar lipid ethers (monoacylglyceryl ether; MAGE) and PL using HPLC analysis. The FFA lipid class was only present in trace amounts. Saturated and monounsaturated 1-*O*-alkylglyceryl ethers ranging from C_{15}-C_{21} were identified. The main components of glyceryl ethers that were distributed in both DAGE and MAGE were 16:0, 16:1, 17:0, 18:0 and 18:1 alkyl chains. The 16:0 and 18:0 alkyl chains were dominant in both DAGE and MAGE respectively. Moreover, unique BCFA including 12-MTA, were also identified in the ether lipid classes. The presence of ether lipids (DAGE and MAGE) as well as the abundance of BCFA, in this SCVL are unique, previously unreported features of SCVL that may be valuable features in the positioning of the lipid in the nutraceutical marine oil market.

CHAPTER 4 EVALUATION OF THE CAROTENOID CONTENT OF SEA CUCUMBER VISCERA LIPID (*CUCUMARIA FRONDOSA*) USING HPLC AND SPECTROPHOTOMETRIC METHODS

4.1 ABSTRACT

The carotenoid content of the pigmented lipid extracted from sea cucumber viscera (SCV) of *Cucumaria frondosa* harvested from the Sable Island Banks off Nova Scotia, Canada was investigated. Using HPLC, the lipid extract of SCV (SCVL) was found to contain canthaxanthin, astaxanthin and neoxanthin, as well as a number of unidentified peaks. Carotenoids previously identified in other sea cucumber species including cucumariaxanthins A, B and C, β-cryptoxanthin, and β-carotene were absent in the profile. Canthaxanthin, the major carotenoid present in SCVL, was present at concentrations 5 times greater than astaxanthin. The total carotenoid content determined using a UV spectrophotometric method (2340-2500 μg/g lipid) was greater than that determined for the oil extracted from Antarctic krill (878 to 1016 μg/g oil). Comparison of the total carotenoid content determined using HPLC and a UV spectrophotometric method indicated that there was a correlation between the two methods and that the spectrophotometric method gave levels that were 35 % greater than those obtained using HPLC. In this study, the carotenoid content of SCVL revealed that it is a unique source of canthaxanthin and astaxanthin, which makes the lipid a potential new commercial nutraceutical lipid for the human or animal health market.

4.2 INTRODUCTION

Lipids isolated from sea cucumbers are highly pigmented with an intense orange-red color (Collin et al., 2003). The color is due to carotenoids, a family of pigments, which includes more than 700 compounds (Biehler et al., 2009). Carotenoids are mainly biosynthesized in plants (Graca Dias et al., 2010) so marine invertebrates such as sea cucumber accumulate carotenoids in their tissues as a result of feeding on algae and other predators rich in carotenoids (Liaaen-Jensen, 1998). In humans, several studies have confirmed the relationship between the consumption of carotenoids and cancer prevention and cardiovascular reduction (FDA-CDER, 1994).

The carotenoid composition of sea cucumber varies with species, tissue type and body part. For instance, Matsuno et al. (1971) isolated astaxanthin, β-carotene, echinenone, and canthaxanthin from the gonads of *Holothuria leucospilota* and *Stichopus japonicas* sea cucumber species, while echinenone, canthaxanthin and phoenicoxanthin have been identified in lipids extracted from the red body wall of *Psolus fabrichii* (Bullock and Dawson, 1970). Canthaxanthin and cucumariaxanthin have been identified in *C. frondosa* (whole and viscera) harvested from Atlantic Canada (Findlay et al., 1983; Pelletier and Mamelona, 2011). There is local interest in producing lipid from the viscera of *C. frondosa* harvested from the Sable Island Banks off Nova Scotia, Canada but the carotenoid content of the lipid obtained from the viscera of this species has not yet been investigated.

Carotenoids in oils have antioxidant activity and provide oxidative stability to oils containing them (Zhong et al., 2007). However, high concentrations of carotenoids and/or high oxygen pressure have been found to decrease the antioxidant effective of carotenoids and, in some cases, have been shown to promote oil oxidation (Kiokias and Gordon, 2004, Subagio and

Morita, 2001). Thus, knowledge of the carotenoid content of lipids extracted from sea cucumber viscera is important in evaluating lipid stability.

The carotenoid composition of marine animals has been determined using HPLC (Ali-Nehari et al., 2012; Ferreres et al., 2010; Mariutti et al., 2012) with C_{18} and C_{30} reverse phase columns using UV/Vis, diode array and mass spectrophotometric detection. Total carotenoids have also been estimated using a rapid UV spectrophotometric method with detection at 450-480 nm (Zhong et al., 2007; Jodlowska and Latala, 2011). However, the relationship between total carotenoids determined with the two methods has not been investigated in sea cucumbers. In the current study, HPLC was used to determine the carotenoid profile and total carotenoid concentration of lipid extracts obtained from the viscera of Atlantic *C. frondosa*. The total carotenoid content was also estimated using a UV spectrophotometric method and the relationship between the total carotenoid content obtained using both HPLC and spectrophotometric methods was investigated.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Materials and Methods

Harvests of sea cucumber viscera from *C. frondosa* were conducted in January 2015 (winter; latitude 4439/ longitude 6041) and March 2015 (spring; latitude 4439/ longitude 6040) from the Sable Island Banks off Nova Scotia, Canada by Ocean Pride Fisheries Limited (Lower Wedgeport, NS). The viscera sample obtained from each harvest timepoint was a pooled sample. The samples were stored in two boxes (each with a dimension of 24, 18, 15 cm), weighing 1.5 kg each, at -30 °C prior to evaluation.

All solvents (Optima grade) were purchased from Fisher Scientific (Ottawa, ON, Canada) except for methyl *t*-butyl ether (MTBE), which was purchased from Caledon (Georgetown, Laboratories Ltd, ON, Canada). Reverse osmosis (RO) water was used in SCVL extractions. Astaxanthin and canthaxanthin standards were purchased from Sigma Aldrich. Carotenoid standards, including fucoxanthin, violaxanthin, lutein, β-carotene, and β-cryptoxanthin, were purchased from Chromadex, Irvine, California, USA. Spectrophotometric data were collected using a UV spectrophotometer (Novaspec II, LKB Biochrom, Holliston, MA, USA). Fish oils (FO) mixed with SCVL to prepare samples for total carotenoid content tests were provided by Ascenta Health (Dartmouth, NS).

4.3.2 Profiling of Carotenoid Content Using HPLC

4.3.2.1 Preparation of SCVL for HPLC Analysis

Lipid samples used in this study were prepared using the Bligh and Dyer (1959) method described in Chapter 2; section 2.3.2. To determine the total carotenoid content in SCVL samples by HPLC, the solid-phase extraction method reported by Mateos and García-Mesa (2006) for use with olive oil was first applied. However, this approach failed to yield a pure carotenoid fraction that did not contain lipids.

As an alternative, lipids were removed by precipitation. SCVL (10-20 mg) was added to 1 mL of a mixture of 81:4:15 methanol: water: MTBE (v/v). The unpigmented precipitate that formed was removed from the sample via filtration through a glass pipette containing a glass wool plug, yielding a highly pigmented solution.

4.3.2.2 HPLC Method for Carotenoid Profiling of SCVL

SCVL was diluted in MTBE to a concentration of 1 to 1.5 mg/mL. A 20 µL aliquot of the solution was injected into an Agilent 1100 HPLC equipped with an YMC_{30} carotenoid column

(250 mm X 4.6 mm I.D.) and an Agilent 1100 diode array detector. The column was eluted with a gradient containing 50 mM ammonium acetate in methanol (solvent A), and 5 % MTBE in methanol (solvent B), at a flow rate of 0.20 mL/min over a run time of 60 min in which the eluent was monitored at 450 nm (Table 4.1). Carotenoids were identified by comparison of retention times and UV-spectra of standards, and co-injection experiments with standards. UV-spectra for the carotenoids that have been previously identified in sea cucumber were obtained from the literature (Britton et al., 2004). Four point calibration curves were constructed for astaxanthin and canthaxanthin to allow for the quantitation of carotenoid content relative to these two standards.

Table 4.1 Gradient HPLC elution system for carotenoid analysis.

Time (min)	Solvents A (%)	Solvent B (%)
0	95	5
5	95	5
35	35	65
40	35	65
41	95	5
60	95	5

Solvent A= 20 mM ammonium acetate in methanol, Solvent B= 5 % MTBE in methanol.

4.3.2.3 Total Carotenoid Content Determined by HPLC

Total carotenoid concentration was determined by summing the content of canthaxanthin, astaxanthin, neoxanthin and unidentified peaks eluting between 5-35 min. Unidentified peaks were quantified using the response factor of canthaxanthin, the dominant peak in the chromatogram. Samples were prepared from winter and spring harvests, and injected in

triplicate. To allow for comparison of the HPLC method with the UV spectrophotometric method (see next page), five dilutions were prepared by mixing SCVL with different percentages of colorless fish oil to obtain different concentrations of carotenoid–based pigmented oil. Samples from both harvests were analyzed at each dilution and results of triplicate injections for each concentration point for each harvest were reported.

4.3.3 Total Carotenoid Determination Using the UV Spectrophotometric Method

The total carotenoid content was determined following Takeungwongtrakul et al. (2012), who modified it from a method reported by Saito and Rigier (1971). A 30 mg sample of SCVL, derived from the same dilutions with fish oil as described above, was dissolved in 10 mL hexane. The mixture was allowed to stand for 30 min at room temperature and then the absorbance was read at 468 nm using a 1-cm path amber glass cuvette. The sample was diluted with hexane as needed to obtain an absorbance between 0.2 and 0.8 Au. Five replicates of each sample were analyzed.

The total carotenoid content was calculated using the equation below in which the constant 0.204 is the absorbance at 468 nm of 1 μg/mL of standard canthaxanthin:

$$Carotenoid\ concentration\ \left(\frac{\mu g}{g}\text{lipid}\right) = \frac{\text{A468} * \text{volume (10mL)} * \text{dilution factor}}{0.204 * \text{weight of oil/lipid sample used in gram}}$$

4.3.4 Statistical Analysis

A graph was constructed to evaluate the relationship between the total carotenoid content determined using the UV spectrophotometric and HPLC methods. This was accomplished by plotting the values obtained from each method for samples containing 20, 40, 60, 80 and 100 % SCVL (from winter and spring) diluted in fish oil. Type II regression was used to fit the data and the correlation coefficient R^2 was obtained. Statistical tests were conducted using Minitab 17. A

two-sample *t* test was used to determine differences in carotenoid content and the % ratio of concentration from UV: concentration from HPLC between winter and spring harvests. Significance was determined at a level of $p < 0.05$.

4.4 RESULTS

HPLC analysis of SCVL revealed eleven peaks eluting between 5 and 35 min (Fig 4.1). Peaks 1 (7.92 min), 2 (11.82 min) and 6 (18.58 min) were initially identified as neoxanthin, astaxanthin and canthaxanthin respectively, based on the retention times obtained from injections of the corresponding standards. Co-injection of neoxanthin, astaxanthin and canthaxanthin standards with SCVL resulted in an increase in height of the corresponding peaks, further supporting the presence of these carotenoids. Comparison of the UV spectra of the standards with those of the corresponding peaks showed agreement in terms of λ_{max} values for neoxanthin, astaxanthin and canthaxanthin. UV spectra and/or retention times of fucoxanthin, violaxanthin, lutein, cucumariaxanthin A, B and C, β-cryptoxanthin and β-carotene, which had previously been identified in other sea cucumber species, were used to show that these carotenoids were absent in the HPLC chromatogram of SCVL.

Using HPLC analysis, the total carotenoid content (μg/g lipid) in lipids extracted from SCV from the winter harvest was less and significantly different ($p < 0.05$) than that determined for the spring harvest (Table 4.2). The concentration of canthaxanthin was significantly greater ($p < 0.05$) in the spring harvest than in the winter harvest.

