ANALYSIS OF PHYTOSTEROLS BY GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY IN ATLANIC SALMON (*SALMO SALAR*) FED NOVEL LIPID SOURCES

by

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A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the requirements for the degree of

Master of Science

in

The Department of Chemistry

Faculty of Science

Memorial University of Newfoundland

October 2022

St. John's Newfoundland and Labrador

Abstract

Fishmeal and fish oil have been considered the most digestible and nutritious ingredients for farmed fish, but there is a shift towards more sustainable alternatives. In this study, fish oil was replaced with four alternative oil sources, oil from *Camelina sativa* seeds, oil from rapeseed, oil from *Pavlova* sp., and a microbial oil produced from *Schizochytrium* sp.. Atlantic salmon (*Salmo salar*) were fed experimental diets, including either microbial oil or camelina oil at low and high levels of inclusion, as well as a control diet containing traditional fish oil. Sterol content was investigated to determine if added phytosterols were incorporated by the salmon, and subsequently transferred to the fish tissue for human consumption. Sterols were identified, and quantified where possible, in the alternative oil sources and then in salmon muscle tissue after feeding trials. Gas chromatography/mass spectrometry (GC-MS) and gas chromatography/flame ionization detection (GC-FID) were used in the analysis.

Five sterols were identified in camelina oil: cholesterol, brassicasterol, campesterol, stigmasterol, and β-sitosterol. In the experimental diets from the 16-week feeding trial containing various inclusion levels of camelina oil, brassicasterol, campesterol, stigmasterol, and β-sitosterol were also identified, however in the muscle tissue from salmon fed these diets, only campesterol and β-sitosterol were detected. In the microbial oil experiment, cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol, 24-methylenelophenol, stigmasterol, and spinasterol were the sterols identified. In the experimental diets containing 5% or 10% microbial oil, cholesterol, brassicasterol, lathosterol and stigmasterol were found, with the addition of campesterol, and two other

sterols (23,24-dimethylcholest-5-en-3β-ol and 24-ethyl-5α-cholest-7-en-3β-ol). In the muscle tissue from salmon fed the diets containing microbial oil, cholesterol, cholestanol, campesterol, stigmasterol, and dinosterol were detected.

Campesterol and β-sitosterol from the camelina oil were transferred from the diet to the tissue, and with the microbial oil, stigmasterol was also detected in both the diets and the salmon tissues. This indicates that sterols in microbial oil can be transferred to the fish by consumption, but not all sterols were found to do so. Also, phytosterols can be metabolized to other sterols by the fish, as was seen with cholesterol and cholestanol.

Acknowledgements

I would like to thank my supervisors, Dr. Bob Helleur (Chemistry) and Dr. Chris Parrish (Ocean Sciences) for their continuous guidance and support throughout my research. Thank you so much for your knowledge, wisdom, encouragement, and patience throughout my studies. I'd also like to thank Dr. Christina Bottaro for being a part of my committee and providing help along the way.

I would also like to thank Jeanette Wells of the Ocean Sciences Centre for her guidance and assistance with my laboratory analysis, as well as Stefana Egli at C-CART for helping me with my instrumental analysis and navigating the computer software. I am also thankful for the assistance of Stefanie Colombo both at the beginning of my research in the lab, and later as part of the OFI research team. I would like to thank Valerie Parsons for allowing me to use her C*amelina* diet data. I would also like to thank Jorge del Angel and Laura Carreon-Palau for sharing some of their extensive knowledge of sterols and for aiding in sterol identification, as well as providing supplemental data.

I would also like to thank my colleagues at the Marine Institute for helping with my research along the way, and for filling in as needed during my leave. I'd also like to thank my partner Bob, and my family, for always being there to offer emotional support and the encouragement I needed to keep going.

A portion of this research was funded by the Ocean Frontier Institute (OFI).

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List of Abbreviations and Symbols

ALA	alpha-linolenic acid
ASG	acylated sterol glycosides
BSTFA	*N,O*-bis(trimethylsilyl)trifluoroacetamide
C-CART	Centre for Chemical Analysis, Research and Training
CEC	capillary electrochromatography
CM	camelina meal
DB-5ms	5%-phenyl, 95%-dimethyl polysiloxane
DESI	desorption electrospray ionization
DHA	docosahexaenoic acid
EI	electron ionization
EPA	eicosapentaenoic acid
FID	flame ionization detection
FM	fish meal
FO	fish oil
GC	gas chromatography
GC-FID	gas chromatography-flame ionization detection

GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
HSE	hydroxycinnamic-acid esters
IR	infrared detection
IS	internal standard
IUPAC	International Union of Pure and Applied Chemistry
LC-PUFA	long chain polyunsaturated fatty acids
LDL	low density lipoprotein
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
MO	microbial oil
MS	mass spectrometry
MUFA	monounsaturated fatty acids
NMR	nuclear magnetic resonance
OFI	Ocean Frontier Institute
OSC	Ocean Science Centre
PAH	polycyclic aromatic hydrocarbons
PUFA	polyunsaturated fatty acids

SE	sterol esters
SG	steryl glycosides
TIC	total ion chromatogram
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
UV	ultraviolet detection
VO	vegetable oil

Chapter 1: Introduction and Literature Review

1.1 Background and Rationale

1.1.1 Aquaculture Industry Significance

Aquaculture continues to be one of the fastest growing food production industries in the world. Global fish production peaked at about 171 million tonnes in 2016, with aquaculture representing 47% of the total. With capture fishery production remaining relatively stable since the late 1980s, aquaculture has been responsible for the continuing growth in the supply of fish for human consumption (FAO, 2018). A steady supply of quality ingredients for fish feed is essential to the aquaculture industry. Fishmeal and fish oil are still considered the most digestible and nutritious ingredients for farmed fish feeds, but their inclusion in aquaculture feeds has shown a downward trend as they are used more selectively (FAO, 2018). This has brought about a shift towards finding more sustainable alternatives to fishmeal and fish oil as an ingredient in farmed fish feed. The challenge is to maintain, if not improve, the benefits of fish to human health while simultaneously maximizing sustainability, fish health, and economic benefits (Turchini et al., 2009). Many research activities in recent years have focused on developing and evaluating alternative lipid sources.

1.1.2 Lipids in Aquaculture

Lipids likely represent one of the most studied nutrient groups in fish nutrition. Lipids are an important source of energy and play a vital role in fish nutrition. They are a major energy contributor in aquaculture nutrition and as such reach high inclusion levels in energy dense feeds (Turchini et al., 2009). Part of the reason for the prominence of lipids in fish nutrition is the unique abundance of long-chain polyunsaturated fatty acids that are found in fish, and the significant impact they have on many aspects of human health (Turchini et al., 2009).

Lipids, along with proteins and carbohydrates, comprise the major macronutrient classes that are required to provide both the essential nutrients for energy production and the building blocks for development of cells and tissues that allow growth and maintenance of homeostasis in all vertebrate organisms (Turchini et al., 2009). Lipids are a diverse and ubiquitous group of compounds which have many key biological functions (Fahy et al., 2011). The diversity in lipid function is reflected by an enormous variation in the structure of lipids. The term lipid has been loosely defined as any of a group of organic compounds that are insoluble in water but are soluble in organic solvents. This includes a broad range of molecules such as fatty acids, phospholipids, sterols, sphingolipids, terpenes, and others. Lipids can be divided into two broad groups that are classified as neutral lipids and polar lipids. The major neutral lipid classes are triacylglycerols (TAG), wax esters, sterols, steryl esters, and free fatty acids. The major polar lipids include phosphoglycerides, sphingolipids, sulpholipids and glycolipids (Turchini et al., 2009). Collectively,

these lipid classes provide the energy and cellular building blocks, in the form of essential and nonessential fatty acids, to maintain growth, health, welfare and reproduction of all organisms including fish (Turchini et al., 2009).

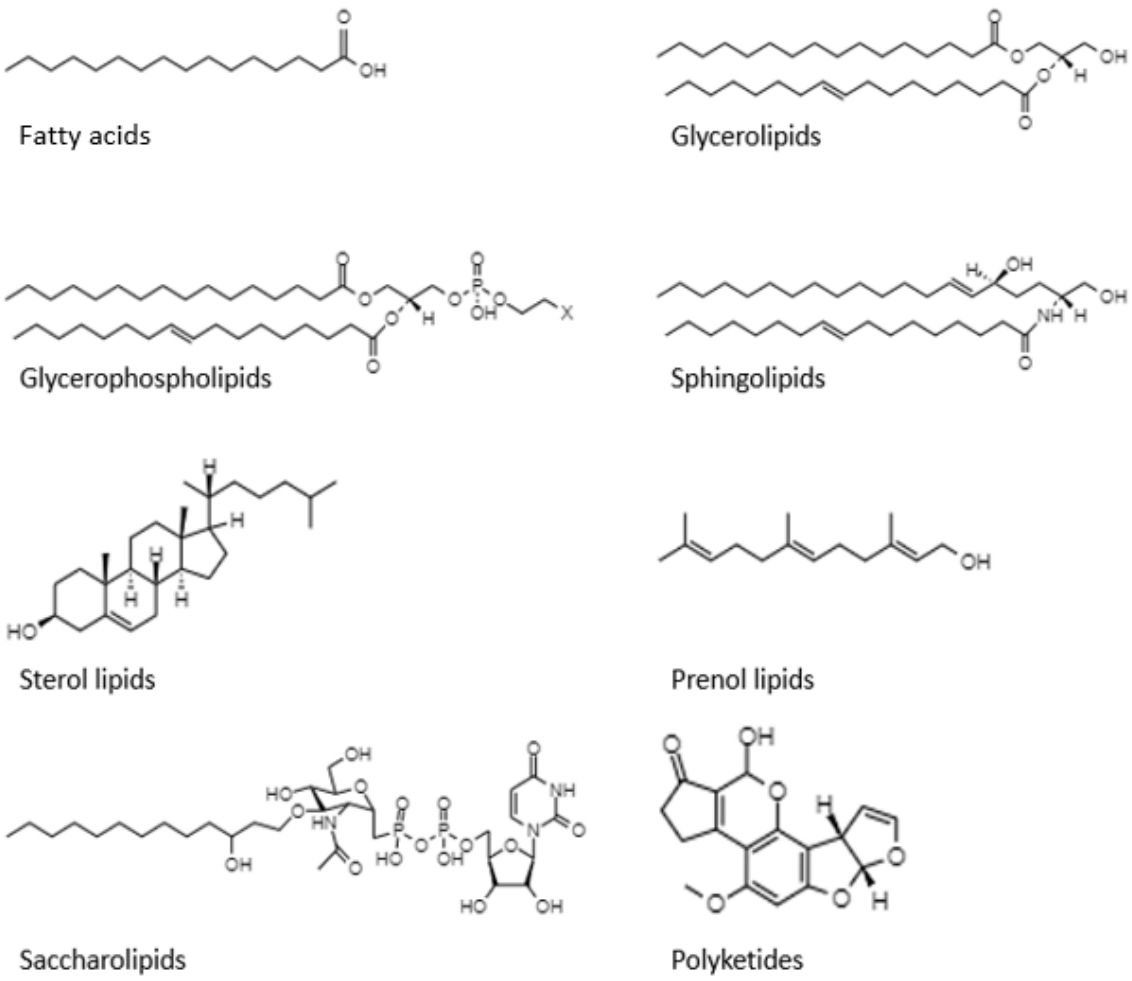

Figure 1.1: Structures of some lipid classes.

Lipids are required by fish as a source of energy, as structural components of biomembranes, as carriers of fat soluble vitamins, as precursors to hormones and vitamins, and as enzyme co-factors. Dietary lipids are essential to fish as they provide fatty acids that are necessary for regular growth, health, and bodily functions. All vertebrates, including fish, have a dietary requirement for n-6 and n-3 polyunsaturated fatty acids (PUFA) (Turchini et al, 2009). Marine fish oils have traditionally been used to provide lipids in fish fed due to their fatty acid composition and availability. However, as the global demand for fish oils increases with growth in the aquaculture industry, other sources will be needed.

1.2 Alternative Lipid Sources in Aquaculture

1.2.1 Fishmeal and Oil Use in Aquaculture

Fishmeal is recognized as a high-quality, very digestible feed ingredient, ideal for diets of most animals, especially fish. Fishmeal can be made from any type of seafood, but it is generally made from small marine fish that are considered not suitable for human consumption, as they contain a high percentage of bones and oil. The major types of fish used for fishmeal are anchovies, herring, sardines, shads and smelts. Most fish used for fish meal are captured for the sole purpose of use in feed and are mainly wild caught at sea (Miles and Chapman, 2006).

Addition of fish meal to aquaculture diets increases feed efficiency and growth and enhances nutrient uptake, digestion and absorption. Fishmeal is a great source of proteins, lipids, minerals and vitamins, while being very low in carbohydrates. High quality fish meal will have a balanced amino acid composition for optimum growth and development. It also complements the effects of other animal and vegetable proteins in the diet (Miles and Chapman, 2006).

Fish oil, produced from wild fisheries, is used more by aquaculture than any other industry (Tacon and Metian, 2008). The inclusion of fish oil in fish feed provides a source of lipids which is highly digestible and an excellent source of essential polyunsaturated fatty acids (PUFA). Fish meal and oil contain more omega-3 fatty acids than omega-6, while most plant oils contain a higher concentration of omega-6 fatty acids. Beneficial effects of lipids in fish meal are evident in cell membrane structure and function. Essential fatty acids are also important in fish reproduction and growth, as well as development of the skin, nervous system, brain, and immune system. The high quality and concentration of essential nutrients, well balanced amino acids, essential fatty acids, and its value as an energy source make fish meal and oil the benchmark ingredient in aquaculture diets (Miles and Chapman, 2006).

1.2.2 Use of Terrestrial Plant Based Oils

Decreasing availability of fish oils as well as variable and often increasing prices have led to the investigation of alternative sources of dietary oil (Turchini et al.,

2009). Several different terrestrial plant oils have been used in fish feed, however most of these are lacking in long chain ω3 polyunsaturated fatty acids (PUFA). A suitable replacement terrestrial oil must be high in precursor fatty acids for biosynthesis of ω3 polyunsaturated fatty acids. It should also have low levels of ω6 polyunsaturated fatty acids to maintain a high ω3: ω6 ratio (Hixson et al., 2014).

Camelina (*Camelina sativa*) is one such terrestrial oil source of interest. *Camelina sativa* is member of the *Brassicaceae* family, along with mustard, rapeseed, broccoli, cauliflower, and other plants. It grows well in cool and semi-arid regions with short growing seasons. It competes well with other plants, is tolerant of insects and weeds, and can survive frost and freeze-thaw cycles (Shukla et al., 2002).

Although mostly underutilized in the past, it has gained interest due to its oil content. The oilseed camelina yields a 40% total lipid content, is low in saturated fatty acids, and high in monounsaturated and polyunsaturated fatty acids (Hixson et al., 2013). Its unusually high alpha-linoleic acid (ALA) content makes it particularly unique. ALA is a precursor to ω3 fatty acids and some fish species, like Atlantic salmon (*Salmo salar*) can convert ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The combination of these characteristics makes Camelina oil particularly intriguing as a replacement for fish oil in fish feed.