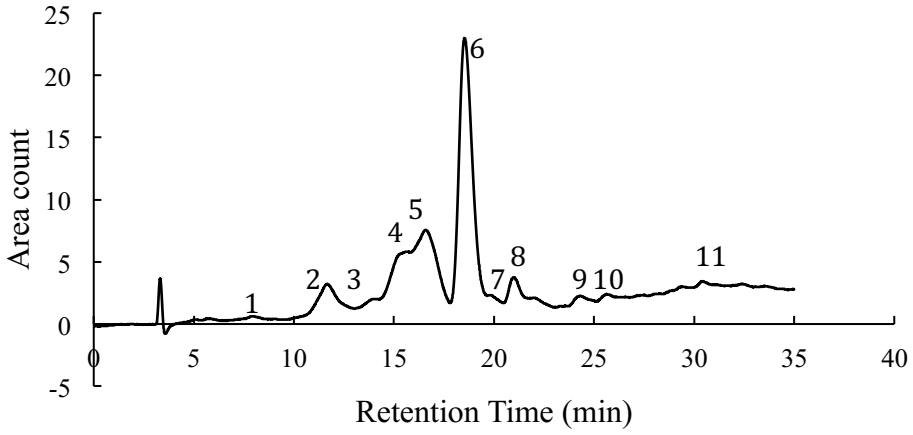


Figure 4.1 HPLC chromatogram of SCVL carotenoids. Peaks 1, 2 and 6 were identified as neoxanthin, astaxanthin, and canthaxanthin, respectively.

Table 4.2 The carotenoid content (μg/g lipid) using HPLC analysis. Values in the same rows with different letters are significantly different (two sample *t* test; $p < 0.05$) (mean +/- SD; n=3).

Carotenoid	**Winter**	**Spring**
Neoxanthin (μg/g lipid)	2.8 ± 0.1a	8.5 ± 1.0b
Astaxanthin (μg/g lipid)	146 ± 11a	146 ± 9a
Canthaxanthin (μg/g lipid)	652 ± 5a	732 ± 6b
The sum of unidentified peaks (μg/g lipid)	740 ± 14a	779 ± 33a
Total carotenoid content (μg/g lipid)	1542 ± 12a	1666 ± 14b

Canthaxanthin represented approximately 50 % of the total carotenoid content and was significantly different in the two harvests. Astaxanthin levels were the same in winter and spring

harvests. The level of neoxanthin detected for the winter harvest was approximately 25 % of that determined for the spring harvest. No significant difference ($p > 0.05$) was observed between the sum of the unidentified peaks in both seasons. However, relative to viscera tissue mass (Table 4.3), the total carotenoid content, the total unidentified carotenoids, and the concentration of astaxanthin and canthaxanthin were significantly higher ($p < 0.05$) in the winter harvest compared to the spring harvest.

Table 4.3 The carotenoid content (μg/g viscera) using HPLC analysis. Values in the same rows with different letters are significantly different (two sample *t* test; $p < 0.05$) (mean +/- SD; n=3).

Carotenoid	**Winter**	**Spring**
Neoxanthin (μg/g viscera)	0.2 ± 0.01a	0.4 ± 0.03b
Astaxanthin (μg/g viscera)	10.7 ± 0.3a	7.2 ± 0.5b
Canthaxanthin (μg/g viscera)	48.0 ± 1.4a	36.1 ± 2.5b
The sum of unidentified peaks (μg/g viscera)	54.2 ± 1.1a	39.0 ± 3.0b
Total carotenoid content (μg/g viscera)	113 ± 3a	82.2 ± 6.0b

The total carotenoid content of SCVL determined using the spectrophotometric method was 2340 ± 50 and 2500 ± 115 μg/g lipid for winter and spring harvests respectively (Table 4.4). The spectrophotometric method overestimated the carotenoid content relative to the HPLC method by 151 ± 3 % for the winter and 149 ± 6 % for the spring harvests. The seasonal difference was not significant for UV but was for the HPLC method. Results of both methods were highly correlated ($R^2 = 0.99$) (Fig 4.2).

Table 4.4 Total carotenoid content (mean +/- SD; n=3; μg/g) of SCVL samples determined by UV spectrophotometric and HPLC methods. Values in the same rows with different letters are significantly different ($p < 0.05$).

Method	Winter	Spring
UV (μg/g of SCVL)	2340 ± 50a	2500 ± 115a
HPLC (μg/g of SCVL)	1540 ± 10a	1670 ± 50b
% ratio of concentration from UV: concentration from HPLC	151 ± 3a	149 ± 6a

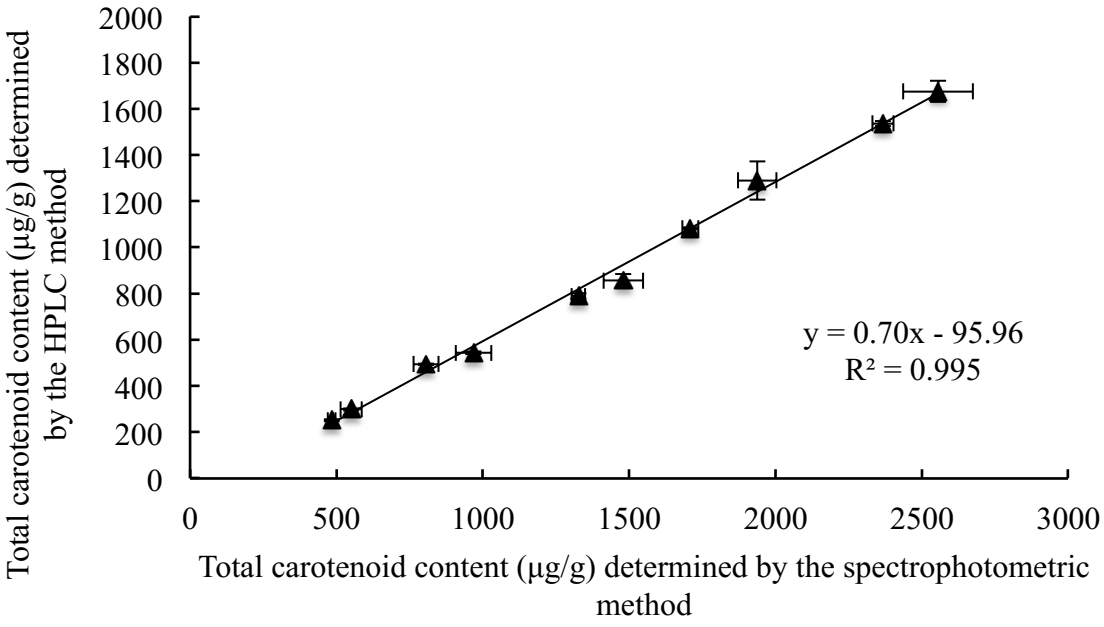


Figure 4.2 Correlation between total carotenoid content determined by UV spectrophotometric and the HPLC method (mean +/- SD; 10 samples on each axis with n=5 for each sample determined by spectrophotometric method and with n=3 for each sample determined by the HPLC method).

4.5 DISCUSSION

4.5.1 Carotenoids in SCVL

4.5.1.1 Carotenoid Content in SCVL

The total carotenoid content of SCVL using HPLC and spectrophotometric analyses was ~1600 and 2500 μg/g of lipid extract, respectively. Comparison of values that were obtained using the spectrophotometric method showed that the SCVL value was much higher than that reported for the total carotenoid content of oil extracted from shrimp (*Feneropenaeus indicus*) waste (200 μg/g lipid) (Sachindra and Mahendrakar, 2010), and farmed Atlantic salmon (*Salmo salar*) (11-44 μg/g lipid) (Saito 1969). The concentrations, however, were comparable to that determined for pigmented oil extracted from Pacific white shrimp *(Litopenaeus vannamei)* (~3000 μg/g lipid) (Takeungwongtrakul et al., 2012) and krill oil (*Euphausiacea sp.)* (878 to 1016 μg/g oil) (Fisher et al., 1955).

Canthaxanthin was the major carotenoid present in SCVL (Table 4.2) and represented 43 % of the total carotenoid content. Its presence was expected as Findlay et al. (1983) identified it in whole body of *C. frondosa* collected in Passamaquoddy Bay, New Brunswick and Pelletier and Mamelona (2011) identified it in the gut of the same species collected in the Maine coastal area of the United States and the Bay of Fundy/ Canada. Canthaxanthin has been also identified in different body parts of other species of sea cucumber including the gonads of *H. leucospilota* and *Stichopus japonicus* (Matsuno et al., 1971), and the red body wall of *P. fabrichii* (Bullock and Dawson, 1970).

Other marine genera including the Mollusca, Arthropoda (Crustaceans) and Echinodermata have been reported to also contain canthaxanthin (Maoka, 2011). It is, however, interesting to note that canthaxanthin is absent in krill (*Euphausia superba*) (Grynbaum et al.,

2005) but was detected in trace amounts in shrimp by-product *(Pandalus borealis)* (Shahidi and Synowieckit, 1991). Canthaxanthin has been detected in some *Chlorella*, *Scenedesmus*, and *Spirulina* species of microalgae (Becker, 2013; Habib et al., 2008) but in relatively low levels compared to other carotenoids present; lipid produced from these species therefore would be expected to contain low levels of canthaxanthan if any at all. The concentration of canthaxanthin in farmed salmon was reported to be 11 mg/kg muscle (Saito and Rigier, 1971), which is well below the levels of canthaxanthin in SCV in this study (30-50 mg/kg SCV; Table 4.2). The low concentrations of canthaxanthin in most organisms in the aquatic environment may occur because canthaxanthin is an intermediate in the metabolism of β-carotene to astaxanthin (Goodwin, 1984). The presence of canthaxanthin in SCVL is therefore a feature that distinguishes it from krill and salmon oils.

Astaxanthin was present at approximately one fifth the concentration of canthaxanthin (146 μg/g lipid); Table 4.2). Like canthaxanthin, astaxanthin has been previously reported in *C. frondosa* (Pelletier and Mamelona, 2011) and in other sea cucumber species including *H. leucospilota* and *S. japonicus* (Matsuno et al., 1969). It has been identified in many other marine animals including jellyfish (Maoka et al., 2010), starfish (Matsuno, 1989), octopus and cuttlefish (Maoka et al., 1989), and crustaceans (Liaaen-Jensen, 1998; Matsuno, 2001). Astaxanthin levels in SCVL were above those observed for lipid extracts of wild (40-62 μg/g lipid) and farmed shrimp (10-21 μg/g lipid) (*Penaeus monodin*) (Latscha, 1989) but five times lower than the astaxanthin levels (including free and esterified forms) found in krill oil (727 μg/g oil) (Lambertsen and Braekkan, 1971).

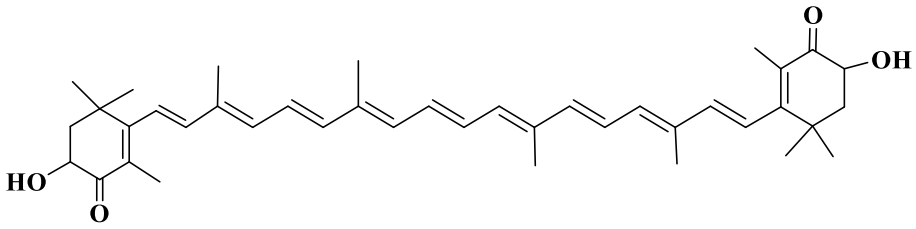
In vitro, astaxanthin has been reported to exhibit 10-times greater antioxidant activity than canthaxanthin, zeaxanthin, lutein and β-carotene, and 100-times greater antioxidant capacity

than α–tochopherol (Miki, 1991). However, canthaxanthin has been shown to exhibit a higher level of antioxidant activity than β-carotene and zeaxanthin (Liebler, 1993) and higher stability towards photodegradation than β-carotene (Delgado-Vargas et al., 2000). Astaxanthin and canthaxanthin (Fig 4.3 A and B) belong to a class of oxygenated forms of carotenoids called xanthophylls (Zaripheh and Erdman, 2016) but astaxanthin is more polar than canthaxanthin as it contains two hydroxyl groups attached to the 2 β-ionone rings (Cote, 2000). This difference in structure offers an explanation for the differences in biological activity observed between these two carotenoids.

Canthaxanthin and astaxanthin in this study comprised > 50 % of the total carotenoid content of the lipid extract. This was expected as the two carotenoids made up 90 % of total carotenoids in the body wall, ovaries and viscera of *Holothuria atra* (Bandaranayake and Des Rocher, 1999). These oxygenated carotenoids, that are obtained directly from the sea cucumber's diet or by metabolism of β-carotene obtained from their diet, are concentrated in the ovaries, gonads, body walls and (Bandaranayake and Des Rocher, 1999).

To date, neoxanthin has not been reported in other sea cucumber species but was a minor contributor to the total carotenoid profile of SCVL here. Neoxanthin is one of the primary carotenoids that capture light in plants during photosynthesis (Lichtenthaler, 1987). During the feeding period of *C. frondosa* in spring, the animal may acquire this carotenoid as a result of consumption of microalgae.

A



B

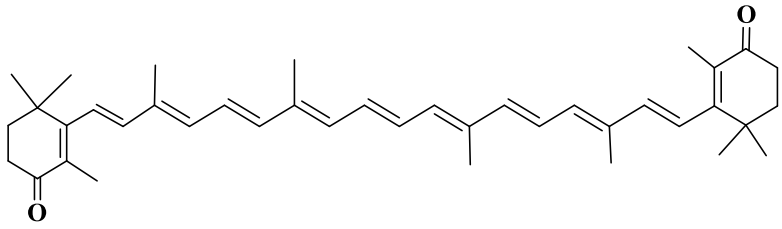


Figure 4.3 Chemical structures for astaxanthin (A) and canthaxanthin (B).

Using the carotenoid values obtained for SCVL, the levels of carotenoids in *C. frondosa* viscera were estimated (82-113 µg/g viscera; Table 4.3) to be higher than those found in Antarctic krill (30-40 µg/g tissue; Yamaguchi et al., 1983). Eighty five to 95 % of the carotenoid content of krill tissue consists of astaxanthin (Yamaguchi et al., 1983). This is quite different from *C. frondosa* viscera that contains a more diversified carotenoid profile that may be marketed as more advantageous for human and animal health applications and oil stability.

When the SCVL extract was exposed to different oxidation conditions (Chapter 2), very low initial PV measurements were obtained that did not increase upon long exposure to oxidative conditions. Carotenoids have the ability to quench and suppress oxygen radical species generated in photooxidation (von Elbe and Schwartz, 1996). The carotenoids, specifically astaxanthin and

canthaxanthin, in SCVL may act as endogenous antioxidants and offer an explanation for the stability of SCVL in oxidation studies monitored by PV measurements.

Several potential health benefits are associated with these two carotenoids. For instance, both astaxanthin and canthaxanthin have been reported to be precursors of 3,4-dehydroretinol (Vitamin A_2) (Matsuno, 1991) and retinol (Vitamin A_1) (Schiedt, 1998), respectively, where their conversion was observed in freshwater fish and Salmonidae. In humans, astaxanthin plays several beneficial roles in cell membranes, such as an inhibitor of PUFA oxidation, a protector against UV-light photooxidation in skin cells, a controller of carcinogenic process and an agent of slowing aging (Guerin et al., 2003; Barros et al., 2011). Both astaxanthin and canthaxanthin were reported also to exhibit anti-inflammatory protection in human cells (Chan et al., 2009).

4.5.1.2 Carotenoid Profile of Sea Cucumbers

Using HPLC analysis I was able to confirm the presence of canthaxanthin, astaxanthin and neoxanthin in SCVL prepared from *C. frondosa* but were unable to identify the remaining peaks in the chromatogram based on the carotenoids which had been previously reported in a number of sea cucumber species. The unidentified peaks that made up at least 48 % of the total carotenoid content may be mono- and di-esterified forms of canthaxanthin and astaxanthin as well as esters of carotenoids which have been previously identified in sea cucumber species. Saponification of the SCVL followed by HPLC analysis or LC-MS-MS analysis of SCVL could be used to confirm their assignment.

Many of the carotenoids observed in other sea cucumber species, including lutein, β-carotene, fucoxanthin, violaxanthin, and β-cryptoxanthin, and cucumariaxanthin A, B and C, were not detected in the SCVL used in this study. For instance, Collin (2000) identified astaxanthin, canthaxanthin, cucumariaxanthin and zeaxanthin in an acetone extract of the gut of

C. frondosa, while Matsuno and Tsushima (1995) found echinenone and cucumariaxanthin A, B and C, in *C. japonica*, a related sea cucumber species and β- carotene in the gonads of *H. leucospilota* (Matsuno et al., 1971). In contrast, these carotenoids were not found in the viscera and whole body extracts of a number of other sea cucumber species, including *S. japonicas*, *H. leucospilota*, *Holothuria moebi*, and *Holothuria pervicas.* These differences may point to species-specific variation, variation in diet or may be due to the different chemical extraction procedures used to isolate the carotenoids. Regardless of cause, they indicate a highly variably carotenoid composition within this group of animals.

4.5.1.3 Factors Affecting Carotenoid Content in Sea Cucumbers

4.5.1.3.1 Metabolic Pathways in Sea Cucumbers

In 1995 Matsuno and Tsushima proposed a metabolic pathway for the biosynthesis of carotenoids from the □□carotene that sea cucumbers obtained in their diet (Fig 4.4) (Matsuno et al., 1995). In many aquatic animals, including the orders Aspidochirotida and Dendrochirotida, the typical metabolic pathway begins with β-carotene, involves intermediary oxidation steps containing β-echinenone, canthaxanthin and phoenicoxanthin, and ends with astaxanthin. However, in the order Dendrochirotida in which *C. frondosa* is classified, canthaxanthin is postulated to undergo reduction steps that result in the formation of cucumariaxanthin C via cucumariaxanthin A and B (Matsuno and Tsushima, 1995). Cucumariaxanthin A contributed 52-60 % and 27-55 % to the total carotenoid content of viscera, gonad and body walls of *C. japonica* and *C. echinata*, respectively, whereas cucumariaxanthin B and C combined contributed 9-14 % and 0-3.4 %, respectively (Matsuno and Tsushima, 1995). In SCV extract, canthaxanthin was the major carotenoid present. Cumariaxanthins (A, B, and C) were not detected, but were expected to be present as they had been identified in *C. frondosa* (viscera)

harvested from Atlantic Canada (Pelletier and Mamelona, 2011). The reason for their absence in SCVL in this study is presently unknown.

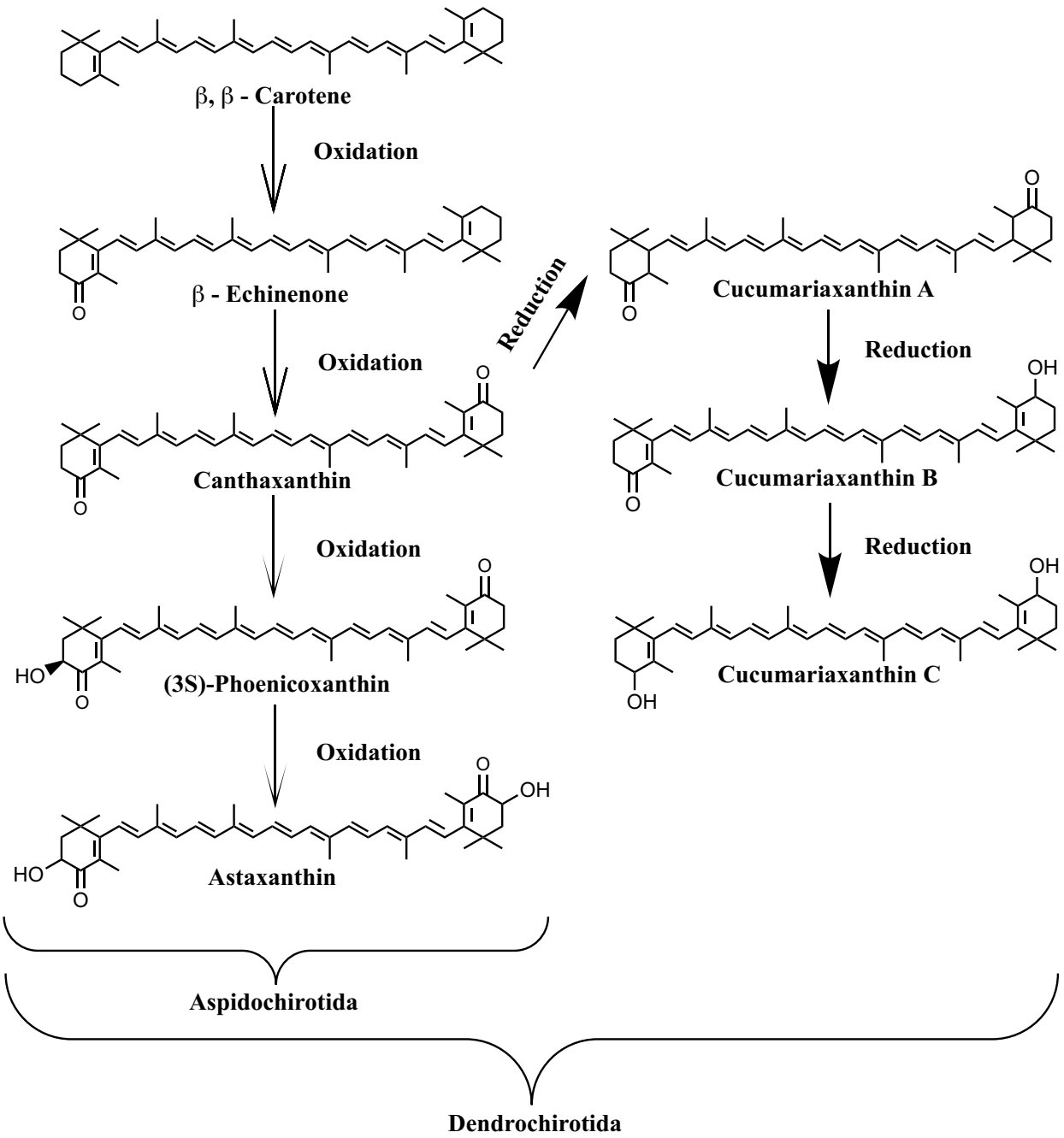


Figure 4.4 Suggested metabolic pathways of carotenoids in sea cucumbers (modified from Matsuno and Tsushima (1995).

4.5.1.3.2 Environmental Effects

Using HPLC analysis, slightly higher levels of carotenoids were observed in SCVL prepared from a spring as compared to the winter harvest (Table 4.2). As mentioned earlier, sea cucumbers accumulate carotenoids in their tissues as a result of feeding on algae and other organisms rich in carotenoids (Liaaen-Jensen, 1998). One factor effecting a seasonal change in carotenoid content might therefore be food availability (Yanar et al., 2004). Also, variation in concentrations of individual and total carotenoids in the same and different sea cucumber species collected in different seasons might be associated with the reproductive status of the sea cucumber (Hamel and Mercier, 1998). The higher levels of carotenoids observed in spring harvests, which occur during gamete production in sea cucumbers, are thought to protect sea cucumber eggs that float to the surface from photooxidation and oxygen toxicity (Matsuno and Tsushima 1995).

4.5.2 Comparison of Total Carotenoid Content Determined by both HPLC and Spectrophotometric Methods

The tendency for the total carotenoid content determined by spectrophotometric methods to overestimate carotenoid content compared to those determined by HPLC has been previously reported (Islam and Schweigert, 2015) but not for sea cucumber. This observation has been explained by the presence of other compounds such as degraded carotenoid and/or chlorophyll products that absorb at the same wavelength as carotenoids (Kimura et al., 2007; Almela et al., 2000). The significant seasonal difference observed from HPLC analysis was obscured in the UV spectrophotometric data by the high variability in its replicates.