1.2.3 Use of Microbial Oils

Marine microorganisms show great potential as an alternative to oil sources for fish feed. The group of microorganisms known as thraustochytrids are often referred to as 'microalgae'; however they are actually classified as marine or brackish fungal protists (Tibbets et al., 2020). Thraustochytrids have demonstrated capability to produce very high lipid concentrations (> 70% of their mass) rich in essential long-chain polyunsaturated fatty acids (LC-PUFA) by using a vast array of carbon and nitrogen feedstocks (Lewis et al., 1999). The level of DHA in the extracted oil has been increased through the optimization of culture conditions (Tibbetts et al., 2020). Thraustochytrids have been of interest as candidates for use in aquaculture feeds, as they are capable of production of high levels of DHA. Additionally, because of their highly controlled fermentation processes, the DHA-rich edible oils produced are more likely to be free of the environmental contaminants often problematic in conventional fish oils (Harwood, 2019).

Very limited microbial oil feeds are available on the market and most of them are for direct human consumption as nutraceuticals or infant formula (Wei et al., 2021). A handful of companies are now involved in this emerging industrial sector, and commercial food-grade DHA products may soon be added to the feedstock portfolios of large aquafeed producers (Tibbetts et al., 2018).

1.3 Sources and Significance of Phytosterols

1.3.1 Chemistry of Sterols

Sterols are part of the vast family of isoprenoids and can be considered to belong to the wider group of compounds collectively referred to as steroids. The conversion of farnesyl diphosphate into squalene marks the channeling of the isoprenoid pathway into the branch that produces sterols (Hartmann, 1998). The majority of sterols contain from 27 to 30 carbon atoms with a cholestane skeleton (steroid nucleus with a side chain in position C-17) and a 3β-hydroxyl group. The aliphatic chain at C-17 has eight carbons or more (Leray, 2013). Sterols are vital components of all eukaryotic organisms. Sterols are cell membrane components and as such regulate membrane fluidity and permeability. The sterols show a fascinating chemical complexity and remarkable diversity of function in living organisms. Their functions range from controlling membrane fluidity and permeability, to cell proliferation, signal transduction and as modulators of the activity of membrane-bound enzymes (Volkman, 2003).

1.3.2 Sterol Nomenclature

Sterol was the name originally proposed to describe a 3β-monohydroxy compound based upon the perhydro-1,2-cyclopentanophenanthrene ring system, with methyl substitution at C10 and C13 and a side chain with 8–10 carbon atoms (Hartmann,

1998). Many of the naturally occurring sterols are known by trivial names and these are widely used in the literature. These trivial names, however, often provide little information about the structure of the compound. Recommendations for the nomenclature of sterols were presented by the International Union of Pure and Applied Chemistry (IUPAC) in 1967 with various subsequent revisions (Goad, 2012). In these recommendations sterols are defined as steroids carrying a hydroxyl group at C-3 and most of the skeleton of cholestane. Additional carbon atoms may be present in the side chain (Hartmann, 1998). The rings of the 1,2-cyclopentanophenanthrene ring system are designated A, B, C and D as shown in Figure 1.2. The numbering of the carbon atoms shown in Figure 1.2 was adopted in 1989 and allowed for more uniformity in the nomenclature of all tetracyclic triterpenoids and their metabolic derivatives including sterols (Goad, 2012).

The designations α and β are used to identify atoms or groups which lie above (β) or below (α) the plane of the rings. The bond to a substituent with the β-orientation is shown as a thickened or wedge-shaped line, while an α-bond is represented by a broken or dashed line. If no atom or group is shown at the end of an α- or β-bond, it implies that a methyl group is attached (Goad, 2012). Many structural variations arise from different substitutions in the side chain and the number and position of double bonds in the tetracyclic skeleton.

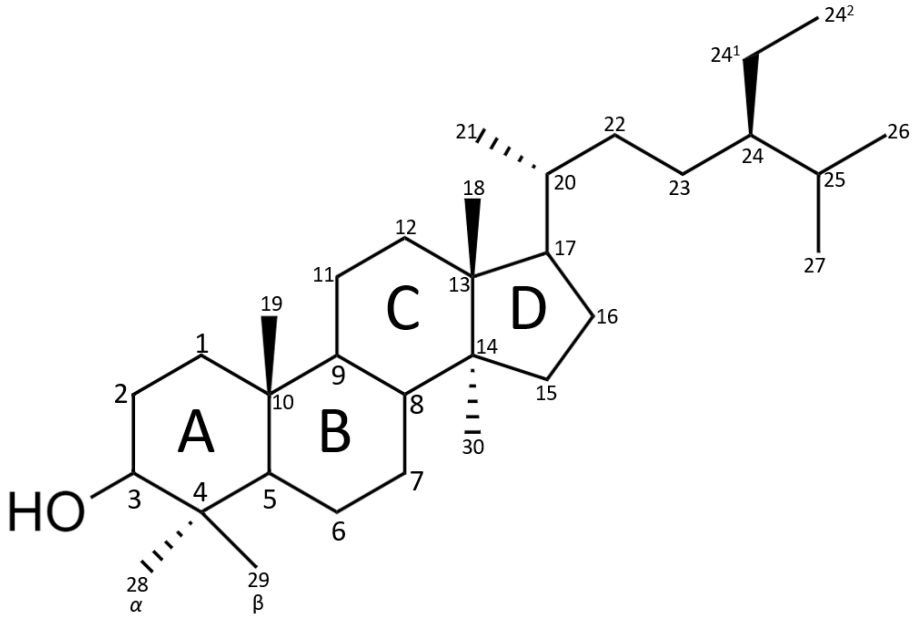

Figure 1.2: Basic structure of a sterol with standard carbon numbering according the 1989 IUPAC-IUB recommendations.

The numbering of the side chain is as shown in Figure 1.2. In previous IUPAC recommendations, the carbons at C-24 were numbered C-28 and C-29 respectively. However, the 1989 recommendations require that an additional carbon atom in the side chain assume the number of the side chain carbon to which it is attached, together with a superscript number, 1, 2, etc. Additional carbon atoms at C-22, C-23, or C-25 would be numbered similarly (Goad, 2012).

Presence of a double bond in a sterol is revealed by changing the –ane ending of the parent hydrocarbon name to –ene, and interposing the locant number (i.e., cholest-5-ene). An acetylenic bond is signified by –yne. When two double bonds

are present, -ane is changed to –adiene. The Greek capital delta (Δ) is used to indicate the location of double bonds in descriptive terminology, such as Δ^5-bond or Δ^8-sterol (Goad, 2012).

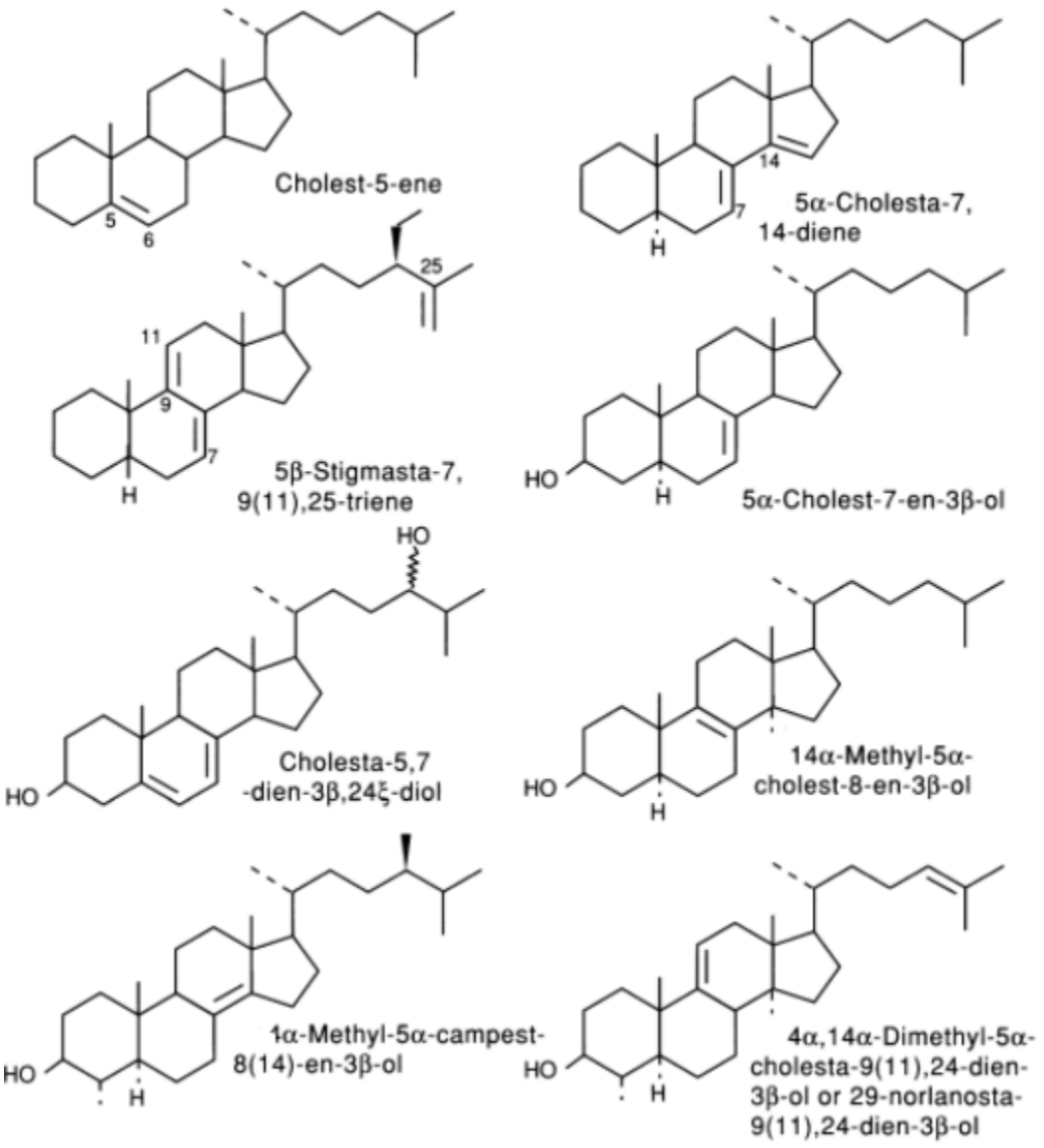

Figure 1.3: Examples of sterols that illustrate the derivation of the systemic name using the 1989 IUPAC recommendations (Goad, 2012).

Phytosterol nomenclature can be confusing as many researchers still use the older naming system. The two main nomenclatures used are the 1989 IUPAC-IUB recommendations as discussed above, as well as the 1976 recommendations. Common or trivial names are often used as well.

1.3.3 Sterol Classifications

A convenient was to catalog sterols is according to the number of methyl groups on carbon number 4. There are three groups; those with two methyl groups, the 4-dimethyl sterols, one methyl group, the 4-monomethyl sterols, or no methyl groups, the 4-desmethyl sterols (Moreau et al., 2002). 4-Dimethyl sterols and 4-monomethyl sterols are metabolic intermediates in the biosynthetic pathway leading to 4-desmethyl sterols, and they are usually present in low levels in most plants (Moreau et al., 2002). Most common sterols belong to the 4-desmethyl group. Most have a double bond between carbons 5 and 6 and are thus called Δ^5 phytosterols. Other phytosterols that are common in plants have a double bond between carbons 7 and 8 and are called Δ^7 phytosterols. Both of these groups can have a second double bond in the side chain (Moreau et al., 2002). The 4-desmethyl group includes cholesterol and all of the common 28-carbon and 29-carbon phytosterols, such as brassicasterol, stigmasterol and campesterol.

The introduction of a methyl group at carbon 24 in 28 or 29 carbon phytosterols renders this position chiral, and therefore two epimers are possible. The epimers

are designated as α or β (or R and S respectively). For 28-carbon phytosterols, they can be either α or β. Campesterol, epibrassicasterol and campestanol are examples of 24α epimers, while brassicasterol and ergosterol are 24β epimers (Moreau et al., 2002). With 29-carbon phytosterols, almost all are 24α epimers.

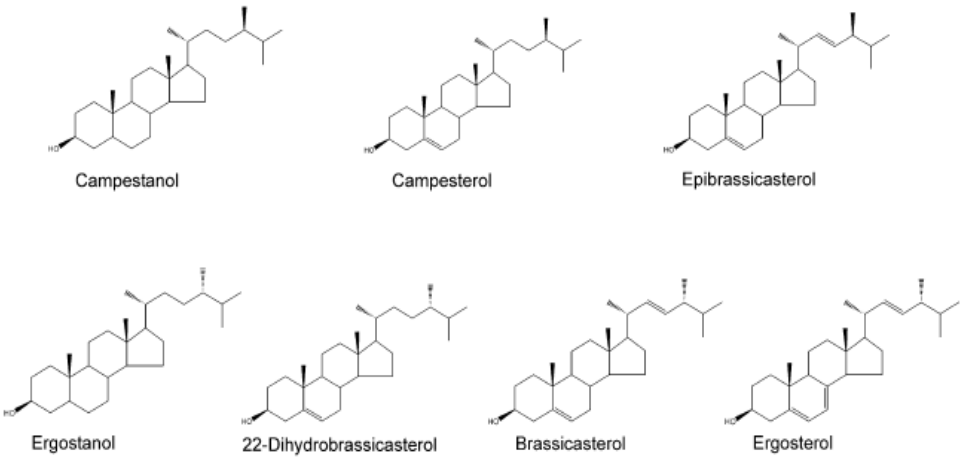

Figure 1.4: Carbon-28 4-desmethyl phytosterols (Moreau et al., 2002).

1.3.4 Conjugated Sterols

In addition to the free form, sterols are frequently found in nature bound to other compounds. Esters of sterols with fatty acids are called steryl esters (SE). The alcohol group is esterified to the fatty acid. They are named by combining the name of the sterol and the fatty acid, such as cholesteryl palmitate. A steryl glycoside (SG) is a sterol in which the C-3 hydroxyl group is replaced by an ether linkage to

the C-1 position of a sugar monomer. An acylated steryl glycoside (ASG) additionally contains a fatty acid esterified to a hydroxyl on the saccharide, most often at C-6 (Schrick et al., 2012). Diversity of chemical structures in these conjugated sterols comes from differences in the type of sterol, fatty acid or sugar and the number of sugar groups.

Sterol conjugation, the conversion of free sterols into steryl esters, steryl glycosides, or acylated steryl glycosides, is a potentially important aspect of membrane lipid metabolism. Steryl glycosides and acylated steryl glycosides are structural components of membranes, while steryl esters appear to be excluded (Moreau et al., 2002). This is likely due to their low solubility in the phospholipid layer. Studies have shown that interconversion of free sterols and their conjugates is quite rapid, suggesting a regulatory function. It has been postulated that they are involved in the regulation of membrane properties in response to changing conditions, such as light, temperature, and water stress (Moreau et al., 2002).

1.3.5 Cholesterol

Cholesterol is the only sterol biosynthesized by animals. It is ubiquitous and predominant in animals, but only present in trace amounts in most plants, with some exceptions (*Camelina* oil). There is a widespread misconception that plant tissues are completely devoid of cholesterol, however it often accounts for 1-2% of the total plant sterols, and can comprise 5% or more in select families, species, organs

or tissues (Moreau et al., 2002). It should be noted however, that this amount is still mainly inconsequential in humans when compared to the amount of cholesterol in meat and dairy products.