The correlation coefficient R^2 (Fig 4.2) of the plot of total carotenoids by the UV spectrophotometric and HPLC methods was >0.99 which indicated a correlation between the

methods and suggested that the UV spectrophotometric method could be used to estimate the total carotenoid content that would be determined by HPLC. While the HPLC method produces a detailed carotenoid profile and quantification of individual carotenoids, it is time consuming and has a high instrumental and operational cost associated with it. Spectrophotometric methods are widely used as they are fast, require low cost instrumentation and do not require a high degree of technical training to operate. The findings in this study suggest that the UV spectrophotometric method could be used in a processing facility to measure the total carotenoid content in SCVL to optimize extraction methods or for quality control.

4.6 CONCLUSIONS

HPLC confirmed the presence of astaxanthin, canthaxanthin, neoxanthin and a number of unidentified peaks in SCVL; it also established the absence of cucumariaxanthins A, B and C, β-cryptoxanthin, lutein and β- carotene. The carotenoid profile of SCVL is distinguished from other pigmented oils such as salmon, shrimp and krill oil as it contains canthaxanthin as the major carotenoid present. The presence of carotenoids in SCVL indicates that it is a rich source of antioxidants that may enhance lipid stability and be marketed as appropriate for human and pet nutrition supplements. The studies that were conducted also showed that the total carotenoid content of SCVL determined by a UV spectrophotometric method correlated well with that determined using an HPLC method. The total carotenoid concentration in SCVL varied seasonally with slightly higher concentration in spring samples, compared to winter. Further work on the carotenoid content in SCVL should focus on the identification and quantification of the unidentified peaks in the SCVL HPLC profile.

CHAPTER 5. VARIATION IN LIPID AND TOTAL CAROTENOID CONTENT OF VISCERA OF *CUCUMARIA FRONDOSA* HARVESTED FROM THE EASTERN COAST OF CANADA

5.1 ABSTRACT

The seasonal variation in the fatty acid (FA) composition and total lipid, moisture, and total carotenoid content (TC) of a lipid extract of the viscera from sea cucumber (*Cucumaria frondosa*) harvested from the Sable Island Banks off of Nova Scotia, Canada was evaluated. The total lipid content was found to vary seasonally from 4.9-7.4 % wet weight of viscera, with the highest lipid content occurring in the winter. All viscera samples contained 74-77 % moisture; the lowest was observed for the winter harvest. Four major lipid classes were identified in the lipid extracts of sea cucumber viscera (SCVL): triacylglycerol (TAG), phospholipids (PL), diacylglyceryl ether (DAGE) and monoacylglycery ether (MAGE). Free fatty acids (FFA) were also present but in trace amounts. DAGE was the predominant lipid class in winter and spring harvests, while TAG was the predominant lipid class present in the summer harvest. The composition of the total FA profile of the lipid extracts of sea cucumber viscera (SCV) varied in composition seasonally. Total polyunsaturated FA (PUFA) varied from 29-32 % by mass of total FA, and was dominated by EPA (~25-28 %); the highest concentration of EPA was observed in the MAGE (> 45 %) and PL (> 30 %) lipid classes. Total branched chain fatty acids (BCFA) ranged from 18 to 31 % and were dominated by 12-methyltetradecanoic acid (12-MTA). BCFA were mostly associated with the DAGE lipid class (~50 %). Total carotenoid content varied from 1.5 to 2.5 mg/g of the lipid extract and was lowest in SCV from the summer harvest. Collectively this information will aid in the development of quality control standards for the development of a SCVL product.

5.2 INTRODUCTION

More than 1250 species of sea cucumber have been identified (Bruckner et al., 2003) that have the ability to adapt to water with varying depths and temperatures. When harvested, sea cucumbers are divided into an outer wall and inner muscle meat, which results in the production of a viscera by-product (Bordbar et al., 2011). Lipid extracts prepared from various body parts of *Cucumaria frondosa* have been reported. For instance, the lipid extracted from the fresh body wall and internal organs of *C. frondosa* harvested near Newfoundland was reported to contain ~43-57 % eicosapentaenoic acid (EPA), ~2.0-5.8 % docosahexaenoic acid (DHA) and between ~2-9 % of the branched chain fatty acid (BCFA), 12-methyltetradecanoic acid (12-MTA) (Zhong et al., 2007). 12-MTA has been shown to inhibit proliferation of cancer cells and the extracellular production of surfactant (Fredalina et al., 1999; Yang et al., 2003; Inoue et al., 2012). Lipid extracted from the fresh body wall and internal organs of *C. frondosa* also contains carotenoids, a class of pigments reported to possess antioxidant properties (Zhong et al., 2007). A number of carotenoids have been identified from extracts of different sea cucumber species such as *Holothuria leucospilota* (Matsuno et al., 1969; Bullock and Dawson, 1970).

Along Nova Scotia and New Brunswick, Canada, the sea cucumber *C. frondosa* is harvested as a by-catch (DFA, 2002). Presently, only the muscular bands and body wall have commercial value, and the viscera, representing 50 % of the sea cucumber biomass, is discarded (Mamelona et al., 2010). There is local interest in producing viscera lipid from the SCV waste stream to develop a nutraceutical product that could compete with other beneficial marine oils but, to date, only a single study has evaluated the compositional content and antioxidant properties of sea cucumber body with and without viscera (Zhong et al., 2007). However, little is known about the lipid class and total carotenoid (TC) content of SCVL; such information is

critical for the development and marketing of SCVL. Most marine invertebrate species show seasonal variation in those parameters; thus, this research focused on evaluation of seasonal variation in lipid class, fatty acid and carotenoid content of SCV of *C. frondosa* harvested off the coast of Nova Scotia.

5.3 EXPERIMENTAL PROCEDURES

5.3.1 Materials and Methods

The following standards were purchased from Sigma Aldrich (Canada): arachidic acid stearyl ester (wax ester; WE), 1-*O*-hexadecyl-2,3-hexadecanoyl glycerol (diacylglyceryl ether; DAGE), tristearin (triacylglycerol; TAG); 1,2-distearine (a diacylglycerol; DAG), 1-monostearin (a monoacylglycerol; MAG), stearic acid (a free fatty acid; FFA), phosphatidyl choline dipalmitoyl (PL), cholesterol (sterol), 2,6-di-tert-butyl-4-methylphenol (BHT). TLC plates (20 X 20 cm, DC-Fertigplatten Kieselgel 60 F-254, layer thickness 0.25 mm) were purchased from Merck (Canada). All solvents (Optima grade) were purchased from Fisher Scientific (Ottawa, ON, Canada). Reverse osmosis (RO) water was used for SCVL extractions. All spectrophotometric data were collected using a UV spectrophotometer (Novaspec II, LKB Biochrom, Holliston, MA, USA). Absorbance measurements were made at 468 nm using a 1-cm path amber glass cuvette.

Harvests of sea cucumber viscera from *C. frondosa* were conducted in January 2015 (winter; latitude 4439/ longitude 6041), March 2015 (spring; latitude 4439/ longitude 6040) and July 2015 (summer; latitude 4439/ longitude 6041) from the Sable Island Banks off Nova Scotia, Canada by Ocean Pride Fisheries Limited (Lower Wedgeport, NS). The viscera sample obtained

from each harvest timepoint was a pooled sample. The samples were stored in three boxes (each with a dimension of 24, 18, 15 cm), weighing 1.5 kg each, at -30 °C prior to evaluation.

Comparisons in lipid characteristics were made between the three harvest dates, corresponding to different reproductive states and levels of feeding activities (Table 5.1).

Table 5.1 Sea cucumber harvests (*C. frondosa*) summarized according to anticipated reproductive and feeding status.

Biological behavior	**January 2015 (Winter)**	**March 2015 (Spring)**	**July 2015 (Summer)**
Feeding*	Non-feeding	Feeding	Feeding
Reproductive**	Pre-spawning	Early spawning /spawning	Post-spawning
Gonad development***	Partially developing	Partially developing	Developing

* (Singh et al., 1999; Hamel and Mercier, 1998); **(Coady, 1973; Singh et al., 1999; Hamel and Mercier, 1996a,b, c); ***(Singh et al., 1999)

5.3.2 Total Lipid and Moisture Content Determination

Extraction of SCV was described in Chapter 2, section 2.3.2 in this thesis. The total lipid content was determined gravimetrically after extraction and expressed as mass percent of SCV. The moisture content was determined by drying ~25 g of homogenized SCV at 100°C (AOCS, 1990) to a constant mass. Three lipid extracts for each collection timepoint were obtained by sampling the SCV in each box from three locations: the two sides and the center of the storage box.

5.3.3 TLC Analysis of Lipid Classes

The TLC analysis was carried out as described in Chapter 3; section 3.3.4.

5.3.4 GC-FID Analysis of FAME

The GC-FID analysis was carried out as described in Chapter 3; section 3.3.7

5.3.5 Identification of BCFA Using GC-MS

The GC-MS analysis was carried out as described in Chapter 3; section 3.3.8

5.3.6 Total Carotenoid Determination Using Spectrophotometer

Total carotenoid content was determined using the method by Takeungwongtrakul et al. (2012), which was a modified version of the method published, by Saito and Regier (1971). SCVL (30 mg) was dissolved in 10 mL hexane and allowed to stand for 30 min at room temperature. The absorbance was read at 468 nm using a 1-cm path amber glass cuvette. Dilutions with hexane were performed when needed. Total carotenoid content (concentration) was calculated using the following modified equation suggested by Takeungwongtrakul et al. (2012):

$$Carotenoid\ concentration\ \left(\frac{\mu g}{g}\text{lipids}\right) = \frac{\text{A468} * \text{volume (10 mL)} * \text{dilution factor}}{0.204 * \text{weight of oil/lipid sample used in gram}}$$

where 0.204 is the measured A_{468} of 1 μg/mL of a canthaxanthin standard.

5.3.7 Statistical Analysis

All samples were run in triplicate and all data were analyzed using one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$ (Minitab 17). Tukey's multiple comparison test was used when significance was found to determine differences between means of the three harvests (significance was determined at a level of $p < 0.05$).

5.4 RESULTS

5.4.1 Total Lipid, Moisture Content and Total Carotenoid Content

The total lipid content of SCV ranged from 4.93 to 7.36 % lipid on a wet weight basis (wwb) of SCV over the three collection time points with the winter harvest showing significantly higher levels (ANOVA; $p < 0.05$) than that observed for spring and summer harvests (Table 5.2). Comparison on a dry weight basis (dwb) demonstrated the same pattern, with lipid content varying between 20.81-28.87 %, with winter samples having the highest values (Table 5.2). The moisture content of SCV varied between 74.47 and 77.75 % with the winter harvest showing the lowest levels. No significant difference ($p > 0.05$) in total lipid and moisture content was observed between spring and summer harvests of SCV. Total carotenoid content varied from ~1.6-2.5 mg/g lipid extract for the three harvest time points. Total carotenoids differed significantly ($p < 0.05$) among the three harvest times with highest concentrations occurring in spring (2.50 ± 0.12 mg/g) and lowest in the summer (1.63 ± 0.06 mg/g).

Table 5.2 Seasonal variation in total lipid and moisture content of SCV and total carotenoid content of SCV lipid extract (n=3; mean +/- SD). Values in the same row with different letters are significantly different ($p < 0.05$).