Many species of the *Solanaceae* (Nightshade) plant family have relatively high levels of cholesterol. A study of the sterol composition of seed oil from 13 species of Solanum showed that in six species the combined free sterols plus steryl ester fractions contained greater than 5% cholesterol (Zygadlo, 1993).

Some tropical oils have relatively large amounts of cholesterol, such as cocoa butter (59 ppm), coconut oil (23 ppm), linseed oil (42 ppm), and palm kernel oil (40 ppm) (Toivo et al., 1998). Vegetable oils from the *Brassicaceae* family, like rapeseed and mustard, can also have modest amounts of cholesterol (Shukla et al., 2002). *Camelina sativa* has an unusually high cholesterol content for a vegetable oil, typically 1 to 5 grams per kilogram of oil (Shukla et al., 2002).

Cholesterol modulates the fluidity of cell membranes, is essential for cell menbranogenesis, growth and differentiation, and is a key structural component of muscles, the brain, and the nervous system, and is the precursor for many physiologically active compounds, including sex and molting hormones, adrenal corticoids, bile acids, and vitamin D (Norambuena et al., 2013). The role of cholesterol in humans has been studied extensively due to its role of cardiovascular disease. However, since fish are capable of synthesizing cholesterol they have no dietary requirement for it, and thus the effects of dietary cholesterol in aquafeeds

had received relatively little scientific research (Norambuena et al., 2013). Due to recent interest in the utilization of alternative sources to fish oil and fish meal in fish feedstocks, there has been a renewed effort to investigate fatty acid nutrition in farmed fish. There is a remarkable difference in the lipid composition of vegetable oils as compared to fish oils. Vegetable oils contain high levels of phytosterols and very little cholesterol, compared to fish oils, which contain large amounts of cholesterol (Norambuena et al., 2013). Thus the addition of vegetable oils to fish feed is responsible not only for the modification of the fatty acid profile, but also for the reduction in cholesterol levels.

Previous studies investigating the effect of dietary cholesterol in fish feed have shown an effect on fatty acid metabolism, however most of the diets contained abundant levels of fish oil in all diets thus providing dietary sources of cholesterol. A study looking at fatty acid metabolism in Atlantic salmon fed a diet based on vegetable oil or fish oil, found that genes of the cholesterol biosynthesis pathway were up-regulated, increasing the amount of cholesterol produced (Leaver et al., 2008). This confirms that the rate of cholesterol biosynthesis is responsive to the amount of available dietary cholesterol, which is known to inhibit biosynthesis by regulating HMG-CoA reductase (Norambuena et al., 2013). This ensures that all vital cholesterol-dependent metabolic pathways are preserved, independent of dietary cholesterol.

It can be argued then, that current fish feeds, containing alternative oils and being low in cholesterol content, are forcing fish to constantly produce cholesterol, and

an important consideration is that cholesterol biosynthesis is a rather expensive metabolic exercise, requiring 18 acetyl-CoA, 18 ATP, 16 NADPH and $4O_2$ molecules per molecule of cholesterol produced (Parish et al., 2008). Limiting dietary cholesterol could lead to effects on fish energy expenditures, and on fatty acid metabolism and tissue content however the possible effects of this are unknown. A study by Norambuena et al. (2013) found that rainbow trout fed a non-fortified vegetable oil diet, actively produced cholesterol to compensate for the reduced dietary intake. Fish fed the cholesterol-fortified diet did not need to biosynthesize cholesterol, and in fact some of the cholesterol was catabolized, i.e. broken down. This was determined by assessing the appearance (biosynthesis) or disappearance (catabolism) of cholesterol using a mass balance equation (Norambuena et al., 2013). There was no difference observed in growth or feed efficiency parameters of fish fed the vegetable oil diet versus the cholesterol diet, indicating that increased energy requirements did not affect fish growth performance (Norambuena et al., 2013).

1.3.6 Phytosterols and Cholesterol Absorption

Dietary phytosterols play an emerging role in human health, specifically their impact on cholesterol. Phytosterols are natural components of human diets, largely derived from vegetable oils, cereals, fruits and vegetables (Moreau et al., 2002). Since the mid-1990s, there has been considerable interest in the use of phytosterols

and their cholesterol lowering effects. There is a scientific consensus that reduction of cholesterol is important for coronary risk reduction (Ostlund, 2007). The concept of using functional foods, as opposed to drugs or dietary restrictions, to reduce cholesterol levels would seem an attractive alternative.

Contrary to popular belief, the use of phytosterols for lowering serum cholesterol is not new. Sitosterol, for instance, has been used since the 1950s as a supplement and as a drug for lowering serum cholesterol levels (Moreau et al., 2002). Issues with poor solubility and bioavailability of free phytosterols produced inconsistent results; however, efforts to improve solubility in recent years has renewed interest.

Non-cholesterol sterols from plants and other dietary sources exhibit poor bioavailability, with less than 5% absorption and efficient biliary elimination (Zein et al., 2019). Since few cells have the capacity to metabolize cholesterol, elimination through biliary and intestinal secretion is essential. Lipid-transport membrane proteins have been shown to be essential for the translocation of sterols and phospholipids to maintain lipid homeostasis, cellular functions, and the structural integrity of lipid bilayers (Zein et al., 2019). ATP-binding cassette transporters are the major sterol exporters responsible for both cholesterol efflux and elimination of excess cholesterol and dietary sterols. Two proteins, ABCG5 and ABCG8, are responsible for the excretion of sterols (Patel et al., 2018). In normal diets, the levels of cholesterol and non-cholesterol sterols are usually equal (Zein et al., 2019). However, 50-60% of dietary cholesterol is absorbed, while less than 5% of non-cholesterol sterols are absorbed. When more plant sterols are

ingested, they compete with cholesterol for solubilization, reducing the dietary absorption of cholesterol and lowering plasma cholesterol levels.

Phytosterols, which are inherently hydrophobic and tend to form stable crystals, must be solubilized to become bioavailable (Ostlund, 2007). Esterified forms with fatty acids were used in margarines and have a demonstrated ability to lower LDL-cholesterol levels in clinical studies (Moreau et al., 2002). These compounds reduce total cholesterol and LDL-cholesterol (low density lipoprotein) levels through a reduction in cholesterol absorption. Phytosterols and phytostanols (primarily 4-desmethyl sterols and stanols) have been shown to inhibit the uptake of both dietary and endogenously produced (biliary) cholesterol from the intestine (Moreau et al., 2002). This results in a decrease in serum and LDL-cholesterol levels. The exact mechanism by which phytosterols decrease serum cholesterol levels is not fully understood, though several theories have been proposed. One suggests that cholesterol in the intestine is less absorbable in the presence of added phytosterols and stanols. Another is based on the fact that cholesterol must enter bile-salt and phospholipid containing micelles in order to be absorbed into the bloodstream. Hence, cholesterol is only marginally soluble in these micelles and is thus displaced by phytosterols preventing its absorption (Moreau et al., 2002).

Although it has been phytosterol and phytostanol esters that have had the most success in lowering cholesterol levels, the physiologically active forms are most likely the free, un-esterified phytosterols and stanols. Sterol and stanol esters are rapidly hydrolyzed by intestinal enzymes, leaving the free phytosterol and

phytostanol forms (Moreau et al., 2002). New formulations and crystallized forms of free phytosterols are now being investigated with the aim of clarifying the true utility of these simpler and less expensive compounds for cholesterol management (Moreau et al., 2002). However, research on free phytosterols and stanols has yielded positive, but inconsistent, results, due to the difficulty in formulating and delivering these relatively insoluble substances (Moreau et al., 2002).

The beneficial effects of phytosterols on serum cholesterol levels has led to many studies on these compounds in food and human diets. Phytosterols are recognized as important components of healthy diets. Increased attention on phytosterol research has prompted the development of functional foods with added plant sterols (Lagarda et al., 2006). Potentially more important than supplements are natural dietary phytosterols, which may be bioactive in their natural food matrices (Ostlund, 2007).

1.3.7 Sources of Phytosterols

In plants, sterols are typically present in a mixture (Hartmann, 1998). Plant sterols (phytosterols) are bioactive components of all vegetable foods. They are 28 or 29 carbon alcohols with one or two carbon-carbon double bonds, typically one in the sterol nucleus and sometimes a second in the side chain (Moreau et al., 2002). Phytosterols contain an extra methyl or ethyl group, or double bond. Most phytosterol side chains contain 9-10 carbon atoms, instead of 8 as in cholesterol

(Lagarda et al., 2006). They are important structural components of cell membranes and are similar to cholesterol in vertebrates in both function and structure (Lagarda et al., 2006).

More than 200 different types of phytosterols have been discovered in plant species, the most abundant being β-sitosterol (24-α-ethylcholesterol), campesterol (24-α-methylcholesterol), and stigmasterol (Δ^{22}, 24-α-ethylcholesterol). The most important natural sources of phytosterols are predominantly oils and margarines, although they are also present in a range of seeds, legumes, vegetables, and unrefined vegetable oils (Dutta, 2003). Ergosterol ($\Delta^{7,22}$, 24-α-methylcholesterol) is the principal sterol of yeast and is found in corn, cotton seed, peanut and linseed oils (Lagarda et al., 2006). Cereal products are a significant source of plant sterols, their content higher than in vegetables (Lagarda et al., 2006). Phytosterols have also been quantified in nuts and seeds, the predominant forms being β-sitosterol, Δ^5-avenasterol, and campesterol (Lagarda et al., 2006).

Among foods of animal origin, particularly bivalve species, 20 different sterols have been detected, with cholesterol present as the major sterol. *Trans*-22-dehydrocholesterol, brassicasterol, 24-methylenecholesterol and campesterol have been detected. This wide range of sterols in bivalves reflects their varied phytoplankton food sources (Lagarda et al., 2006).

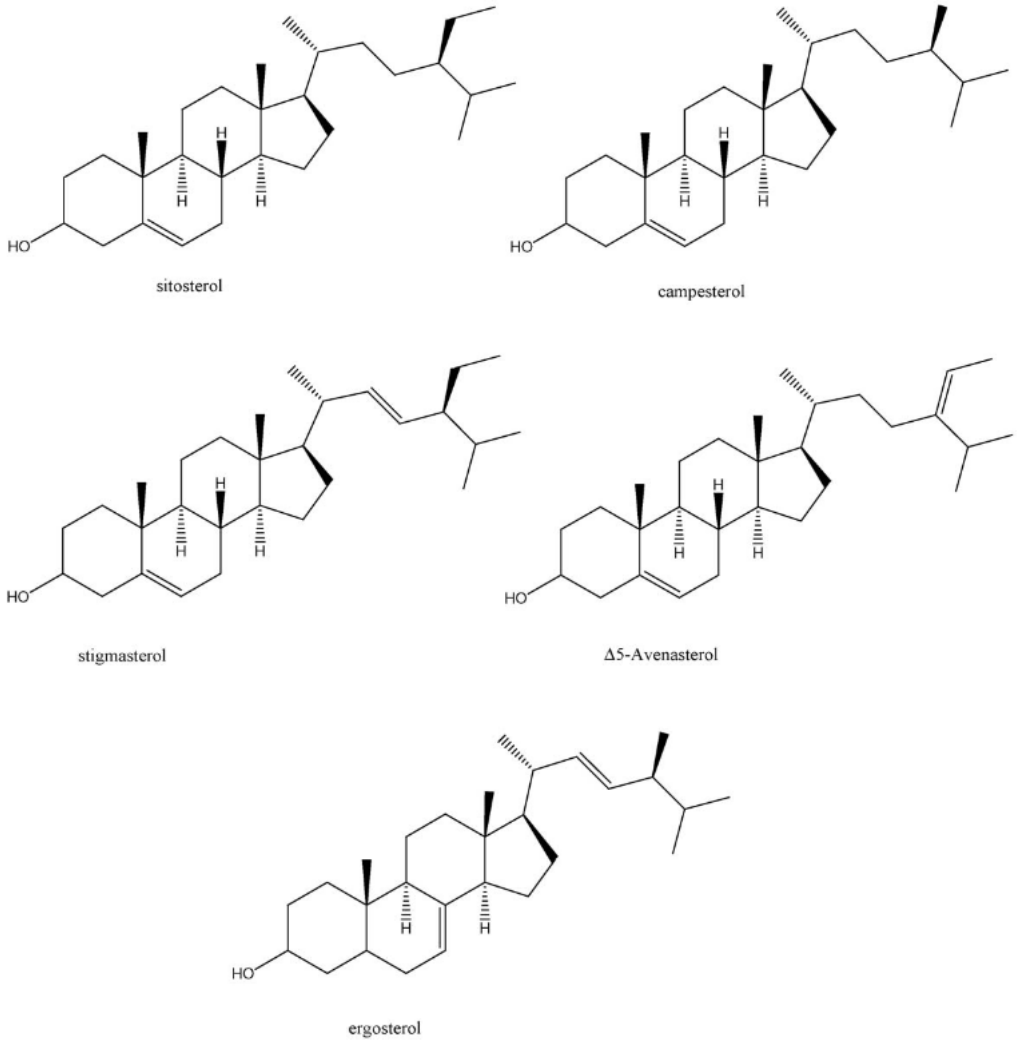

Figure 1.5: Structures of some of the most common phytosterols found in food (Lagarda et al., 2006).

There is a fascinating diversity of sterol distribution in microalgae. Some sterols are widespread, but others appear to be restricted to just a few algal classes (Volkman, 2003). The diversity of structures includes the presence or absence of a methyl group at C-4 and differences in the various positions of the double bonds in both the nucleus and side chain, the degree and positions of alkylation in the side

chain, the presence of an additional hydroxyl group and the different stereochemistry of the alkyl group at C-24 (Volkman, 2003). The distributions found range from a predominant single sterol such as cholesterol to mixtures of 10 or more 4-desmethyl and 4-monomethyl sterols. The sterol content of microalgae varies between strains and can be influenced by factors such as light intensity, temperature, and growth stage (Volkman, 2003).

All microalgae contain sterols, with Δ^5 double bond sterols with alkylation at C-24 and sterols with lack of methyl groups at C-4 being dominant. Most 4-desmethyl sterols have C-27 to C-29 carbon skeletons, but trace amounts of a C-23 steroid have been detected in a dinoflagellate from the genus *Prorocentrum* and C-30 sterols have been found in some chrysophytes (Volkman, 2003). Cholesterol is usually considered an animal sterol; however, it is surprisingly common in microalgae, and has been found in many species. C-28 sterols are major sterols in many microalgae, a common constituent of which is 24-methylcholesta-5,22E-dien-3β-ol, known as diatomsterol because of its occurrence in many diatoms. C-29 sterols such as sitosterol and stigmasterol, which are common in plants, but have also been found as a major sterol in a number of microalgae. C-29 sterols with a C-24 ethyl group and a Δ^5 double bond are also found in many *Chlorophyceae* microalgae. 4-methyl sterols are biosynthetic intermediates in the formation of 4-desmethyl sterols; however, their presence in microalgae is rare, with the exception of dinoflagellates (Volkman, 2003). The most abundant sterol, dinosterol, is derived from lanosterol and contains an unusual pattern of side chain alkylation

with methyl groups at C-23 and C-24. Several 4α-methyl sterols have been identified in microalgae from the genus *Pavlova*, thus they are not unique to dinoflagellates. Microalgae from the genus *Pavlova* contain novel 3,4-dihydroxy-4α-methylsterols are termed pavlovols. Pavlovols contain an additional hydroxyl group at C-4, a methyl group at C-4 and no nuclear double bonds. The most common compounds are 24-methylpavlovol and 24-ethylpavlovol (Volkman, 2003). Sterols have also been found in yeast, fungi and protozoans, as well as cyanobacteria, and some other bacterial strains (Volkman, 2003).

1.3.8 Characterization of Free and Total Sterols

In nature, phytosterols can be found as free sterols or fall into one of four types of conjugates. These conjugates include sterol esters (SE), steryl glycosides (SG), and acylated sterol glycosides (ASG). Phytosteryl hydroxycinnamic-acid esters (HSE), in which the sterol hydroxyl group is esterified to ferulic or *p*-coumaric acid make up the fourth type of phytosterol conjugate, and are found in seeds of corn, rice and other grains (Moreau et al., 2002). Structures of the phytosterol conjugates are found in Figure 1.6.

Sterols are frequently found in cells and plasma bound to a fatty acid as sterol esters. The cholesterol esters are the most common forms in animals. They are the form in which cholesterol is transported to and stored in various tissues. Oxygenated cholesterol esters are considered largely responsible for the development of atherosclerosis, as they

are generated in macrophages, transported, and then deposited in the atherosclerotic plates (Leray, 2013).

In plants, several sterol esters are present in cellular membranes and in seed oils. Their importance is variable, as more than 50% of sterols are esterified in groundnut and rapeseed oils, whereas less than 30% are esterified in soya or palm oil (Leray, 2013). In yeasts, all sterols are esterified by a large variety of saturated or unsaturated fatty acids. Steryl glycosides are present in most plants and in many marine invertebrates. They are linked with a carbohydrate unit, which varies depending on the plant or animal. In plants, the carbohydrate is generally glucose, xylose or arabinose, and the sterol is generally sitosterol, but campesterol, stigmasterol, brassicasterol or even cholesterol have been observed (Leray, 2013). Acylated derivatives of steryl glycosides are also present in many plants, formed by esterification of a fatty acid on the alcohol group of the carbohydrate (Leray, 2013).

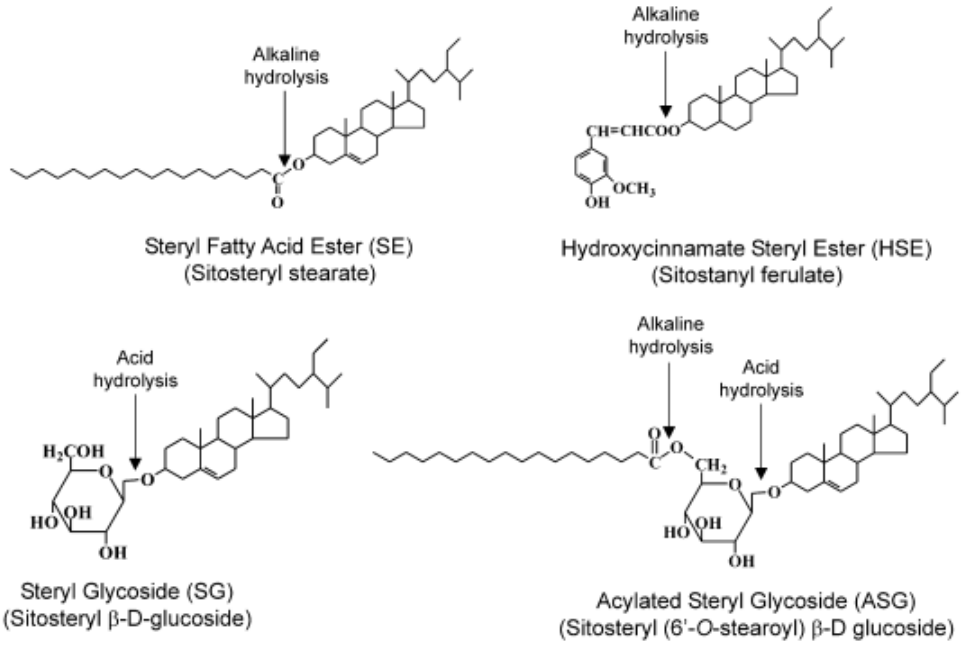

Figure 1.6: Structures of phytosterol conjugates. The sites of cleavage via alkaline hydrolysis and acid hydrolysis are indicated with arrows (Moreau et al., 2002).

1.4 Analysis of Phytosterols by Gas Chromatography

1.4.1 Lipid Extraction

Isolation, or extraction, of lipids from tissues is performed with the use of various organic solvents. Folch et al (1957) developed the chloroform/methanol/water phase system (the "Folch" method) which is considered the classic and most reliable means for quantitatively extracting lipids (Iverson et al., 2001). The Bligh and Dyer method of extraction was developed as a rapid but effective method for determining total lipid content in fish muscle (Bligh et al., 1959). The primary

advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio (Iverson et al., 2001). Since the Folch and Bligh and Dyer methods for total lipid extraction were published, there have been numerous modifications to both methods to improve recovery, such as that developed by Parrish (1999). This method is a variation of the Folch et al. method (1957) (Parrish, 1999).

1.4.2 Gas Chromatography

In the last few decades, research on gas chromatography-mass spectrometry (GC-MS) based sterol structure determination and quantification has been the most common (Singh et al., 2020). The characterization and quantitation of free phytosterols can also be achieved by several other chromatographic techniques including gas chromatography (GC), normal phase high performance liquid chromatography (HPLC), reversed phase HPLC, and capillary electrochromatography (CEC), coupled with a suitable detection method for sterols, such as flame ionization detection (FID), mass spectrometry (MS), ultra-violet detection (UV), infrared detection (IR) or nuclear magnetic resonance (NMR) (Abidi, 2001). However, many of these techniques were unable to differentiate between closely related sterol structures, such as phytosterols (Singh et al., 2020).

1.4.3 Gas Chromatography/Mass Spectroscopy

In gas chromatography, components of a sample are separated as a consequence of being partitioned between a mobile gaseous phase and a stationary solid phase (Skoog, 2007). Separation of molecules occurs based on their affinity for the stationary phase, thus they are eluted at different times and detected by the mass spectrometer. The mass spectrometer fragments the molecules into predictable ionized fragments which are detected and measured using their mass-to-charge ratio (Sparkman et al., 2011).

The combined use of gas chromatography-mass spectrometry (GC-MS) in the analysis and characterization of sterols has been studied extensively. Although the relative retention times of most common phytosterols can be found in the literature, differences in the GC columns, operating parameters, phytosterol forms, and standards can complicate their identification. GC-MS can be utilized for structural identification and confirmation, as well as quantitation (Abidi, 2001). When high temperature (>390˚C) capillary GC columns are used, sterol assays can be achieved with higher degrees of molecular weight detection sensitivity and component resolution, making GC-MS the most frequently used technique for the analysis of sterols (Abidi, 2001).

1.4.4 Gas Chromatography/Flame Ionization Detection

Gas chromatography is also frequently coupled with flame ionization detection (FID) for more initial studies and routine sterol analysis. The FID monitors analytes in the column effluent, producing a current that varies in proportion to the amount of solute present. Using gas chromatography-flame ionization detection (GC-FID), phytosterols can be partially identified by comparing retention times to that of standards. The elution order of sterols depends on their structural characteristics and molecular weight (Abidi, 2001). Although FID has proven to be a robust tool for lipid analysis, the lack of selectivity can limit the usefulness of this detector when applied to complicated samples, since only instrument response and retention time information may be gathered (Dodds et al., 2005). Complex biological extracts may contain other components which elute at similar times as sterols and which elicit an FID response, interfering in the quantification. Hence, FID may prove inadequate in some situations, leading to the need for coupling of GC to mass spectrometry for identification and quantification (Dodds et al., 2005).

1.4.5 Derivatization of Phytosterols

Direct GC analysis of phytosterols is possible (Morton et al., 1995), however, derivatization prior to analysis has been shown to provide better resolution. Un-derivatized phytosterols have low volatility and are thermally labile, making GC analysis at high temperatures unreliable (Dutta, 2004). Thus, prior to GC, sterols are usually transformed into derivatives that yield improved peak shape, resolution

and sensitivity, and a greater stability for the thermally labile unsaturated sterols (Lagarda et al., 2006).

Previous derivatization procedures employed acetylation with acetic anhydride, however silylation is now the more common method used (Abidi, 2001). The majority of sterol determinations use trimethylsilyl (TMS) ether derivatives, as the TMS derivatives are more suitable for GC-MS characterization and quantification of sterols (Abidi, 2001). Sterols are treated with bis(trimethylsilyl)-trifluoroacetamide (BSTFA), with 1% trimethylchlorosilane (TMCS) as a catalyst (Lagarda et al., 2006). These TMS-ether derivatives give enhanced component resolution and improve sample volatility (Abidi, 2001).

1.5 Project Objectives

The aim of this research was to analyze phytosterol content of alternative sources of oil for aquaculture feed, and to determine their phytosterol composition. Both qualitative and quantitative evaluation of the phytosterol content of Atlantic salmon (*Salmo salar*), that were fed alternative lipid sources will be carried out. This research will indicate which sterols, if any, are catabolized by salmon and if they can be detected in both the diets made with alternative oils and in muscle tissues. This is important in determining whether or not the addition of phytosterols to aquaculture diets provides benefits for human consumption. Current research is underway into replacing the fish oil in aquaculture fish fed with more available and cost-effective alternative oils.

Chapter 2 of this thesis explores the use of phytosterol-containing plant oils as an alternative to fish oil in fish feeds. The sterol content of camelina seed oil, a microalgae oil (*Pavlova*), and a rapeseed-based feed were examined. Diets were also examined containing varying levels of Camelina oil, rapeseed oil, or *Pavlova*, as well as tissue samples from fish fed these experimental diets.

Chapter 3 looks at the inclusion of *Schizochytrium* microbial oil in fish diets. The sterol content of the lipid extract from this microbial oil was obtained, as well as the sterol content of lipid extracts from experimental diets containing microbial oil and salmon muscle tissue lipid extracts from the experimental trials.

Chapter 2: Phytosterol oils as a replacement for fish oil in cold water fish aquafeeds: Effect on sterol composition

2.1 Introduction

Plant oils generally contain significant amounts of phytosterols, particularly campesterol, stigmasterol, and sitosterol, usually in the range of 0.2 to 2% of total sterols in the oil (Shukla et al., 2002). Most vegetable oils are thought to contain very little cholesterol, although trace amounts have been found in many vegetable oils. A few of the tropical fats and oils, such as palm oil, coconut oil, and cocoa, and a few of the field crop oils (peanut, cottonseed, linseed, and rapeseed) have modest amounts of cholesterol, although the concentrations vary depending on the source of the oil (Shukla et al., 2002). A study by Shukla in 2002 found that Camelina oil has an unusually high cholesterol content for a vegetable oil (188 ppm). They also found relatively large amounts of brassicasterol, campesterol, stigmasterol, sitosterol, and Δ^5-avenosterol (Shukla et al., 2002). Studies by Mansour et al (2014) and Kolenc et al (2020) both found significant levels of cholesterol, brassicasterol, campesterol, stigmasterol, and β-sitosterol. Additionally, Mansour et al. identified isofucosterol in their analysis of Camelina oilseed (Mansour et al., 2014).

The oilseed *Camelina sativa* is of interest from both an aquaculture and agriculture perspective. Camelina has recently been reintroduced to Canadian agriculture on account of its agriculturally robust nature (Hixson et al., 2013). In the United States, Camelina has been primarily known as a weed although it is best described as an underutilized oilseed

(Shukla et al., 2002). The plant has seen renewed interest in recent years because of its potential as an oilseed crop and research into new sources of fatty acids. Camelina's ability to grow in semi-arid and low-fertility soils, its tolerance to insects, frost and low temperatures has drawn the attention of researchers in both agriculture and aquaculture.

Camelina is also particularly unique due to its lipid content. It has an unusually high lipid content (40%) and a high amount of α-linolenic acid (ALA), which is a precursor to longer chain ω-3 fatty acids that can be synthesized by fish. Oil from Camelina seeds contains greater levels of ω-3 polyunsaturated fatty acids (PUFA) than most plant oils commonly used in aquaculture feeds. This gives it nutritional and commercial advantages over currently used plant oils such as corn, canola, soybean or palm oil (Hixson et al., 2014). It also contains a high amount of γ-tocopherol, the most potent antioxidant tocopherol isomer, thus it is naturally protective against lipid oxidation, despite increased PUFA content (Hixson et al., 2013). Camelina has more PUFA and monounsaturated fatty acids (MUFA) than other terrestrial plant oils and has a lower amount of saturated fatty acids. This is beneficial for both fish and humans. Camelina oil is sufficiently stable to constitute a technically and economically competitive alternative to fish oil as a source of ω-3 polyunsaturated fatty acids (Hixson et al., 2014).

Camelina meal (CM) is a potential protein source in aquaculture feeds, because of its crude protein level (39%) and essential amino acids such as methionine, lysine, phenylalanine, threonine, leucine, isoleucine and valine (Hixson et al., 2016). The lipid fraction of the meal is high in ω-3 and ω-6 fatty acids. It is also high in polyunsaturated and monounsaturated fatty acids. The Camelina meal used in this study was solvent-extracted

to model the oilseed by-product if most of the oil was to be removed for other purposes (Hixon et al., 2016). Camelina oil was also added to the feed to increase dietary lipids and to retain the Camelina signature in the diet, as Camelina oil is being considered as a potential lipid source in aquaculture feeds.

Algae are a diverse group of aquatic, photosynthetic organisms generally categorized as either macroalgae (seaweed) or microalgae (unicellular). Microalgae are primarily photoautotrophic (make energy using light) and can grow in a wide range of habitats. Successful commercial utilization of microalgae has been established in the production of numerous products, and a portion of this biomass has been used in the fish and shellfish cultivation industries (Hemaiswarya et al., 2011).

Combinations of different algal species have been used to provide balanced nutrition and improve animal growth. In order to be used in aquaculture, a microalgae strain has to meet various criteria such as ease of culturing, lack of toxicity, high nutritional value, and a digestible cell wall to make nutrients available (Hemaiswarya et al., 2011). Protein and vitamin content are factors in determining nutritional value, as well as polyunsaturated fatty acid content, such as DHA and EPA. Species of *Pavlova* have shown promise for aquaculture nutrition through high polyunsaturated fatty acid content and are worthy of further study including sterol content.

2.2 Methods

2.2.1 Materials

Camelina seeds were generously provided by the Faculty of Agriculture, Dalhousie University. Camelina was grown and harvested by the Department of Plant and Animal Sciences, Faculty of Agriculture, Dalhousie University at an off-campus location (Canning, Nova Scotia, Canada). The seeds were single pressed using a KEK 0500 press at the Atlantic Oilseed Processing, Ltd. (Summerside, Prince Edward Island, Canada) to extract the oil, and ethoxyquin was added to the final product as an antioxidant (Hixson et al., 2014). Prior to use, Camelina seeds were stored at approximately -20°C.

Sterol standards (brassicasterol, campesterol, cholestanol, β-sitosterol and stigmasterol) were purchased from Steroloids Inc., Newport, RI, USA. The cholesterol standard was purchased from Sigma Aldrich, St. Louis, MO, USA. All sterol standards were stored at approximately 4°C prior to use. The silylating agent (BSTFA + 0.1% TMCS) was purchased from Supelco (Newport, RI, USA) and stored at room temperature prior to use. All organic solvents used were of analytical grade.