Component	Winter	Spring	Summer
Total lipid content (% lipid (wwb))	7.36 ± 0.21a	4.93 ± 0.34b	5.30 ± 0.29b
Total lipid content (% lipid (dwb))	28.87 ± 1.92a	20.81 ± 1.22b	23.83 ± 1.46b
Moisture content (%)	74.47 ± 1.09b	76.29 ± 0.08a	77.75 ± 0.37a
Total carotenoid Total carotenoid (mg/g lipids)	2.32 ± 0.04b	2.50 ± 0.12a	1.63 ± 0.06c

5.4.2 Lipid Classes and FAME

Visualization of TLC plates after separation of lipid extracts showed the presence of five bands in addition to un-recovered pigments. These bands included PL, TAG, DAGE, MAGE and a faint FFA band. MAG and DAG were not detected by TLC analysis; they however were detected using HPLC and GC/MS analysis (Chapter 3). The main lipid class in SCV harvested in the winter and spring was DAGE which represented ~50 % and ~40 % of total recovered lipids respectively (Fig 5.1). TAG was the main lipid class found in the summer and represented ~45 % of total recovered lipids. Polar lipids including PL represented ~20 % of total recovered lipids in the three harvests and MAGE represented ~10 % of the total in the spring harvest. Cholesterol was not detected in the three harvests.

The total PUFA varied between 29-32 % by mass of total FAME identified; the highest levels were observed in the summer harvest (Table 5.3; full FA profiles in Appendix A1). The weight percentage of EPA in the total recovered lipid in the three harvests was ~24-28 % where the levels were significantly higher ($p < 0.05$) in summer. No significant difference ($p > 0.05$) was observed between proportions of DHA or total PUFA excluding EPA and DHA (~4 %) over the three seasons.

Total BCFA varied between 18-31 % and included (4,8,12-Me-13:0), (*i*-15:0), (8,12-Me-14:0), (12-Me-15:0), 8,12-Me-15:0), (15-Me-16:0), (14-Me-16:0), and branched FA containing 15 carbon atoms (Me-14:0a, Me-14:0b, Me-14:0c). The weight percentage of 12-MTA varied significantly ($p < 0.05$) among the three harvests, with the highest proportion (~21 %) occurring in the winter and the lowest proportion (~11 %) in the summer. The proportions of BCFA excluding 12-MTA followed a similar pattern and were significantly lower in summer (winter and spring harvests ~9 % vs summer harvest ~6 %).

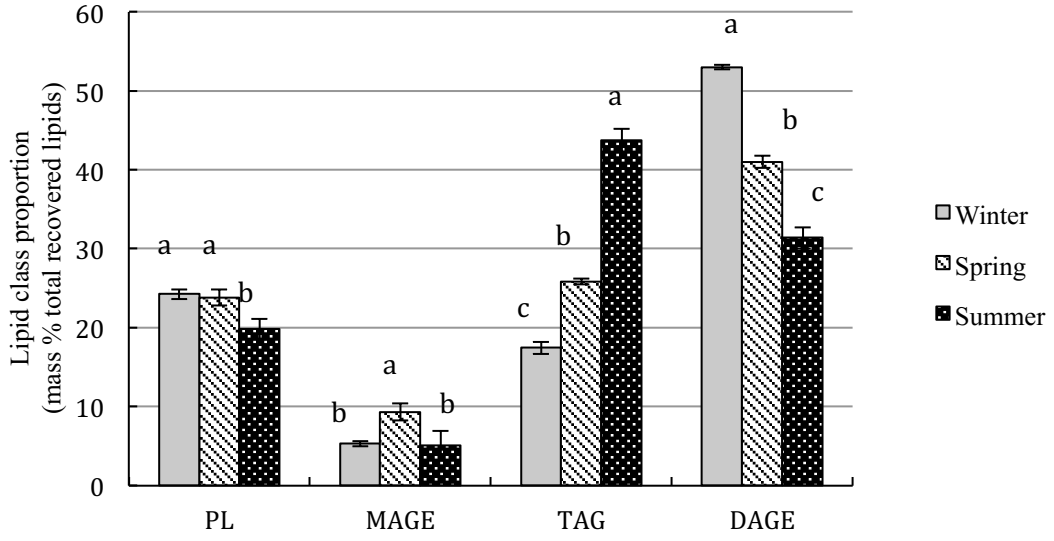


Figure 5.1 The mass percentage of each lipid class relative to total lipids recovered from TLC plates for SCVL (mean +/- SD, n=3). Values with different letters for each series are significantly different (ANOVA; $p < 0.05$). The percentage of FFA was below detection limits.

Table 5.3 Seasonal variation in the major FA (weight percentage of total FAME identified; mean +/- SD; n=3) in the total lipids of SCV of *C. frondosa.* Values in the same row with different letters are significantly different ($p < 0.05$).

Fatty acid	Winter	Spring	Summer
EPA	24.74 ± 0.68b	25.38 ± 0.18b	28.23 ± 0.40a
DHA	0.73 ± 0.01b	0.84 ± 0.02a	0.00 ± 0.00c
Σ PUFA without EPA & DHA	4.08 ± 0.09a	4.56 ± 0.21b	3.97 ± 0.14a
Σ PUFA	29.56 ± 0.62a	30.78 ± 0.27b	32.20 ± 0.42c
Σ MUFA	23.37 ± 0.20a	23.58 ± 0.55a	30.18 ± 0.35b
Σ Saturated FA	4.32 ± 0.05b	4.45 ± 0.18b	7.70 ± 0.22a
12-MTA	21.27 ± 0.77a	19.17 ± 0.93b	11.79 ± 0.78c
Σ BCFA without 12-MTA	9.89 ± 0.13a	8.67 ± 0.44b	6.42 ± 0.37c
Σ BCFA	31.13 ± 0.75a	27.84 ± 0.94b	18.21 ± 0.87c
Others	11.64 ± 0.35b	13.35 ± 0.27a	11.74 ± 0.65b

5.4.3 FA Composition of Lipid Classes

Total PUFA was highest in PL and MAGE (Table 5.4; Appendix A2) with the highest levels occurring in the winter and spring harvests. EPA was present at levels > 50 % in MAGE and between 30-47 % in the PL lipid class. The lowest level of EPA (~6 %) was found in DAGE. EPA levels were significantly greater ($p < 0.05$) in winter and spring (~ 46 %) than in summer (~30 %) in the PL lipid class. MAGE EPA was significantly lower in the summer harvest than the other seasons. DHA was detected at < 2 % in all lipid classes. The levels of PUFA, excluding EPA and DHA, were 2-9 % in the four lipid classes, and the highest proportion (~9 %) was observed in PL of the spring harvest. The lowest level (~2 %) was detected in DAGE.

Total BCFA were present at levels greater than 50 % in DAGE, with the highest occurring in winter harvest (~59 %). The dominant BCFA in MAGE, TAG and DAGE was 12-MTA (Table 5.4). It was present between 36 and 41 % in DAGE where the highest proportion was found in the winter and spring (40 %) compared to 36 % in the summer harvest. The levels of BCFA excluding 12-MTA were 1-17 % in the four lipid classes. The highest levels (~17 %) were in DAGE in both winter and spring harvests. The lowest levels of BCFA were observed in PL (1-4 %); 12-MTA was only found in trace amounts in PL. The winter harvest contained the lowest levels of BCFA in the PL lipid class.

Table 5.4 Seasonal variation in the major FA (weight percentage of total FAME identified, mean +/- SD, n=3) in lipid classes of viscera of *C. frondosa)*. Values in the same rows for each three harvests (winter, spring and summer) with different letters are significantly different (ANOVA; $p < 0.05$).

	PL			MAGE			TAG			DAGE		
Fatty acid	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
EPA	47.80 ± 2.61a	46.01 ± 0.60a	30.66 ± 0.36b	57.67 ± 0.31a	57.87 ± 0.25a	45.06±0.66b	16.43 ± 0.27b	13.84 ± 0.81c	19.46 ± 0.43a	5.21 ± 0.28c	6.21 ± 0.84b	8.85 ± 0.21a
DHA	1.53 ± 0.22a	1.44 ± 0.09a	0.97 ± 0.23b	0.99 ± 0.02a	1.04 ± 0.06a	0.91±0.04b	0.90 ± 0.07a	0.84 ± 0.03a	0.76 ± 0.02b	0.30 ± 0.02b	0.39 ± 0.08a	0.42 ± 0.01a
Σ PUFA without EPA+DHA	7.92 ± 0.24b	9.31 ± 0.15a	7.10 ± 0.38c	2.65 ± 0.1a	2.66 ± 0.15a	2.81±0.26a	5.15 ± 0.66a	5.05 ± 0.23a	5.11 ± 0.20a	1.84 ± 0.06a	1.96 ± 0.07a	1.99 ± 0.19a
Σ PUFA	57.25 ± 2.09a	56.76 ± 0.54a	38.73 ± 0.54b	61.31 ± 0.36a	61.56 ± 0.30a	48.78±0.72b	22.48 ± 0.70b	19.73 ± 0.76a	25.33 ± 0.45c	7.35 ± 0.27a	8.55 ± 0.76b	11.26 ± 0.26c
Σ MUFA	15.77 ± 0.56a	23.66 ± 0.82b	38.47 ± 1.24c	21.51 ± 0.54a	21.86 ± 0.76a	31.76±0.70b	31.36 ± 0.42a	32.72 ± 0.40b	37.87 ± 0.86c	26.31 ± 0.51a	26.86 ± 0.40a	29.62 ± 0.83b
Σ Saturated FA	18.02 ± 0.84c	9.18 ± 0.26a	10.80 ± 0.68b	1.92 ± 0.07a	2.00 ± 0.17a	5.25±0.36b	10.38 ± 0.24a	11.61 ± 0.35b	14.05 ± 0.52c	2.05 ± 0.09a	3.34 ± 0.23b	4.61 ± 0.85c
12-MTA	0.42 ± 0.03a	0.27 ± 0.03b	0.32 ± 0.10b	4.48 ± 0.18a	4.60 ± 0.26a	4.90±0.27a	18.80 ± 0.89a	19.53 ± 0.96a	9.66 ± 0.72b	41.75 ± 0.61a	40.62 ± 0.87a	36.11 ± 0.12b
Σ BCFA without 12-MTA	1.14 ± 0.09c	2.64 ± 0.67b	5.53 ± 0.29a	3.50 ± 0.34a	3.41 ± 0.24a	5.15±0.18b	11.13 ± 0.40a	10.79 ± 0.61a	7.06 ± 0.37b	17.24 ± 0.45a	15.36 ± 0.54c	13.33 ± 0.34b
Σ BCFA	1.56 ± 0.10a	2.91 ± 0.67b	3.84 ± 0.30c	7.98±0.38a	8.01 ± 0.34a	10.05±0.29b	29.93 ± 0.83a	30.33 ± 1.06a	16.72 ± 0.75b	58.99 ± 0.72b	55.98 ± 0.96a	49.43 ± 0.42c
Others	7.42 ± 0.53a	7.51 ± 0.57a	8.15 ± 0.51a	7.28±0.40a	6.56 ± 0.41a	4.16±0.58b	5.65 ± 0.16a	5.62 ± 0.14a	6.02 ± 0.28b	5.30 ± 0.15a	5.26 ± 0.22a	5.08 ± 0.36a

5.5 DISCUSSION

5.5.1. Variation in Lipid Composition with Environmental and Biological Conditions in *C. frondosa*

Changes in food availability surrounding an animal and its biological reproductive status affect its biochemical composition, including lipid content, lipid classes, FA profile and total carotenoid content (Annous et al., 1997; Bandarra et al., 1997). The high lipid content (associated with lower moisture content) in winter as compared to the other two harvest time points might be due to the need of the sea cucumber to accumulate energy, in the form of lipids, to allow for its survival during the non-feeding period in winter (David and MacDonald, 2002). When the temperature changes around an organism, the physical state of the organism's membrane adjusts to accommodate this change so that the membrane can function properly (Annous et al., 1997). Cholesterol normally regulates this function but when there is a deficiency or no cholesterol, such as in bacteria (de Mendoza and Cronan, 1983; Suutari and Laakso, 1994), and in the SCVL, PL (Bandarra et al., 1997), FA (Annous et al., 1997) and carotenoids (Chintalapati et al., 2004) regulate this function.