Diets were formulated (Table 2.1) and produced at the Faculty of Agriculture, Dalhousie University (Truro, Nova Scotia, Canada). The lipid component of the diets was either from fish oil (FO) in the control diet or from camelina oil (CO). The experimental trial was conducted with salmon smolts at the Ocean Sciences Centre, Memorial University of Newfoundland. The smolts were randomly distributed into experimental tanks with triplicate tanks per treatment and 50 fish per tank. Fish were fed to apparent satiation twice

daily and feed consumption was recorded weekly, for a total of 16 weeks (Hixson et al., 2014).

In a second study by Hixson et al (2016), Camelina meal was used as the protein source in the fish feeds. Diets were formulated at the Faculty of Agriculture, Dalhousie University (Truro, Nova Scotia, Canada). Camelina meal was grown and harvested by the Department of Plant and Animal Sciences, Faculty of Agriculture, Dalhousie University (Truro, NS). Seeds were single pressed to extract the oil, and the remaining meal was ground with a hammer mill into a pre-pressed meal cake at Atlantic Oilseed Processing (Summerside, PE), then solvent extracted with petroleum ether. Diets were formulated according to published values for digestible protein and energy of nutrients, and the proximate composition and nutrient digestibility values of solvent-extracted Camelina meal (Hixson et al., 2016). The control diet (0% CM) was fish oil/fish meal based. Camelina meal was included at 15% and 40% of the diet.

Table 2.1: Formulation and proximate composition of control and experimental diets for Camelina meal experiment (Hixson et al., 2016)

Ingredient (% of diet)	**Control (0% CM)**	**15% Camelina Meal**	**40% Camelina Meal**
Fish oil	5.43	5.43	5.43
Fish meal	50.49	45.08	36.05
Wheat middlings	27.13	16.98	0.07
Wheat gluten	5.0	5.0	5.0
Whey powder	5.0	5.0	5.0
Krill hydrolysate	2.5	2.5	2.5
Corn starch	0.25	0.25	0.25
Vitamin premix	1.95	1.95	1.95
Mineral premix	1.95	1.95	1.95
Choline chloride	0.3	0.3	0.3
Camelina meal	-	15.0	40
Camelina oil	-	0.56	1.5

2.2.2 Instrumentation

For identification of sterols, lipid extracts were analyzed using an Agilent 6890 N Gas Chromatograph coupled to an Agilent 5973 Mass Spectrometer. Use of this instrument was provided by the Centre for Chemical Analysis, Research and Training (C-CART) at Memorial University. Separation of sterols was achieved using an Agilent DB-5ms (95% dimethyl-, 5% diphenyl-polysiloxane) GC column (30 m, 0.25 μm thickness, and 0.25 mm i.d.). All analyses were run in splitless mode, using helium as the carrier gas at a pressure

of 14 psi. 1.0 μL samples were injected into the inlet at a temperature of 290°C. The temperature of the column was held at 80°C for 1 min, followed by ramping to 200°C at a rate of 50°C/min. The temperature was finally increased to 305°C at a rate of 4°C/min and held for 5 min, for a total analysis run time of 34.65 min. The quadrupole MS ion source was operated in electron ionization (EI) mode at 70 eV. The ion source temperature was 200˚C and the interface temperature was 180 ˚C. The scan parameters were set to detect m/z values between 50-550, with a scan rate of 2.94 scans/s.

2.2.3 Sample Preparation and Derivatization

Lipid extraction was performed using a modified Folch procedure (Parrish, 1999). In this procedure, the fish feed, or the tissue sample, was placed in a test tube and a mixture of chloroform/methanol 2:1(v/v) was added. The content of the tube was homogenized using a polytron PCU-2-110 homogenizer (Brinkmann Instruments, Ontario, Canada). The metal-ended rod of the homogenizer was washed into the tubes with 1 mL 2:1 chloroform/methanol, followed by 0.5 mL chloroform-extracted water. This gave a final ratio of chloroform/methanol/water (8:4:3, by vol) which was followed by sonication for 4 min. Samples were then centrifuged at a speed of 3000 rpm for 2-3 min. The lower organic layer, which contained the lipids, was then drawn through a Pasteur pipette using a double pipetting technique, into a vial. The extraction process was repeated four times to ensure that all lipids were extracted. Upon removal of the organic layer, the pipettes were each rinsed with 1.5 mL ice-cold chloroform. The extracts were then concentrated to near dryness by roto-evaporation in a water bath heated between 40 - 45°C and transferred to

sample vials. Vials were then flushed with nitrogen gas and sealed. Samples were kept at -20°C until analysis.

Derivatization of free sterols was performed by silylation with *N, O*-bis-trimethylsilyl trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) to form their corresponding trimethylsilyl (TMS)-ethers. Samples were evaporated until dryness under a stream of nitrogen. 100µL of BSTFA containing 1% TMCS was added to the lipid extract and heated at 85ºC for 15 minutes. Samples were then cooled to room temperature and excess reagent was evaporated under nitrogen gas. 500 µL of hexane/dichloromethane (1:1, by vol) was added followed by addition of 100 µL of 100 mg/L of 5α-androstanol as internal standard and then stored at -20˚ until analysis by GC-MS and GC-FID.

2.3 Results

2.3.1 Camelina oil

Camelina oil for analysis was extracted from a seed sample using a modified Folch method, and the sterols present derivatized to their corresponding TMS-ethers prior to analysis by GC-MS. The peaks were identified by comparing their relative retention times with those of the standards, as well as literature values and elution patterns, and confirmed by their unique mass spectra. The initial class of compounds which elutes from the column is primarily long chain fatty acid TMS esters, with the sterol region beginning at approximately 23 minutes retention time.

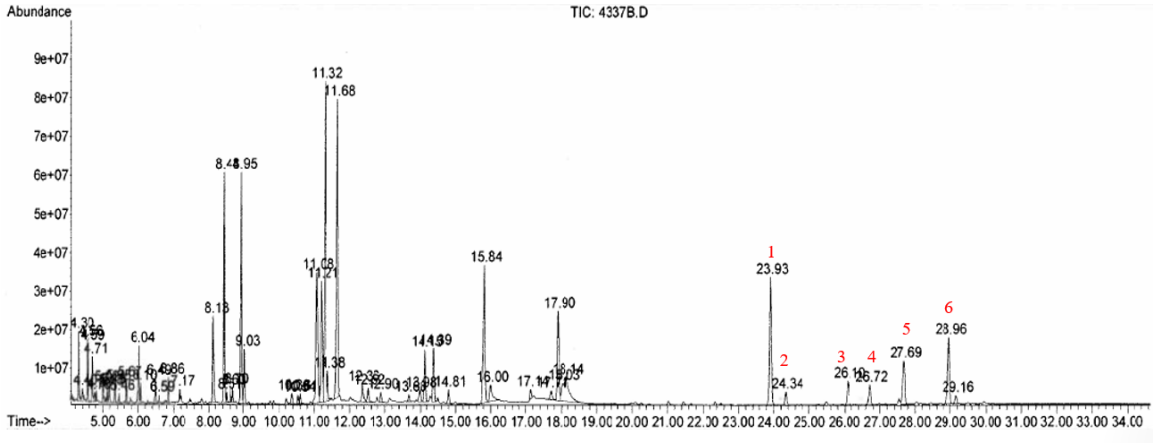

Figure 2.1: Total ion chromatogram of the lipid extract from Camelina seed, indicating sterols present as TMS-ethers. 1. β-tocopherol 2. Cholesterol 3. Brassicasterol 4. Campesterol 5. Stigmasterol 6. β-sitosterol.

A total of six major sterols were identified. There is a significant peak at 23.93 minutes which was identified as β-tocopherol TMS ether. β-tocopherol, a form of vitamin E, is commonly found in plant oils. Cholesterol TMS ether elutes next, although it is a relatively small peak in comparison to the other sterols. The remaining sterol TMS ethers in order of elution are brassicasterol, campesterol, stigmasterol, and β-sitosterol. The order of elution of the sterols identified is in agreement with the literature on sterols typically present in Camelina. An analysis of peak heights indicated that β-sitosterol is in greatest abundance, which is similar to the study of Camelina by Shukla et al (2002). Stigmasterol is the second most abundant, followed by brassicasterol, campesterol, and cholesterol respectively. This is typical of Camelina, and similar results have been seen in other studies (Shukla et al., 2002; Kolenc et al., 2020).

2.3.2 Experimental Diets

GC-MS analysis of the Camelina meal experimental diets was performed by Valerie Parsons, Department of Chemistry, Memorial University. The total lipid extracts of all samples were extracted using a modified Bligh and Dyer procedure. The free sterol content of each type of Camelina meal was determined by comparison of peak areas to calibration data (Parsons, 2013).

The analysis of the commercial fish feed indicated the presence of some common phytosterols, including campesterol, stigmasterol and β-sitosterol. The presence of these phytosterols can likely be attributed to the high wheat content of the commercial feed, 27.13% wheat middlings (leftover particles of bran, germ and flour produced during the milling process). No detectable levels of brassicasterol were found, as expected, as brassicasterol is unique to plants of the Brassicaceae family, to which wheat does not belong. Although the cholesterol content was not quantitatively measured, an analysis of the chromatograms using triangulation of peak area provided an estimation of cholesterol content. The highest cholesterol content was observed in the commercial feed as was expected given the higher inclusion of fish meal.

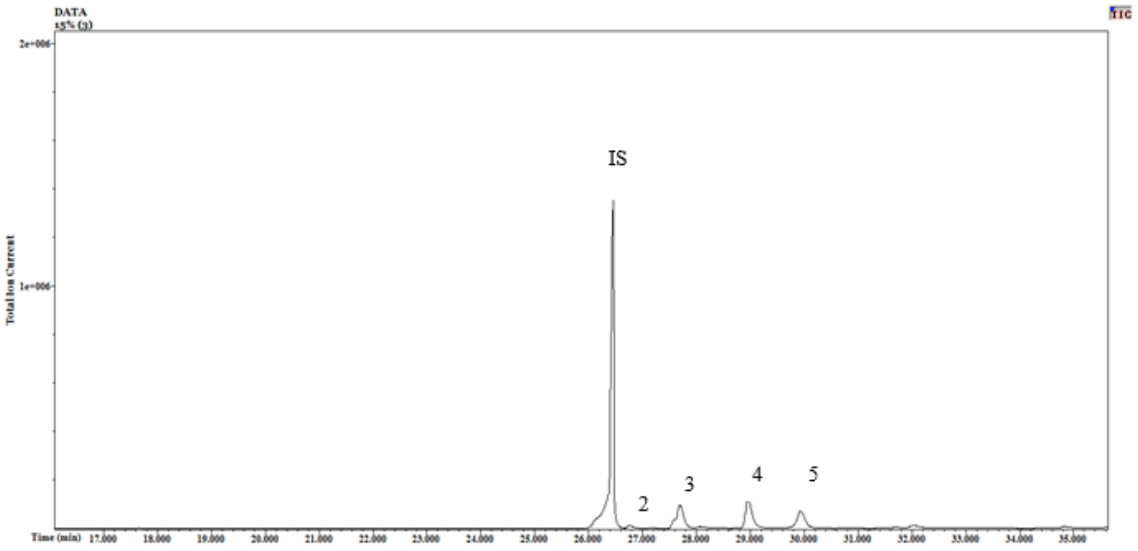

Figure 2.2: Selected ion mode chromatogram of the lipid extract from diet containing 40% Camelina meal. Sterols (TMS ethers) identified: 2. Brassicasterol (m/z 470) 3. Campesterol (m/z 472) 4. Stigmasterol (m/z 484) 5. β-sitosterol (m/z 486) (Parsons, 2013).

The phytosterols identified in the Camelina feed used in the experimental diets were brassicasterol, campesterol, stigmasterol, and β-sitosterol. The concentration of each sterol is summarized in the Table 2.2, as well as the total sterol content and phytosterol: cholesterol ratio. The total sterol content increased with the increased inclusion of Camelina meal. The ratio of phytosterols to cholesterol also increased with the inclusion of Camelina meal, as would be expected with the increase in phytosterol added to the diets.

Table 2.2: Concentrations of phytosterols present in experimental camelina meal diets (μg/g)[1].

	Control (0% CM)	**15% Camelina Meal**	**40% Camelina Meal**
Cholesterol	77.8 ± 5.6	68.9 ± 7.9	70.1 ± 8.4
Brassicasterol[2]	N/A	10.8 ± 0.6[c]	13.1 ± 1.7 [c]
Campesterol[2]	32.2 ± 0.7[b]	35.3 ± 2.1 [b]	38.4 ± 1.8 [b]
Stigmasterol[2]	8.55 ± 1.30 [c]	9.32 ± 0.97 [c]	6.44 ± 0.36 [c]
β-Sitosterol[2]	66.9 ± 2.8[a]	88.0 ± 2.2 [a]	93.1 ± 3.9 [a]
Total Sterols	185.5 ± 1.8	212.3 ± 1.6	221.1 ± 2.3
Phytosterol:Cholesterol Ratio	1.38	2.08	2.15

[1] Values are means ± standard deviation (n=3). Means with different superscripts indicate significant differences. [2] Phytosterol data provided by V. Parsons.

In the study by Hixson et al (2016), lipid class composition was determined using an Iatroscan Mark VI TLC-FID (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), silica coated Chromarods and a three-step development method (Hixson et al., 2016). The total lipid content increased with greater inclusion of Camelina meal (Table 2.3). Sterols were present in all diets but did not differ significantly among inclusion levels.

Table 2.3: Total lipid and lipid class composition of Camelina meal diets[1] (Hixson et al., 2016).

	Control (0% CM)	15% Camelina Meal	40% Camelina Meal
Total Lipid (mg/g)	8.9 ± 0.2[a]	12.2 ± 0.3[b]	14.5 ± 0.8[c]
Lipid Class (% total lipid)			
Triacylglycerol	85.0 ± 2.7	87.0 ± 2.7	88.9 ± 2.7
Sterols	2.5 ± 1.2	2.6 ± 0.9	3.0 ± 1.2
Phospholipid	1.1 ± 0.3	0.9 ± 0.1	1.1 ± 0.3

[1] Values are expressed as % total lipid. Values are means ± standard deviation (n=3). Means with different superscripts indicate significant differences.

2.3.3 Muscle Tissue Extracts

Salmon from the 16-week feeding trial were sampled for lipid analysis. Muscle tissue was subsampled, and lipids were extracted according to Parrish (1999). Lipid extracts were analyzed using GC-MS as described in the instrumentation section.

In the control group, which was fed diets containing fish oil, the only sterol detected was cholesterol. Two additional sterols were identified, campesterol and β-sitosterol, in salmon fed the diet containing 40% Camelina oil. Campesterol and β-sitosterol were also identified in salmon fed the diet with 100% of fish oil substituted with Camelina oil. The other sterols that were detected in the camelina seed extract and the diets, brassicasterol and stigmasterol, were not detected in the muscle tissue. Total sterol amounts increased in

salmon fed the 100% Camelina diet, which can be attributed to the additional phytosterols detected, as well as an increase in cholesterol content.

The phytosterol: cholesterol ratio of the muscle tissue from salmon fed the 100% Camelina diet was 0.41. Since there has been limited research focused specifically on the effects of phytosterols, it is difficult to isolate the specific effects of phytosterols from other dietary compounds such as cholesterol. Phytosterol rich ingredients are generally used as replacement for cholesterol rich ingredients, leading to an inverse relationship between amounts of phytosterols and cholesterol. It is not clear whether it is the absolute amounts of phytosterols and cholesterol or the ratio between these that may affect fish lipid metabolism (Sissener et al., 2017). Since there were no phytosterols detected in the muscle tissue from the control diet, we are unable to compare the phytosterol: cholesterol ratio in this case.

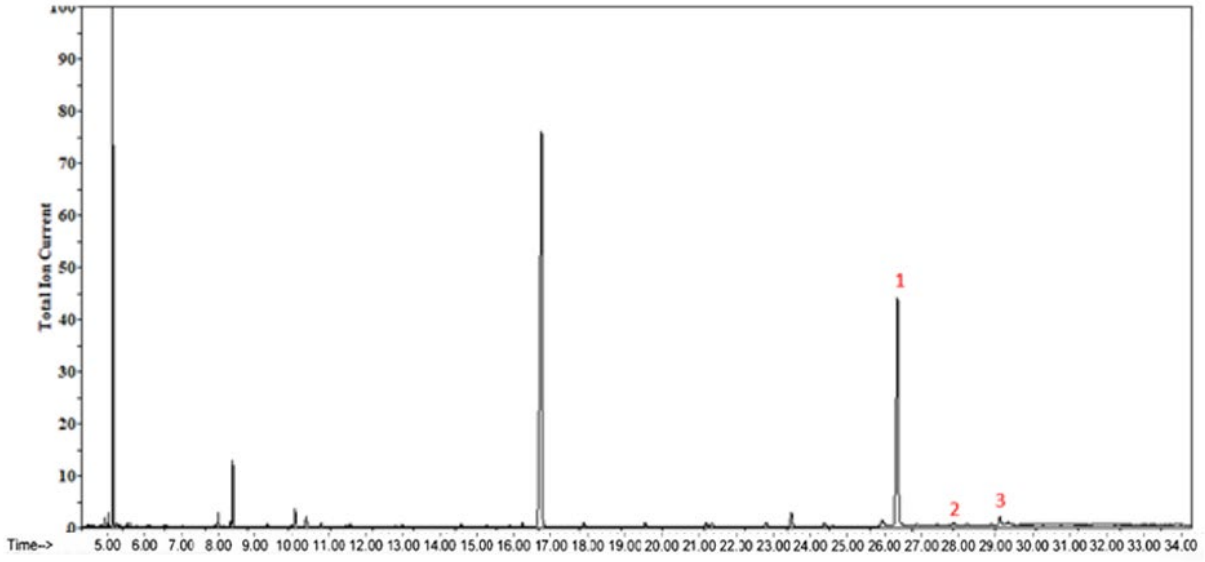

Figure 2.3: Total ion chromatogram of salmon muscle tissue lipid extract from week 16 of the experimental trial with 100% camelina oil substitution. Peaks identified: 1. Cholesterol 2. Campesterol 3. β-sitosterol.

Table 2.4: Sterol composition of salmon muscle tissue lipid extract from week 16 of the experimental trial with 100% Camelina oil substitution (μg/g) [1].

	Control (100% FO)	**100% Camelina Oil**
Cholesterol	140 ± 60	120 ± 60
Campesterol	-	12 ± 30
β-Sitosterol	-	38 ± 20
Total Sterol	140 ± 60	170 ± 40
Phytosterol:Cholesterol Ratio	-	0.41

[1] Values are means ± standard deviation (n=9).

In the study by Hixson et al (2014), lipid class composition was determined using an Iatroscan Mark VI TLC-FID (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), silica coated Chromarods and a three-step development method (Hixson et al., 2014). When comparing Atlantic salmon from the experimental feeding trial with 100% fish oil or 100% Camelina oil, salmon fed the Camelina oil diet had significantly lower total lipid content (Table 2.5) in the white muscle tissue relative to the fish oil diet (Hixson et al., 2014). Total free sterol content in the fish oil diet was low at 0.02 mg/g, and free sterols were not detected in the Camelina oil diet. Iatroscan data (Table 2.5) for sterols does not include conjugated sterols. This could explain the difference in the total sterol amounts determined by GC-MS versus the Iatroscan amounts.

Table 2.5: Total lipid and lipid class composition by Iatroscan of muscle tissue in Atlantic salmon fed fish oil and Camelina oil containing diets (mg/g)[1] (Hixson et al., 2014).

	Control (100% FO)	**100% Camelina Oil**
Total Lipid (mg/g)	90.4 ± 24^{a}	55.7 ± 16^{b}
Triacylglycerol	83.9 ± 2.3^{a}	52.3 ± 5.3^{b}
Free Sterols	0.02 ± 0.001	-
Phospholipid	3.28 ± 0.71^{a}	1.46 ± 0.44^{b}

[1] Values are means ± standard deviation (n=9). Means with different superscripts indicate significant differences.

2.3.4 Pavlova

A lipid extract from a *Pavlova* species (*Pavlova* sp. CCMP 459) was analyzed via GC-MS for sterol composition. The lipid extract was derivatized with BSTFA+TMS into its TMS ethers as in the previous procedure described above. A total of 9 sterols were identified. In order of retention time, the sterols identified were cholesterol, campesterol, stigmasterol, stigmastanol, spinasterol, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, 24-methylpavlovol, and 24-ethylpavlovol.

24-methylpavlovol and 24-ethylpavlovol are 3,4-dihydroxy-4α-methyl sterols which are unique to the genus *Pavlova* and have been termed pavlovols (Volkman, 2003). These compounds have a number of unusual features including the presence of an additional hydroxyl group at C-4, a methyl group at C-4 and no nuclear double bonds (Volkman, 2003).

The mass spectrum of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol is almost identical to that of the closely related sterol dinosterol and the mass spectrum of 4α-methyl-24-ethyl-5α-cholestan-3β-ol is almost identical to that of dinostanol. Dinosterol is a 4α-methyl sterol that is an intermediate in the formation of 4-desmethyl sterols. It is expected in small amounts in microalgae and is very closely related to 4α-methyl-24-ethyl-5α-cholestan-3β-ol. The presence of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol *versus* dinosterol is confirmed by looking at the intensity of the ion at m/z 83. According to Volkman et al. (1990), m/z 83 was the second major peak after the base peak. As this is also the case in

this mass spectrum (Figure 2.4), we can confirm the identity of the phytosterols as 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol and 4α-methyl-24-ethyl-5α-cholestan-3β-ol.

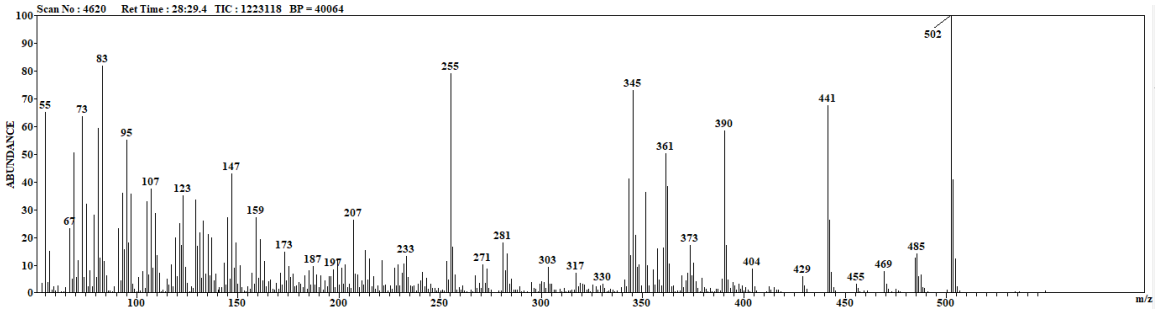

Figure 2.4: Mass spectrum of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol-TMS ether.

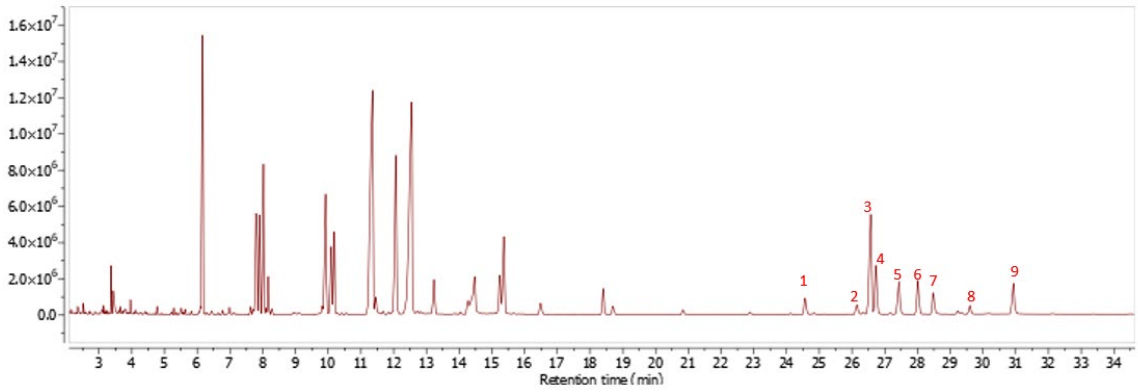

Figure 2.5: Total ion chromatogram of the lipid extract from *Pavlova*, indicating sterols present as TMS-ethers. 1. Cholesterol 2. Campesterol 3. Stigmasterol 4. Stigmastanol 5. Spinasterol 6. 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol 7. 4α-methyl-24-ethyl-5α-cholestan-3β-ol 8. 24-Methylpavlovol 9. 24-Ethylpavlovol

Table 2.6: Sterols identified in *Pavlova* lipid extract.

Sterol	**Common Name**	**Formula**	**MW(+TMS)**
Cholest-5-en-3β-ol	Cholesterol	$C_{27}H_{46}O$	458
24-methylcholest-5-en-3β-ol	Campesterol	$C_{28}H_{48}O$	472
23,24-dimethylcholest-5,22E-dien-3β-ol	Stigmasterol	$C_{29}H_{48}O$	484
4, 24-dimethyl-5α-cholest-22E-en-3β-ol	Stigmastanol	$C_{29}H_{48}O$	484
23,24-dimethylcholest-5-en-3β-ol	Spinasterol	$C_{29}H_{50}O$	486
4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol	N/A	$C_{30}H_{52}O$	500
4α-methyl-24-ethyl-5α-cholestan-3β-ol	N/A	$C_{30}H_{54}O$	502
4α,24-dimethyl-5α-cholestan-3β,4β-diol	24-Methylpavlovol	$C_{30}H_{56}O$	504
4α-methyl-24-ethyl-5α-cholestan-3β,4β-diol	24-Ethylpavlovol	$C_{31}H_{58}O$	518

Guerra et al. (2022) conducted a feeding trial that incorporated algal biomass (AB) derived from *Pavlova* sp. strain CCMP459 (Pav459) into experimental diets. Fish meal and fish oil were partially replaced with the Pav459 algal biomass. Two experimental diets were used; one composed of 50/50 fish meal and Pav459 algal biomass, containing reduced fish oil (FM/AB); and a second composed of full replacement of fish meal with Pav459 algal biomass (AB), also containing reduced fish oil. Lipids were extracted and lipid classes were determined using TLC on silica-coated Chromarods and a three-step development method, followed by Iatroscan analysis.

The total lipid content of the Pav459 algal biomass was 109.6 mg/g wet weight, with a sterol content of 10.9%. There were no significant differences in total lipid composition among the treatments. In the experimental diets, sterol content ranged from 3.5% to 7.5%. The sterol content of the diet with partial replacement of FM with AB was significantly higher than the control diet.

Table 2.7: Lipid class composition of Pav459 and experimental diets[1] (Guerra et al., 2022).

	AB Pav459	**FM Diet**	**FM/AB Diet**	**AB Diet**
Total Lipid (mg/g)	109.6 ± 10.4	144.2 ± 23.6	200.0 ± 38.2	174.5 ± 34.8
Triacylglycerol	24.7 ± 0.4	74.1 ± 1.8	75.1 ± 1.1	72.7 ± 2.2
Sterols	10.9 ± 1.0	3.5 ± 0.4^{b}	7.5 ± 0.7^{a}	4.7 ± 1.7^{b}
Phospholipid	16.6 ± 1.4	6.3 ± 1.7	3.1 ± 0.5	6.1 ± 2.2

[1] Data expressed as mg/g. Values are means ± standard deviation (n=3). Means with different superscripts indicate significant differences. FM = fish meal (control); FM/AB = fish meal/AB Pav459; AB = AB Pav459

After 12 weeks of feeding the experimental diets to the salmon, tissue samples from liver and muscle were analyzed. The total lipid concentration of the liver increased across all dietary treatments but there was no significant difference in any lipid class. In the muscle tissue, there again was no significant difference in the total lipid content across the dietary treatments; however the sterol content in salmon fed the AB diet was low and significantly different from that of salmon fed the FM and FM/AB diets.

Table 2.8: Lipid class and composition of Atlantic salmon liver and muscle tissues after 12-week experimental trial[1] (Guerra et al., 2022).

	Liver FM	**Liver FM/AB**	**Liver AB**	**Muscle FM**	**Muscle FM/AB**	**Muscle AB**
Total Lipid (mg/g)	28.3 ± 4.9	24.4 ± 4.5	26.3 ± 8.3	70.4 ± 15.9	64.5 ± 2.7	63.4 ± 12.5
Triacylglycerol	12.4 ± 16.3	10.1 ± 10.6	6.8 ± 7.0	43.9 ± 10.3	54.8 ± 4.9	49.2 ± 8.6
Sterols	20.4 ± 4.1	23.9 ± 3.5	21.9 ± 3.6	11.0 ± 2.2[a]	11.9 ± 3.8[a]	6.2 ± 4.3[b]
Phospholipid	23.4 ± 5.4	25.4 ± 5.3	24.1 ± 4.3	20.3 ± 5.1[b]	8.1 ± 5.2[b]	18.4 ± 7.4[a]

[1] Data expressed as mg/g. Values are means ± standard deviation (n=9). Means with different superscripts indicate significant differences. FM = fish meal diet (control); FM/AB = fish meal/AB Pav459 diet; AB = AB Pav459 diet

2.4 Discussion

2.4.1 Camelina

The sterol composition of the Camelina oil was determined to contain 5 major sterols: cholesterol, brassicasterol, campesterol, stigmasterol and β-sitosterol. Kolenc et al., 2020, Mansour et al., 2014, Schwartz et al., 2008, and Shukla et al., 2002 also found the same 5 major sterols. Mansour et al. (2014) also detected isofucosterol which was not detected here; while Shukla et al. (2002) found Δ^5-avenosterol which was also not detected in this study. Schwartz et al. (2008) also found other minor sterols such as Δ^7-avenosterol, citrostadienol, cycloartenol and 24-methylene cycloartenol. Campesterol and stigmasterol are usually present in vegetable oils which can differ in amount over a large range depending on the source of the oil. Sitosterol is present in large amounts in the majority of

vegetable oils, including Camelina. Brassicasterol is typically absent, or present in trace amounts, in most other oils, but is present in substantial amounts in vegetable oils from the Brassicaceae family, such as Camelina (Shukla et al., 2002). The level of cholesterol found in Camelina oil has been found to be larger than in most vegetable oils (Shukla et al., 2002).