The highest levels of BCFA (~31 %) and 12-MTA (~21 %) in the total lipids of SCV of *C. frondosa* were detected in the winter (Table 5.3). These levels were associated with the DAGE lipid class (Table 5.4). It has been reported that BCFA are involved in the fluidity in bacterial membranes, and their concentration is associated with environmental temperature (Annous et al., 1997; de Mendoza and Cronan, 1983). To maintain cell membrane fluidity at low temperatures, an increase in the concentration of FA that have low melting points such as 12-MTA and PUFA is therefore expected. In this study, BCFA proportions excluding 12-MTA (Table 5.3) were also

found to be the highest in winter and spring. These results indicated that FA other than 12-MTA were involved in increasing the percentage of BCFA in winter and spring harvests.

The lipid content in this study was highest in the winter, which corresponded with the pre-spawning phase of sea cucumber (Hamel and Mercier, 1996a). It is unlikely that seasonal differences in total lipid content is related to the lipid content of sea cucumber gonads as their lipid content does not vary over the year in *C. frondosa* (David and MacDonald, 2002). However, David and MacDonald (2002) did report higher lipid content in female gonads than in male gonads, which was possibly related to lipid nutrition in developing eggs. The percentage of male and female viscera is unknown in the samples in this study but an unequal distribution in the winter samples may have contributed to the higher lipid content in that season.

Proportions of TAG were the highest in the summer harvest which corresponded with the post-spawning period of sea cucumber (Table 5.1). This phase requires a higher storage of energy than the pre-spawning period (Jayasinghe et al., 2003). These high levels of TAG might also be associated with the type of food that is available to sea cucumber at this time (Kayama et al., 1969, 1971). In this study, while total lipid increased in the winter, TAG was not observed to accumulate during this period. Instead, elevated levels of DAGE were observed, which suggests that DAGE might be in some way associated with the pre-spawning period. A noticeable drop in total PUFA was observed when oyster gonads were in the development stage (Mclean and Bulling, 2005), which might explain the lower levels of PUFA observed at this time point (winter) in the SCV lipid extract (Table 5.1).

The differences in carotenoid levels that were observed in this study may be associated with the reproductive state and food availability of the sea cucumber at different times of the year. The winter and spring harvests are believed to contain higher levels of carotenoids as

carotenoids are thought to protect the eggs in sea cucumbers from photooxidation and oxygen toxicity (Matsuno and Tsushima 1995) during spawning (DFO, 2009). The low levels of total carotenoids in the summer harvest (~1.5 mg/g) (Table 5.2) may reflect the correspondingly low levels of carotenoids that are available in the food sea cucumber are consuming at this time. Marine invertebrates do not have the ability to synthetize carotenoids (Liaaen-Jensen, 1998) or modify them through metabolic reactions (Matsuno, 2001), which means that they accumulate carotenoids in their tissues as a result of feeding on algae and other predators rich in carotenoids (Liaaen-Jensen, 1998). Hamel and Mercier (1998) have reported that *C. frondosa* in St Lawrence/ Eastern Canada feed on algae, phytoplankton and other suspended particles. Carotenoid content and concentration in algae vary seasonally (Yanar et al., 2004), which in turn might cause variation in concentration and content of carotenoids accumulated in the tissues of marine invertebrate.

5.5.2 Marketable Features of SCVL

5.5.2.1 Lipid Content

Total lipid content in the viscera of the three sea cucumber harvests was far above the total lipid content of freeze-dried SCV (~2 %) of *C. frondosa* collected from Rimouski, Quebec, Canada (Mamelona et al., 2010). However, the total lipid content determined in this study were lower than lipids in some fish by-products, including Catfish viscera (33 % wwb) and the liver of cold-water fish species such as Pacific halibut (8 % wwb), but within the same range of fish viscera of cod and salmon (2-8 % wwb) (Sathivel et al., 2002; Bechtel and Oliveira, 2006; Bechtel, 2003). The SCV lipid content levels in this study were higher than the total lipid content reported for other marine commercial oils extracted from Northern and Antarctic Krill (2-5 %) (Mayzaud et al., 1999, Phleger et al., 2002; Martin, 2007), and pink salmon viscera and liver (2-3

%) (Bechtel, 2003; Bechtel and Oliveira, 2006). The lipid extracted from the viscera by-product obtained from processing of *C. frondosa* may therefore be used for commercial products.

5.5.2.2 Fatty Acid Composition

The total PUFA levels in the lipid extract in this study (~30 % of total FA) were higher than the PUFA levels determined for other marine by-products such as herring processing waste (18 %), mackerel and Atlantic salmon viscera (11-28 %), and closely comparable to liver of some Alaska fish (16-36 %) and North Atlantic squid (~35 %) (Aidos et al., 2003; Zuta et al., 2003; Bechtel and Oliveira, 2006; Sun et al., 2006). When compared to commercial oils, the PUFA content of SCVL was found to be lower than that for Northern krill (53 %), comparable to that of Antarctic krill species (11-45 %) and higher than the PUFA content for menhaden oil (28 %) (Ackman, 1982; Mayzaud et al., 1999; Martin, 2007). The lipid extract from SCV therefore is a rich source of PUFA.

This study showed that EPA was the predominant FA in SCVL at all three harvest time points (~24 – 28 % of the total FA). Zhong et al. (2007) found that EPA (45 % of total FA content) was the main FA in the extract of fresh, whole *C. frondosa* harvested in Newfoundland. When compared with other marine by-products, the proportion of EPA in this studies SCV lipid extracts was higher than those in herring processing waste (5 %), mackerel and Atlantic salmon viscera (6-8 %), livers of some Alaska fish (6-16 %) and North Atlantic squid (~14 %)) (Aidos et al., 2003; Zuta et al., 2003; Bechtel and Oliveira, 2006; Sun et al., 2006). Comparison of the EPA content in SCV lipid extracts to that of commercial marine oils shows that the extracts contained higher levels of EPA than that of Northern krill oil (KO) (15 %), some Antarctic krill species (12-20 %) and menhaden oil (11 %) (Ackman, 1982; Mayzaud et al., 1999, Phleger et al., 2002). Lipid extracts of SCV from *C. frondosa,* therefore, are a source of high levels of EPA

which is important as it has been associated with improvements in cardiovascular and immunological health (Nestel, 2000; Ryan et al., 2010), and has been reported to be involved in blood clotting (Gibson, 1983) and is thought to possess antithrombotic activity (Croft et al., 1987).

Levels of DHA were detected at a much lower concentrations than EPA in SCV lipid extracts. Similar results were reported by Zhong et al. (2007) when fresh, whole *C. frondosa* harvested in Newfoundland were analyzed. High EPA and a low DHA content is not typically observed in oils prepared from fish species (Mamelona et al., 2010).

Previous to this study, 12-MTA levels in fresh *C. frondosa*, containing its internal organs, were reported to be in the range of 4 % (Zhong et al., 2007). This level is far below the weight percentage of 12-MTA detected in our study which ranged between 11-21 %. BCFA and specifically 12-MTA have a number of promising health benefits including anti-inflammation and anti-cancer activity, and wound healing ability (Collin et al., 2003). High proportions of BCFA and especially 12-MTA in SCV lipid extracts may make them attractive to the nutraceutical market as these unique FA are not found in other marine oils (Gigliotti et al., 2011).

5.5.2.3 Unique Lipid Class Composition

Similar to krill oil, and in contrast to the traditional omega-3 supplements that are based on omega-3 FA associated with TAG (such as cod liver oil and fish oil), high levels of omega-3 FA associated with PL were observed in the lipid extract of SCV in this study. PUFA associated with PL have been reported to have greater health benefit than those associated with TAG (Fukunaga et al., 2016). Burri et al. (2012) reported that PUFA (in particular n-3) associated with PL provided improved metabolic profiles than those associated with TAG. In another study,

consumption of PL in krill oil, which is rich in PUFA (n-3), resulted in greater accumulation of PUFA in the liver, white adipose tissue, and heart tissue in mice than with TAG consumption (Batetta et al., 2009). Also, PL have been shown to exhibit higher antioxidant capacity (Cho et al., 2001) and to be more bioavailable than TAG (Wijendran et al., 2002).

Ether lipids such as 1-*O*-alkylglycerol are considered to be the main constituents in the membranes of marine and some terrestrial animals (Karnovsky and Rapson, 1946). In the last two decades, ether lipids have been used in cancer therapy (Andreesen, 1988; Berdel, 1991; Diomede et al., 1993). Recently, Torres et al. (2009) and Martin et al. (2011) showed that ether lipids improved the bioavailability of other lipid molecules such as butyric acid or omega-3 FA. In this study, it was found that ether lipids, specifically DAGE and MAGE, were abundant in the SCV lipid extracts; ether lipids have not been previously reported in krill oil. The level of DAGE in SCVL (> 50 %) was comparable to that in liver oil of deep-water sharks (79 %) (Deprez et al., 1990). The biological role of ether lipids remains unknown (Hayashi and Kishimura, 2002).

Cholesterol was not detected in SCVL in its free form. Echinodermata including Holothuroidae, similar to many other invertebrates (Crustaceans for example) are unable to synthesize cholesterol *de novo* (Goad, 1981). The levels and types of sterols present can vary greatly in the diet of invertebrates (Véron et al., 1996). Goad (1978) reported that free sterols were detected at very low concentrations in Echinodermata including sea cucumbers. The sterol content of *C. frondosa* is composed of free sterols, sterol glycosides and sterol sulfates in which the sterol sulfate mixture consists mainly of cholesterol and cholestenol (Findlay et al., 1983). Further study of the cholesterol content of *C. frondosa* which focuses on cholesterol sulfate might reveal SCVL to be more favourable to consumers, particularly when relatively high levels of cholesterol have been reported in krill oil (17-76 mg/g oil) (Watanabe et al., 1979).

The trace amounts of FFA, DAG and MAG in the SCV lipid extract indicates that samples were properly stored and extracted. Hydrolysis of FA from the glycerol backbone might be accelerated if animal tissues were allowed to thaw too long prior to extraction, which was not the case in this study. A low level of FFA is a good quality indicator of the lipid as high concentration leads to greater measures of acidity in the lipid.

5.5.2.4 Total Carotenoid Content

The total carotenoid content of SCVL (~1.5-2.5 mg/g lipid) was higher than that reported for oil extracted from shrimp waste (*Feneropenaeus indicus*) (0.2 mg/g lipid) (Sachindra and Mahendrakar, 2010) and Antarctic krill (*Euphausia superba*) (1.1 mg/g lipid) (Kolakowska, 1989). It was, however, comparable to that of the pigmented oil extracted from Pacific white shrimp *(Litopenaeus vannamei)* (~3 mg/g lipid) (Takeungwongtrakul et al., 2012). The presence of carotenoids in the lipid extracted from the viscera of *C. frondosa* is responsible for its red color. SCVL might therefore be marketed in a similar fashion to krill oil as it too has an attractive red color.

5.6 CONCLUSIONS

Information gained from this study is valuable in positioning the lipid prepared from SCV in the market so that it can compete with other commercial marine oils. Lipid extracts from SCV are easily marketable as they contain high levels of PUFA such as EPA that are associated with PL. SCVL is a unique lipid compared to other commercial oils as it contains ether lipids (DAGE and MAGE), which have the ability to improve the bioavailability of other bioactive lipids. Moreover, the lipid extracted from the viscera is an important source of BCFA such as 12-MTA,

which has been shown to exhibit important health benefits. SCV lipid extracts are highly pigmented, as they are rich in carotenoids, which exhibit high antioxidant activity.

Lipids extracted from the viscera of *C. frondosa* harvested during the winter season showed the highest levels of lipid, and carotenoid content, PUFA, EPA, 12-MTA, and BCFA. Knowing the lipid class content and FA profile for each lipid class can aid in the development of quality control parameters for a SCVL product.