Camelina meal was investigated to determine if it could be used as a protein source in aquaculture diets. Sterols identified in the camelina meal diets were brassicasterol, campesterol, stigmasterol and β-sitosterol. Three of these, campesterol, stigmasterol and β-sitosterol, were also present in the control diet. This is likely due to the presence of wheat middlings in the formulation of the diets, as these sterols are commonly present in wheat (Piironen et al., 2002). Brassicasterol can be attributed to the presence of the Camelina meal as it is not present in wheat. For campesterol, stigmasterol and β-sitosterol, there was no significant difference in the amounts of each sterol with the increase in Camelina meal inclusion. The total amount of phytosterols increased as the amount of camelina meal was increased. This is in agreement with the total sterol content from the Iatroscan data in Table 2.3. The total lipid content, which includes all lipid classes, also increased with camelina meal inclusion.

The replacement of fish oil in fish feed with Camelina oil was evaluated to determine if any of the sterols present in the Camelina seed would be transferable to the diets, and in turn, to the fish flesh. The sterol profile of the Camelina seed included cholesterol, brassicasterol, campesterol, stigmasterol, and β-sitosterol, as well as β-tocopherol. The results from the Camelina meal diet analysis indicated that most of the sterols detected in the seed oil were also detected in the feed samples, brassicasterol, campesterol,

stigmasterol, and β-sitosterol. Cholesterol was also present in the Camelina meal diets, however it co-eluted with the internal standard used (cholestenol) and the peak could not be easily distinguished from the internal standard peak. In the muscle tissue samples from fish fed diets containing Camelina oil, only two sterols (campesterol and β-sitosterol) were detected. β-sitosterol and campesterol were detected in fish muscle tissue from both experimental diets, with 40% and 100% substitution with Camelina oil. Since the diet results indicated the β-sitosterol and campesterol were the most abundant sterols present, it is possible that the other sterols may have been present, but at levels too small to be detected in the muscle tissue. The Iatroscan data failed to detect any free sterols in the diet containing 100% Camelina oil. This can possibly be attributed to the trace level of sterols present in the muscle tissue of the fish, which are below the Iatroscan detection limits. The total lipid content of salmon fed a diet containing 100% camelina oil was significantly lower than the salmon fed the fish oil diet. This can be attributed to the differences in lipid utilization, metabolism, and storage, with the use of alternative oils (Hixson et al., 2014).

Total sterol amounts (Table 2.4) were 140 μg/g for muscle tissue from fish fed the control diet and 0.17 mg/g for muscle from fish fed the diet with 100% substitution of Camelina oil. In Miller et al. (2007) the average sterol content in white muscle tissue across four treatments (control, Canola oil, Echium oil, and fish oil) was 160 μg/g. There was no significant difference in the sterol content in the white muscle tissue among dietary treatments, as was also the case with the Camelina oil treatments. Miller et al. (2007) detected five phytosterols in the white muscle from the canola oil diet, with brassicasterol, campesterol, and β-sitosterol being the most abundant. As mentioned above, campesterol

and β-sitosterol were the only two sterols detected in the muscle tissue from the Camelina oil substituted diet, which coincides with findings by Miller et al. (2007), except for the lack of brassicasterol. The cholesterol content observed by Miller et al. averaged 170 μg/g across all treatments with no significant difference between the experimental diets and the control (fish oil). The cholesterol content in the salmon muscle tissue from the Camelina trial averaged 130 μg/g, also with no significant difference amongst treatments. The total phytosterol concentration in the white muscle tissue in the study by Miller et al. was significantly higher in the canola oil diet as compared to the control (FO) diet with a fourfold increase (Miller et al., 2007). In the Camelina study presented here, phytosterols were not detected in the control (FO) diet, but were present in the 100% Camelina oil substituted diet at an average of 25 μg/g.

2.4.2 Pavlova

Microalgae have diverse uses in aquaculture. Their applications are mainly to provide nutrition and to enhance the colour of the flesh of salmonids (Hemaiswarya et al., 2011). Successful commercial utilization of microalgae has been established in the production of nutritional supplements, antioxidants, cosmetics, natural dyes, and polyunsaturated fatty acids (Hemaiswarya et al., 2011). The most frequently used species are *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema* and *Thalassiosira* (Hemaiswarya et al., 2011). Several microalgae species, including *Pavlova lutheri*, have been claimed to possess great nutritional value owing to

their ability to synthesize and accumulate great amounts of polyunsaturated fatty acids (PUFA). The use of *Pavlova lutheri* as a feedstock has been reported to enhance the growth rate of fish larvae (Meireles et al., 2003). A great diversity of sterol structures have been found in microalgae indicating the potential benefits of their inclusion in aquafeed diets. Diets including *Pavlova* sp. have shown promise for sea scallop (*P. magellanicus*) larvae and postlarvae, as well as bay scallop (*Argopecten irradians*) larvae, postlarvae and juveniles (Milke et al., 2008).

The sterols detected in the Pavlova oil were cholesterol, campesterol, stigmasterol, stigmastanol, spinasterol, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, 24-methylpavlovol, and 24-ethylpavlovol. C-29 sterols such as stigmasterol and spinasterol are commonly associated with plant oils but a number of microalgae have been found to contain these sterols as well (Milke et al., 2008). Campesterol is a common C-28 plant sterol and has also been found in microalgae (Milke et al., 2008; Parrish et al., 2011). Cholesterol is usually considered to be an animal sterol, yet it is surprisingly common in microlagae (Parrish et al., 2011). Cholesterol is the major sterol present in many red algae (Volkman, 2003) and was also previously identified in *Pavlova* species by Milke et al (2008) and Parrish et al (2011). A study by Xu et al. (2007) detected 5 sterols in *Pavlova viridis*, stigmasterol, sitosterol, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol, as well as 24-methylpavlovol and 24-ethylpavlovol. This is consistent with results seen here, with the exception of sitosterol which was not detected in the Pav 459 sample.

2.4.3 Other plant sources

Numerous plant sources have been investigated for their potential use in aquafeeds, replacing fish oil and/or fish meal with alternative sustainable sources of lipids and protein. Scientific researchers and feed manufacturers have made significant progress by looking at alternative protein sources for use in fish diets in order to develop feeds that provide adequate nutrition for animals' growth, while reducing to a minimum the use of traditional sources (Gasco et al., 2018). Plant oils are great candidates as they are rich in linoleic and α-linolenic acids, are less expensive than fish oil, and have not shown to be detrimental to fish health or growth (Gasco et al., 2018).

A study by Foroutani et al. (2020) evaluated the effects of using such alternative diets containing low levels of marine resources on the lipid class as well as the fatty acid and elemental compositions of liver and head kidney tissues of farmed Atlantic salmon. Seven diets were used with different inclusion levels of fish meal, fish oil, animal by-products, vegetable oil, vegetable protein and DHA + EPA (Foroutani et al., 2020). The vegetable protein diets contained high levels of vegetable proteins such as corn gluten, soy protein and wheat gluten meal. The vegetable oil diet used rapeseed oil as the plant oil source, at varying levels of inclusion. The vegetable oil diets were classed according to the level of ω-3 long chain fatty acids (ω3LC), with 0%, 1.0% or 1.41% of the ω-3 long chain fatty acids included. The experiment was conducted on Atlantic salmon smolts (*Salmo salar*) for a 14 week trial.

Table 2.9: Experimental diet ingredients (Foroutani et al., 2020).

Ingredients (%)	**Marine Diet**	**Vegetable Protein**	**ω3LC 0%**	**ω3LC 1.0%**	**ω3LC 1.41%**
Fish meal	35	5	5	5	5
Animal by-products[1]	15	10	22	21	22
Corn gluten	1.4	7.0	5.1	5.0	5.0
Soy protein concentrate	7	35	25	25	25
Wheat gluten meal	0.9	4.7	3.4	3.3	3.3
Fish oil	12	5	0	5	7
Vegetable Oil[2]	6	17	27	22	20
Premix[3]	0.5	3.0	2.4	2.3	2.4
EPA + DHA	2.91	1.00	0.09	1.00	1.41

[1] Poultry, feather, and blood meal. [2] Rapeseed oil.
[3] Vitamins, trace elements, and inorganic phosphorous.

The composition of lipid classes was determined through a three-step development thin-layer chromatography method (Parrish, 1987). Lipid extracts were then scanned in an Iatroscan (Mark VI; Iatron Laboratories inc., Tokyo, Japan) using a flame ionization detector (Foroutani et al., 2020). Lipid composition in the diets was mainly triacylglycerols, sterols and phospholipids (Table 2.8). Sterol content did not increase significantly with the inclusion of ω-3 long chain fatty acids.

Table 2.10: Lipid class proportions in experimental diets[1] (Foroutani et al., 2020).

Lipid Classes (% total lipid)	**Marine Diet** (*n*=6)	**Vegetable Protein**(*n*=6)	**ω3LC 0%** (*n*=9)	**ω3LC 1.0%** (*n*=9)	**ω3LC 1.41%** (*n*=9)
Triacylglycerol	54.5 ± 15 ^{ab}	72.2 ± 10.7^{ab}	75.0 ±8.4^{a}	72.4 ± 5.0 ^{ab}	71.3 ± 13.8 ^{ab}
Sterol	4.2 ± 1.4 ^{ab}	2.7 ± 1.9 ^{ab}	2.7 ± 1.9 ^{ab}	2.2 ± 1.3 ^{b}	3.2 ± 1.5 ^{ab}
Phospholipid	6.3 ± 1.4 ^{ab}	8.6 ± 4.9 ^{ab}	5.1 ± 3.5 ^{ab}	6.7 ± 4.2 ^{ab}	3.8 ± 1.9^{b}

[1] Data expressed as % of total lipid of diet dry weights. Values are means ± standard deviation (n=6). Means with different superscripts indicate significant differences.

Cholesterol and phytosterols in the diets, and in the muscle tissue from the fish fed these diets, were determined using GC-MS. Diets containing the highest level of fish oil and fish meal (marine diet) as well as the diet with a high level of rapeseed oil and low fish oil (ω3LC 1.0%) were analyzed. Sterols detected in the diets were cholesterol, occelasterol, brassicasterol, and stellasterol. Lower cholesterol proportions and higher phytosterol proportions were found in the rapeseed oil treatment (ω3LC 1.0%). Occelasterol (5α-ergosta-7,22-dien-3β-ol) is 27 carbon, Δ-5 sterol, characteristic of phytoplankton. Occelasterol was likely trophically transferred by zooplankton. It was present in both the marine and the vegetable oil diets as well as the muscle tissue samples from both diets. Brassicasterol is commonly found in vegetable oils, and seed oils extracted from rapeseed which typically has a higher brassicasterol content than other plant sterols. Brassicasterol was found in both the marine diet and the ω3LC 1.0% diet, with higher amounts in the ω3LC 1.0% diet. Brassicasterol was not detected in the muscle tissue of salmon fed the

marine diet samples, but it was found in the salmon tissues fed the ω3LC 1.0% diet which coincides with the lower amount present in the marine diet versus the ω3LC 1.0% diet. Stellasterol (5α-ergosta-7,22-dien-3β-ol) is a 28 carbon, Δ-7 sterol originally isolated from starfish. This sterol was found in both diets, marine and ω3LC 1.0%, but was only present in the muscle tissue from fish fed the marine diet. Since the occelasterol content in muscle tissue of salmon fed the ω3LC 1.0% diet was higher than the amount found in muscle tissue of salmon fed the marine diet (while in the diets themselves there was no significant difference), it is possible that the stellasterol is being metabolized into a different sterol, likely the occelasterol. The structures of both can be seen in Figure 2.7. They differ in the position of the double bond in the ring structure and the addition of a methyl group on stellasterol side chain.

Table 2.11: Total phytosterol content present in experimental diets and muscle tissue (μg/g)[1] (Carreón-Palau, unpublished data).

	Marine Diet	**ω3LC 1.0%**	**Muscle tissue from Marine Diet**	**Muscle tissue from ω3LC 1.0%**
Cholesterol	78.6 ± 0.4	48.7 ± 1.8	96.6 ± 1.3	91.1 ± 3.8
Occelasterol	1.4 ± 0.7	1.0 ± 0.1	1.2 ± 0.3	3.4 ± 1.4
Brassicasterol	1.6 ± 0.2	4.3 ± 0.7	-	1.6 ± 0.6
Stellasterol	5.0 ± 0.4	14.3 ± 0.1	1.5 ± 1.1	-

[1] Data expressed as μg/g. Values are means ± standard deviation (n=3).

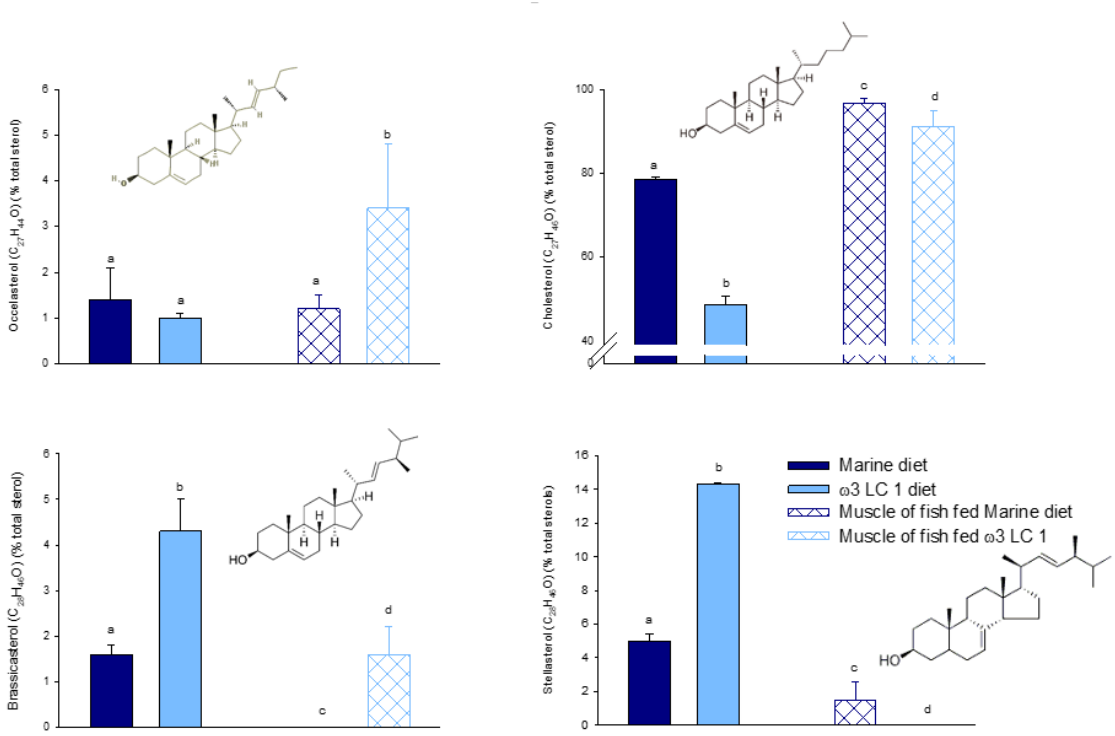

Figure 2.6: Total sterol proportions in salmon diets and in muscle after 14 weeks feeding (Carreón-Palau, unpublished data).