CHAPTER 6 CONCLUSIONS

6.1 GENERAL CONCLUSIONS

As SCVL is highly pigmented lipid, a modified FOX method was developed to determine its PV. The strong color in pigmented oils/lipids rich in carotenoids such as SCVL complicates color-dependence analyses such as the iodometric AOCS official method, as well as in other spectroscopy-based methods. The method developed here incorporated a water extraction step after the formation of the XO-complex to remove the carotenoids from the aqueous phase containing the Fe(III)-XO complex. The linearity, accuracy, precision and repeatability parameters were tested for the modified FOX method and data indicated an acceptable validity. This method can therefore be successfully used to determine the PV of pigmented lipids including solvent extracted SCVL. In this study, and while developing this method, SCVL showed resistance to different oxidation conditions. This might be due to the high concentration of carotenoids in this extract (1.5-2.5 mg/g of the lipid extract) that was dominated by a high concentration of canthaxanthin.

This study showed that SCV is a good source of lipids (4.9-7.4 % wwb; 20.8-28.8 % dwb) that contains high levels of PUFA (29-32 % by mass of total FA). The presence of carotenoids in SCVL might suggest that SCVL is a rich source of antioxidants that improve the lipid stability and might be targeted for use in human and pet nutrition supplements, competing with other commercial pigmented oils such as salmon, shrimp and krill oil. The EPA in SCVL was found to be mostly associated with MAGE (> 50 %) and PL (> 30 %). EPA associated to phospholipids have been reported to be more beneficial in terms of health effects than those associated with TAG. Moreover, a number of BCFA, including 12-MTA, were also identified in DAGE of this extract. The ether lipids DAGE and MAGE, which have the ability to improve the

bioavailability of other lipid molecules such as butyric or omega-3 fatty acids, are abundant in SCV lipid extracts. BCFA, specifically 12-MTA have proven to have several health benefits. The highest levels of lipid, carotenoid, PUFA, EPA, 12-MTA, and other BCFA were found in *C. frondosa* from the winter harvest. Winter harvest of *C. frondosa* collected near Nova Scotia represented the best quality in terms of these biochemical constituents.

6.2 FUTURE WORK

In this work, when attempts were to deliberately oxidize SCVL, PV remained surprisingly constant and near zero. Further research on SCVL should investigate the oxidation products produced when SCVL is exposed to different oxidation conditions as hydroperoxides do not seem to be produced when SCVL is oxidized. Further work on the carotenoid content in SCVL by focusing on the assignment of structures to the unidentified peaks in the SCVL HPLC profile is recommended. This might be achieved by conducting in depth LC-MS-MS analysis of the carotenoid profile. Last, lipid content and composition may vary with storage duration and condition; thus, such influences on lipid quality should be investigated before a commercial nutraceutical product is launched.

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APPENDIX A. FATTY ACID PROFILES FOR EXTRACTED LIPIDS AND LIPID CLASSES RECOVERED FROM SCVL

Appendix A1 The fatty acid profile (weight percentage of total FAME identified; mean +/- SD; n=3) of lipid extract of SCV of the three harvest times of *C. frondosa.* Values in the same rows with different letters are significantly different ($p < 0.05$).

Fatty acid	Winter	Spring	Summer
14:0	1.26 ± 0.04b	1.38 ± 0.09b	2.93 ± 0.13a
4,8,12-Me-13:0	2.49 ± 0.06b	2.30 ± 0.11a	2.06 ± 0.10c
*Me-14:0 (a)	0.45 ± 0.01a	0.44 ± 0.01a	0.32 ± 0.14a
Me-14:0 (b)	0.45 ± 0.01a	0.43 ± 0.02a	0.22 ± 0.02b
i-15:0	0.07 ± 0.00a	0.07 ± 0.01a	0.07 ± 0.06a
12-MTA	21.27 ± 0.77a	19.17 ± 0.93b	11.79 ± 0.78c
8,12-Me-14:0	1.52 ± 0.08a	0.99 ± 0.10b	0.99 ± 0.10b
Me-14:0 (c)	0.31 ± 0.01a	0.27 ± 0.03b	0.16 ± 0.02c
12-Me-15:0	3.52 ± 0.13a	3.12 ± 0.41a	1.66 ± 0.27b
8,12-Me-15:0	0.21 ± 0.01a	0.19 ± 0.03a	0.07 ± 0.06b
16:0	1.18 ± 0.02b	1.25 ± 0.14b	2.19 ± 0.17a
16:1n-7	14.99 ± 0.15a	14.36 ± 0.32b	20.60 ± 0.17c
**17:1(a)	0.02 ± 0.00a	0.01 ± 0.00b	0.00 ± 0.00c
i-17:0	0.06 ± 0.03b	0.08 ± 0.00b	0.25 ± 0.01a
ai-17:0	0.63 ± 0.01a	0.61 ± 0.04a	0.65 ± 0.01a
17:1(b)	0.04 ± 0.00a	0.03 ± 0.00b	0.00 ± 0.00c
17:1(c)	0.22 ± 0.01a	0.21 ± 0.01a	0.00 ± 0.00b
17:1(d)	3.17 ± 0.12a	4.02 ± 0.49a	3.99 ± 0.29a
16:4n-1	1.09 ± 0.02a	1.07 ± 0.13a	0.17 ± 0.07b
18:0	1.88 ± 0.01b	1.83 ± 0.12b	2.59 ± 0.07a
18:1n-9	1.15 ± 0.06b	1.25 ± 0.11b	1.81 ± 0.04a
18:1n-7	2.76 ± 0.02a	2.69 ± 0.15a	2.71 ± 0.03a
18:4n-3	0.75 ± 0.01b	0.74 ± 0.04b	1.32 ± 0.07a
EPA	24.74 ± 0.68b	25.38 ± 0.18b	28.23 ± 0.40a
22:4n-6	2.01 ± 0.10b	2.51 ± 0.19a	1.43 ± 0.09c
22:5n-3	0.24 ± 0.01b	0.24 ± 0.00b	1.05 ± 0.04a
DHA	0.75 ± 0.01b	0.85 ± 0.02a	0.00 ± 0.00c
24:1	1.02 ± 0.05a	1.00 ± 0.04a	1.04 ± 0.02a
Others	11.64 ± 0.35b	13.35 ± 0.27a	11.74 ± 0.65b
Σ Saturated FA	4.32 ± 0.05b	4.45 ± 0.18b	7.70 ± 0.22a
Σ MUFA	23.37 ± 0.20a	23.58 ± 0.55a	30.18 ± 0.35b
Σ PUFA	29.56 ± 0.62a	30.78 ± 0.27b	32.20 ± 0.42c
Σ BCFA	31.13 ± 0.75a	27.84 ± 0.94b	18.21 ± 0.87c

*Me-14:0 (a, b and c), and ** 17:1 (a, b, c and d) are isomers

Appendix A2 The fatty acid profile (weight percentage of total FAME identified; mean +/- SD; n=3) of the five recovered bands of SCV of the three harvest times of *C. frondosa.* Values in the same rows for each three columns (winter, spring and summer) with different letters are significantly different ($p < 0.05$).

	PL			MAGE			TAG			DAGE		
Fatty acid	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
14:0	0.23 ± 0.10a	0.13 ± 0.02b	0.20 ± 0.08ab	0.15 ± 0.01b	0.16 ± 0.02b	0.47 ± 0.04a	3.37 ± 0.07c	3.75 ± 0.10b	5.75 ± 0.24a	0.52 ± 0.03b	0.60 ± 0.01b	1.36 ± 0.16a
4,8,12-Me-13:0	0.07 ± 0.01a	0.05 ± 0.01a	0.07 ± 0.03a	0.39 ± 0.00b	0.35 ± 0.02c	0.48 ± 0.04a	3.91 ± 0.21a	4.03 ± 0.52a	2.78 ± 0.36b	3.61 ± 0.05a	3.28 ± 0.19b	2.44 ± 0.33c
*Me-14:0(a)	0.00 ± 0.00b	0.00 ± 0.00b	0.31 ± 0.02a	0.09 ± 0.00a	0.08 ± 0.00a	0.09 ± 0.01 a	0.56 ± 0.04a	0.60 ± 0.03a	0.28 ± 0.03b	0.84 ± 0.03a	0.83 ± 0.04a	0.78 ± 0.01b
Me-14:0(b)	0.13 ± 0.01a	0.12 ± 0.01a	0.04 ± 0.03b	0.16 ± 0.03b	0.16 ± 0.02b	0.33 ± 0.03a	0.51 ± 0.02a	0.54 ± 0.02a	0.41 ± 0.13a	0.74 ± 0.01a	0.74 ± 0.02a	0.72 ± 0.04a
i-15:0	0.08 ± 0.00a	0.00 ± 0.00c	0.04 ± 0.02b	0.02 ± 0.00b	0.01 ± 0.00b	0.07 ± 0.01a	0.15 ± 0.00c	0.15 ± 0.00b	0.18 ± 0.00a	0.07 ± 0.02a	0.06 ± 0.02a	0.08 ± 0.00a
12-MTA	0.42 ± 0.03a	0.27 ± 0.03b	0.32 ± 0.10b	4.48 ± 0.18a	4.60 ± 0.26a	4.90 ± 0.27a	18.80 ± 0.89a	19.53 ± 0.96a	9.66 ± 0.72b	41.75 ± 0.61a	40.62 ± 0.87a	36.11 ± 0.12b
8,12-Me-14:0	0.04 ± 0.02b	0.02 ± 0.01b	0.12 ± 0.04a	0.41 ± 0.03a	0.28 ± 0.03b	0.44 ± 0.02a	1.72 ± 0.25a	1.25 ± 0.12b	1.01 ± 0.08c	3.29 ± 0.44a	2.11 ± 0.29b	2.66 ± 0.14c
15:0	0.03 ± 0.02a	0.03 ± 0.01a	0.00 ± 0.00b	0.03 ± 0.01b	0.12 ± 0.01a	0.00 ± 0.00b	0.44 ± 0.24b	0.89 ± 0.27a	0.00 ± 0.00c	0.23 ± 0.02b	1.35 ± 0.22a	0.00 ± 0.00c
Me-14:0(c)	0.02 ± 0.01a	0.00 ± 0.00b	0.02 ± 0.01a	0.07 ± 0.00b	0.06 ± 0.00c	0.09 ± 0.00a	0.29 ± 0.02a	0.27 ± 0.05a	0.12 ± 0.02b	0.66 ± 0.03a	0.56 ± 0.02b	0.44 ± 0.04c
12-Me-15:0	0.19 ± 0.03b	1.99 ± 0.73a	2.52 ± 0.21a	1.95 ± 0.35b	2.14 ± 0.22b	3.37 ± 0.17a	2.51 ± 0.28a	2.46 ± 0.29a	1.29 ± 0.10b	6.98 ± 0.22a	6.76 ± 0.47a	5.05 ± 0.02b
8,12-Me-15:0	0.00 ± 0.00b	0.03 ± 0.01a	0.02 ± 0.00ab	0.12 ± 0.04ab	0.10 ± 0.01b	0.16 ± 0.03a	0.18 ± 0.04a	0.17 ± 0.04a	0.06 ± 0.02b	0.47 ± 0.01a	0.43 ± 0.07a	0.30 ± 0.01b
16:0	4.67 ± 0.22c	1.74 ± 0.26a	3.65 ± 0.75b	0.71 ± 0.05b	0.70 ± 0.07b	2.67 ± 0.14a	3.05 ± 0.07c	3.37 ± 0.13b	4.21 ± 0.31a	0.67 ± 0.05b	0.71 ± 0.07b	1.94 ± 0.14a
16:1n-9	0.25 ± 0.07c	2.47 ± 0.71a	1.36 ± 0.18b	1.11 ± 0.42ab	1.29 ± 0.27a	0.76 ± 0.05b	0.14 ± 0.01b	0.15 ± 0.01ab	0.16 ± 0.00a	0.15 ± 0.03b	0.17 ± 0.03b	0.26 ± 0.02a
16:1n-7	1.71 ± 0.22b	1.26 ± 0.05c	2.17 ± 0.05a	10.49 ± 0.38b	10.45 ± 0.35b	11.08 ± 0.42a	23.37 ± 0.43b	23.74 ± 0.35b	26.77 ± 0.68a	20.40 ± 0.56a	20.66 ± 0.34a	19.00 ± 0.84b
**17:1(a)	0.16 ± 0.10a	0.17 ± 0.03a	0.06 ± 0.03a	0.05 ± 0.01b	0.01 ± 0.00c	0.08 ± 0.03a	0.04 ± 0.03a	0.02 ± 0.02ab	0.01 ± 0.00b	0.04 ± 0.01a	0.02 ± 0.01b	0.01 ± 0.02c
i-17:0	0.20 ± 0.06a	0.12 ± 0.04ab	0.08 ± 0.03b	0.20 ± 0.05a	0.22 ± 0.02a	0.00 ± 0.00b	0.06 ± 0.03a	0.04 ± 0.02b	0.05 ± 0.00ab	0.04 ± 0.02b	0.02 ± 0.02b	0.11 ± 0.04a
ai-17:0	0.41 ± 0.08a	0.32 ± 0.00a	0.30 ± 0.15a	0.10 ± 0.01ab	0.08 ± 0.01b	0.11 ± 0.00a	1.23 ± 0.07c	1.29 ± 0.01a	0.88 ± 0.02b	0.55 ± 0.02b	0.56 ± 0.03b	0.75 ± 0.02a

	PL			MAGE			TAG			DAGE		
Fatty acid	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
17:1(b)	0.09 ± 0.04a	0.03 ± 0.00b	0.04 ± 0.01b	0.03 ± 0.00a	0.03 ± 0.02a	0.02 ± 0.01b	0.04 ± 0.01a	0.04 ± 0.01a	0.03 ± 0.00a	0.03 ± 0.01ab	0.03 ± 0.00a	0.02 ± 0.01b
16:2n-4	0.09 ± 0.02a	0.12 ± 0.02a	0.06 ± 0.01a	0.31 ± 0.03a	0.33 ± 0.03a	0.35 ± 0.00a	1.18 ± 0.11b	1.07 ± 0.03c	1.64 ± 0.02a	0.72 ± 0.01a	0.74 ± 0.02a	0.61 ± 0.03b
16:3n-4	0.09 ± 0.04b	0.19 ± 0.04a	0.04 ± 0.03b	0.22 ± 0.01a	0.17 ± 0.03b	0.18 ± 0.01b	0.60 ± 0.13b	0.48 ± 0.04c	1.05 ± 0.05a	0.23 ± 0.02a	0.21 ± 0.02a	0.16 ± 0.01b
17:1(c)	0.03 ± 0.00b	0.03 ± 0.01b	0.09 ± 0.05a	0.20 ± 0.01a	0.20 ± 0.02a	0.20 ± 0.01a	0.11 ± 0.02a	0.07 ± 0.04ab	0.05 ± 0.02b	0.44 ± 0.01a	0.42 ± 0.01b	0.32 ± 0.00c
17:1(d)	0.36 ± 0.20c	8.04 ± 0.87b	24.09 ± 0.97a	4.72 ± 0.14b	5.18 ± 0.53b	14.01 ± 0.37a	0.00 ± 0.00b	0.13 ± 0.02b	2.50 ± 0.29a	0.29 ± 0.05b	0.21 ± 0.15b	1.95 ± 0.25a
16:4n-1	0.14 ± 0.09a	0.10 ± 0.00a	0.15 ± 0.05a	0.13 ± 0.02a	0.11 ± 0.01a	0.03 ± 0.00b	1.02 ± 0.32a	1.07 ± 0.18a	0.02 ± 0.01b	0.17 ± 0.03b	0.17 ± 0.04b	0.38 ± 0.02a
18:0	11.23 ± 0.69a	5.36 ± 0.11b	5.63 ± 0.78b	0.92 ± 0.02b	0.85 ± 0.10b	2.06 ± 0.07a	2.95 ± 0.06b	2.91 ± 0.10b	3.59 ± 0.43a	0.49 ± 0.07b	0.48 ± 0.07b	1.11 ± 0.46a
18:1n-9	1.08 ± 0.04a	1.09 ± 0.05a	1.25 ± 0.44a	0.45 ± 0.03b	0.41 ± 0.05b	0.86 ± 0.01a	1.95 ± 0.05c	2.14 ± 0.07b	2.66 ± 0.16a	0.85 ± 0.04b	0.95 ± 0.14b	2.10 ± 0.12a
18:1n-7	3.89 ± 0.39a	3.47 ± 0.21a	2.38 ± 0.17b	2.33 ± 0.13b	2.24 ± 0.27b	2.63 ± 0.07a	2.45 ± 0.04ab	2.74 ± 0.14a	2.26 ± 0.26b	2.71 ± 0.06a	2.81 ± 0.17a	2.69 ± 0.11a
18:2n-7	0.00 ± 0.00b	0.00 ± 0.00b	1.16 ± 0.09a	0.02 ± 0.00b	0.02 ± 0.00b	0.52 ± 0.08a	0.02 ± 0.01a	0.02 ± 0.00a	0.03 ± 0.01a	0.02 ± 0.00b	0.02 ± 0.00b	0.08 ± 0.03a
18:4n-3	0.31 ± 0.04a	0.30 ± 0.02a	0.37 ± 0.01a	0.28 ± 0.06a	0.30 ± 0.08a	0.43 ± 0.00a	0.93 ± 0.19b	0.85 ± 0.09b	1.78 ± 0.12a	0.28 ± 0.02a	0.30 ± 0.04a	0.32 ± 0.06a
20:0	0.95 ± 0.08a	1.01 ± 0.02a	0.16 ± 0.05b	0.03 ± 0.01b	0.05 ± 0.04b	0.16 ± 0.05a	0.26 ± 0.02b	0.35 ± 0.01a	0.25 ± 0.02b	0.04 ± 0.01b	0.08 ± 0.02a	0.03 ± 0.02b
20:1n-11	2.12 ± 0.07a	2.28 ± 0.05a	1.34 ± 0.33b	0.20 ± 0.02a	0.20 ± 0.02ab	0.16 ± 0.01b	0.64 ± 0.05b	0.83 ± 0.04a	0.37 ± 0.04c	0.20 ± 0.01c	0.26 ± 0.03b	0.31 ± 0.01a
20:1n-9	0.95 ± 0.09a	0.85 ± 0.03a	1.25 ± 0.41a	0.41 ± 0.01a	0.40 ± 0.05a	0.45 ± 0.02a	0.59 ± 0.01a	0.69 ± 0.04a	0.61 ± 0.08a	0.29 ±0.01c	0.40 ± 0.04b	0.52 ± 0.03a
20:1n-7	1.09 ± 0.17a	0.50 ± 0.11b	0.69 ± 0.23b	0.07 ± 0.01b	0.01 ± 0.01c	0.31 ± 0.01a	0.20 ± 0.08b	0.12 ± 0.01b	1.03 ± 0.22a	0.07 ± 0.01c	0.04 ±0.04b	1.38 ± 0.50a
20:4n-6	1.73 ± 0.17b	2.10 ± 0.07a	0.94 ± 0.26c	1.39 ± 0.07a	1.31 ± 0.13a	1.06 ± 0.00b	0.38 ± 0.08a	0.37 ± 0.03a	0.36 ± 0.01a	0.13 ± 0.03b	0.20 ± 0.03a	0.18 ± 0.02a
EPA	47.80 ± 2.61a	46.01 ± 0.60a	30.66 ± 0.36b	57.67 ± 0.31a	57.87 ± 0.25a	45.06 ± 0.66b	16.43 ± 0.27b	13.84 ± 0.81c	19.46 ± 0.43a	5.21 ± 0.28c	6.21 ± 0.84b	8.85 ± 0.21a
22:0	0.89 ± 0.05b	0.90 ± 0.03b	1.16 ± 0.17a	0.08 ± 0.06b	0.13 ± 0.11ab	0.20 ± 0.00a	0.30 ± 0.01ab	0.34 ± 0.09a	0.24 ± 0.02b	0.10 ± 0.04b	0.12 ± 0.02ab	0.16 ± 0.02a
22:1n-9	1.11 ± 0.32a	1.00 ± 0.03a	0.82 ± 0.26a	0.13 ± 0.05a	0.11 ± 0.03a	0.16 ± 0.00a	0.46 ± 0.03b	0.56 ± 0.03a	0.46 ± 0.01b	0.09 ± 0.02b	0.14 ± 0.03b	0.25 ± 0.03a
22:1n-7	1.27 ± 0.16b	1.22 ± 0.03b	1.46 ± 0.04a	0.10 ± 0.04a	0.09 ± 0.02a	0.13 ± 0.00a	0.49 ± 0.03b	0.55 ± 0.03a	0.37 ± 0.02c	0.13 ± 0.02b	0.15 ± 0.02ab	0.18 ± 0.01a

	PL			MAGE			TAG			DAGE		
Fatty acid	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**	**Winter**	**Spring**	**Summer**
22:4n-6	5.55 ± 0.58b	6.49 ± 0.10a	4.39 ± 0.29c	0.30 ± 0.01b	0.41 ± 0.05a	0.24 ± 0.09c	1.02 ± 0.10a	1.19 ± 0.09a	0.23 ± 0.01b	0.29 ± 0.03a	0.34 ± 0.01a	0.27 ± 0.11a
DHA	1.53 ± 0.22a	1.44 ± 0.09a	0.97 ± 0.23b	0.99 ± 0.02a	1.04 ± 0.06a	0.91 ± 0.04b	0.90 ± 0.07a	0.84 ± 0.03a	0.76 ± 0.02b	0.30 ± 0.02b	0.39 ± 0.08a	0.42 ± 0.01a
24:1	1.66 ± 0.14a	1.25 ± 0.10c	1.47 ± 0.03b	1.20 ± 0.07a	1.23 ± 0.45a	0.90 ± 0.02a	0.88 ± 0.07a	0.94 ± 0.05a	0.60 ± 0.18b	0.61 ± 0.04a	0.64 ± 0.07a	0.60 ± 0.07a
Others	7.42 ± 0.53a	7.51 ± 0.57a	8.15 ± 0.51a	7.28 ± 0.40a	6.56 ± 0.41a	4.16 ± 0.58b	5.65 ± 0.16a	5.62 ± 0.14a	6.02 ± 0.28b	5.30 ± 0.15a	5.26 ± 0.22a	5.08 ± 0.36a
Σ Saturated FA	18.02 ± 0.84c	9.18 ± 0.26a	10.80 ± 0.68b	1.92 ± 0.07a	2.00 ± 0.17a	5.25 ± 0.36b	10.38 ± 0.24a	11.61 ± 0.35b	14.05 ± 0.52c	2.05 ± 0.09a	3.34 ± 0.23b	4.61 ± 0.85c
Σ MUFA	15.77±0.56a	23.66±0.82b	38.47 ± 1.24c	21.51 ± 0.54a	21.86 ± 0.76a	31.76 ± 0.70b	31.36 ± 0.42a	32.72 ± 0.40b	37.87 ± 0.86c	26.31 ± 0.51a	26.86 ± 0.40a	29.62 ± 0.83b
Σ PUFA	57.25 ± 2.09a	56.76 ± 0.54a	38.73 ± 0.54b	61.31 ± 0.36a	61.56 ± 0.30a	48.78 ± 0.72b	22.48 ± 0.70b	19.73 ± 0.76a	25.33 ± 0.45c	7.35 ± 0.27a	8.55 ± 0.76b	11.26 ± 0.26c
Σ BCFA	1.56 ± 0.10a	2.91 ± 0.67b	3.84 ± 0.30c	7.98 ± 0.38a	8.01 ± 0.34a	10.05 ± 0.29b	29.93 ± 0.83a	30.33 ± 1.06a	16.72 ± 0.75b	58.99 ± 0.72b	55.98 ± 0.96a	49.43 ± 0.42c

*Me-14:0 (a, b and c), and ** 17:1 (a, b, c and d) are isomers