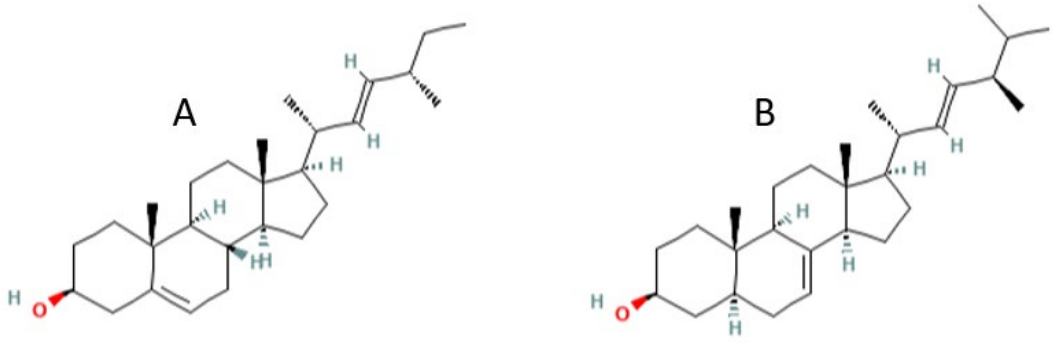

Figure 2.7: Structures of occelasterol (A) and stellasterol (B).

2.5 Conclusion

The free sterol content of Camelina seed oil, Camelina meal, and Camelina-based fish feed was determined in this study by GC-MS with derivatization to TMS ethers. The sterol profile of the Camelina seed indicated that the most abundant phytosterols present were β-sitosterol, stigmasterol, brassicasterol, campesterol and cholesterol. The analysis of diets containing Camelina oil indicated the presence of β-sitosterol, campesterol, brassicasterol, stigmasterol, and cholesterol, the same sterols that were found in the oil. In the muscle tissue of fish fed these diets, only cholesterol, β-sitosterol and campesterol were detected. These two phytosterols were in greatest abundance in the feeds tested. Brassicasterol and stigmasterol were not detected in fish from the experimental trials, indicating that the salmon were either unable to absorb them or they were in such small quantities as to be undetectable via this method. Total sterol content increased in the fish fed the diet containing 100% substitution with Camelina oil, which can be attributed to the increase in cholesterol.

The *Pavlova* oil analysis by GC-MS gave a sterol composition of 9 unique sterols: cholesterol, campesterol, stigmasterol, stigmastanol, spinasterol, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, 24-methylpavlovol, and 24-ethylpavlovol. These sterols are consistent with other literature research on *Pavlova* species. Work performed by Guerra et al. (2022) showed that there was no significant difference in total lipid composition for muscle and liver tissue in a *Pavlova* algal biomass diet trial, while salmon fed the FM/AB diet had the highest proportions of sterols in the liver. The muscle tissue sterol content was similar in salmon fed the FM and FM/AB diets,

but significantly different than in salmon fed the AB diet. Differences in sterol content can be attributed to effects on membrane composition, as determined by differences in membrane phospholipid fatty acid composition. Replacing FM with Pav459 had clear effects on the membrane composition of both liver and muscle tissues, however, the magnitude of the effect varied between tissues. Overall, the research demonstrated that replacing fish meal with *Pavlova* (Pav459) will likely not significantly affect the growth parameters of Atlantic salmon.

Based on these results, it appears that plant oils and microbial oils have potential for replacement of fish oils in aquaculture feeds. The availability, sustainability, cost, and nutritional value of the ingredients are factors that should be considered when including novel lipid sources in fish feed. Currently, fish meal and fish oil remain important ingredients for farmed fish feed but given their cost and sustainability, other sources should be further investigated.

Chapter 3: *Schizochytrium* microbial oil as a replacement for fish oil in Atlantic Salmon: Effect on sterol composition

3.1 Introduction

Fish oil and fish meal have primarily been used as the major nutrient source in Atlantic salmon (*Salmo salar*) feeds. In recent years, with increasing costs and reduced availability, commercial salmon feeds have moved towards incorporating terrestrial plant-based protein and lipid sources (Mowi, 2019). While the replacement of dietary fish meal protein with plant protein has been widely successful, the replacement of dietary fish oil has been more challenging (Tibbetts et al., 2020). Maintaining ω-3 long chain polyunsaturated fatty acid (LC-PUFA) content of the final product is essential, as it supports fish growth and development. In both fish and consumers, dietary LC-PUFAs play important and physiologically complex roles in several key metabolic functions. These include cell membrane structure, cholesterol metabolism, prostaglandin synthesis, plasma triacylglycerol transport and efficient intestinal absorption of fat-soluble vitamins (Tibbetts et al., 2020). Diets lacking in these LC-PUFAs, resulting in a low ω-3/ω-6 ratio, can compromise fish and human health. High ω-3/ω-6 ratios promote an anti-inflammatory response in humans and animals resulting in improved cardiac and nervous system health and increased cell membrane fluidity (Hixson, 2014). The LC-PUFAs, docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3), are primarily aquatic-based nutrients and are found in limited amounts in terrestrial plant-based sources (Colombo et al., 2017). With the lipid fraction of many farmed salmon feeds containing

double the amount of terrestrial derived oils as opposed to conventional marine fish oils, fish health and consumer products have been compromised in recent years (Tibbetts et al., 2020). The outcome is a systematic reduction of the dietary ω-3/ω-6 ratios of the feed and a reduced LC-PUFA content in favour of elevated levels of C18 n-6 fatty acids such as linoleic acid (LA; 18:2n-6) (Sprague et al., 2016).

Marine microorganisms show great potential to produce these LC-PUFAs (DHA and EPA) in an efficient and sustainable way (Wei et al., 2021). The group of microorganisms known as thraustochytrids are often referred to as 'microalgae' in the published literature and for marketing purposes (Tibbetts et al., 2020). However, they are not microalgae (Armenta and Valentine, 2013; Leyland et al., 2017). They are classified taxonomically as marine or brackish fungal protists, composed of the 5 genera, *Thraustochytrium, Aplanochytrium, Japonochytrium, Ulkenia and Schizochytrium* (Barclay et al., 1994; Burja et al., 2006). More specifically, three different genera within the genus *Schizochytrium* sensu lato have been proposed by Yokoyama and Honda (2007), which has been gaining acceptance. They are *Schizochytrium* sensu stricto*, Aurantiochytrium* and *Oblongichytrium* gen. nov. (Yokoyama and Honda, 2007). Under optimized heterotrophic cultivation conditions, many thraustochytrids have demonstrated their capability to produce very high lipid concentrations (>70% of their mass), rich in essential LC-PUFAs by using a vast array of carbon and nitrogen feedstocks (Lewis et al., 1999). The level of DHA in the extracted oil has been increased through the optimization of culture conditions (e.g. Tibbetts et al., 2020). Since these organisms are capable of industrial production of DHA at very high

levels, they have become a particularly interesting group of organisms for mass fermentation, oil extraction, and use in aquaculture feeds (Sprague et al., 2017).

In comparison to terrestrial crops, the production of marine microorganisms does not require arable land and uses minimum quantities of freshwater resources (Tibbetts et al, 2018). Additionally, because of their highly controlled fermentation processes, the DHA-rich edible oils produced are more likely to be free from environmental contaminants often problematic with conventional fish oils. Fish consumption, particularly oily fish, is a potential dietary route for human exposure to persistent organic pollutants, including dioxins, dioxin-like polychlorinated biphenyls and polybrominated diphenyl ethers. These lipophilic compounds are easily absorbed and rapidly distributed to lipid-rich organs and tissues which can result in their bioaccumulation (Sprague et al., 2015). A significant advantage of microbial biomass is that it can be harvested year round under industrial production. These products have tremendous potential to reduce pressures on wild fish stocks harvested for fish meal and fish oil reduction (Tibbetts et al., 2020). *Schizochytrium* sp. is commercially available as an oil and whole-cell meal (Wei et al., 2021). Several studies have been conducted to evaluate the use of whole cell thraustochytrids meal in farmed salmonid feeds (Tibbets et al., 2020). Only three published studies have demonstrated the effect of the extracted *Schizochytrium* sp. oil on growth, digestibility, and fatty acid composition of Atlantic salmon (Lee Chang et al., 2020; Miller et al., 2007; Tibbetts et al., 2020). These studies have shown positive results without being detrimental to the growth of salmon, however, the absence of EPA in the oil may be of concern with respect to growth, composition, and immune response (Miller et al., 2007).

A handful of companies are now involved in this emerging industrial sector, and commercial food grade DHA products may soon be added to the feedstock portfolios of large aquafeed producers (Tibbetts et al., 2018). New thraustochytrid strains with good potential continue to be isolated and explored for development (Shene et al., 2019). Although product processing such as getting concentrated cells, extracting target nutrients, rupturing cell walls, and removing other anti-nutritional factors are more energy intensive and costly than the microbe culture, advances in oil-producing technologies have gradually made microbial oil more economical and comparable to fish oil (Tibbetts, 2018).

The use of plant based sources for use in aquafeeds is not without its challenges. Certain plant components may interfere with fish health and welfare. It is well known that increasing the levels of plant protein in fish diets reduces the levels of dietary cholesterol (Kortner et al., 2014). Cholesterol is an essential component in the cell membranes of animals. Cholesterol has multiple effects on the physical properties of membranes including membrane stabilization, reduction of membrane permeability, facilitation of morphological characteristics and interactions between cells, influencing phase transitions, and providing a suitable microenvironment for membrane-associated proteins (Crockett, 1998). The cholesterol level of cellular membranes is a factor influencing the sensitivity of cells to heat shock. For some types of cells there is a linear relationship between cholesterol content and resistance to heat shock (Ortega et al., 1996). Cholesterol serves as a precursor to many physiologically active compounds, such as sex hormones, adrenal corticoids, bile acids, and vitamin D (Deng at al., 2013). Vertebrates such as fish have the ability to synthesize sterols from acetate, thus only limited research had been carried out to address

the need for dietary cholesterol. However, recent studies have shown that fish fed diets containing high levels of plant-derived protein sources had lower levels of blood cholesterol and were more susceptible to infectious disease and occurrences of green liver (Deng et al., 2013).

The inclusion of plant based protein and oils in fish fed, replacing fish oil, may be associated with decreased lipid digestibility and cholesterol levels, which could lead to inadequate supplies of cholesterol in fish (Kortner et al., 2014). Cholesterol, although commonly considered an animal sterol, is common in many microalgae, and there are several examples where it is the major sterol present (Volkman, 2003). Most macrophyte red algae contain primarily C-27 sterols with cholesterol as the predominant sterol. Several species contain large amounts of desmosterol (cholesta-5,24-dien-3β-ol) which is closely related to cholesterol. Sterols of the phylum Haptophyta usually contain one to five major sterols, and cholesterol commonly predominates (Volkman, 2016).

The goal of this study was to determine the identity of and quantify the phytosterol content of microbial oil (MO), the feed containing microbial oil, and the muscle and liver tissues of salmon fed diets containing microbial oil. Fish fed traditional diets containing fish oil (FO) and fish meal (FM) were compared to fish fed diets that were substituted with different amounts of microbial oil. The purpose of this study was to investigate the viability of microbial oil as a potential alternative to fish oil in commercial fish feed. This portion of the research focused on the sterol content of the microbial oil and whether or not the sterols are transferable to the fish for consumption.

3.2 Methods

3.2.1 Materials

The microbial oil (MO) used in this experiment was provided by Mara Renewables (Dartmouth, Nova Scotia, Canada).

Sterol standards (brassicasterol, campesterol, cholestanol, stigmasterol, β-sitosterol and androstanol) were purchased from Steroloids Inc., Newport, RI, USA. The cholesterol standard was purchased from Sigma Aldrich, St. Louis, MO, USA. All sterol standards were stored at approximately 4°C prior to use. The silylating agent (BSTFA + 0.1% TMCS) was purchased from Supelco (Newport, RI, USA) and stored at room temperature prior to use. All organic solvents used (chloroform, methanol, diethyl ether, n-hexanes, and dichloromethane) were of analytical grade.

Diet samples were provided by the Chute Nutrition Lab at Dalhousie University, Faculty of Agriculture, Truro, Nova Scotia. Four diets were formulated to meet the nutritional requirements of Atlantic salmon. The control diet contained 20% FO as the lipid source. Since most current commercial feeds use a lower FO level, a secondary control was included that contained 10% FO and 10% vegetable (canola) oil (FO/VO diet). The two experimental diets included MO at 5% (low inclusion-MO) and 10% (high inclusion-MO) of the diet, with canola oil as the other lipid source (Wei et al., 2021). Table 3.1 gives the formulation and composition of the experimental diets.

Table 3.1: Diet formulation and composition (g/kg as fed basis) of experimental diets containing microbial oil (MO), fish oil (FO), or FO/vegetable (VO) blend, fed to Atlantic salmon (Wei et al., 2021).

Ingredient (g/kg)[1]	**FO**	**FO/VO**	**Low MO**	**High MO**
Fish meal	150	150	150	150
Fish oil (Herring)	200	100	0	0
Microbial oil (MO)[2]	0	0	50	100
Canola oil	0	100	150	100
Ground wheat	117.5	117.5	117.5	117.5
Empyreal (corn protein concentrate)	250	250	250	250
Poultry byproduct	170	170	170	170
Soybean meal	80	80	80	80
Vitamin/mineral mix[3]	2	2	2	2
Dicalcium phosphate	20	20	20	20
Special premix[4]	2.5	2.5	2.5	2.5
Lysine HCL	5	5	5	5
Choline chloride	3	3	3	3

[1] All ingredients were supplied and donated by Northeast Nutrition (Truro, Nova Scotia, Canada). [2] Produced by Mara Renewables (Dartmouth, Nova Scotia, Canada). [3] Vitamin/mineral mix contains zinc, manganese, iron, copper, iodine, vitamin A, vitamin D, vitamin K, vitamin B12, thiamine, riboflavin, pantothenic acid, niacin, folic acid, biotin, pyridoxine, inositol, ethoxyquin, wheat shorts. [4] Special premix contains selenium, vitamin E, vitamin C, astaxanthin, wheat shorts.

The feeding trial was performed at the Aquaculture lab at Dalhousie University Faculty of Agriculture, Truro, Nova Scotia. Salmon were fed 3 mm pellets at the beginning and then switched to 5 mm pellets when they grew larger. Fish were hand-fed to satiation with experimental feed for 16 weeks after the initial sampling (week 0) twice a day at 9:00 and 15:00. Hand feeding was performed carefully to ensure minimum feed was unconsumed and feed consumption was recorded weekly (Wei et al., 2021). Five fish per tank were randomly sampled at week 0, and at the end of the trial at week 16. White dorsal muscle

tissues, as well as liver samples, were taken for subsequent analysis. Samples were flash frozen in liquid nitrogen immediately after sampling and stored at -80ºC until analysis.

3.2.2 Sample Preparation and Derivatization

Lipid extraction was performed using a modified Folch procedure (Parrish, 1999). In this procedure, the fish feed, or the tissue sample, was placed in a test tube and a mixture of chloroform/methanol 2:1(v/v) was added. The content of the tube was homogenized using a polytron PCU-2-110 homogenizer (Brinkmann Instruments, Ontario, Canada). The metal-ended rod of the homogenizer was washed into the tubes with 1 mL 2:1 chloroform/methanol, followed by 0.5 mL chloroform-extracted water. This gave a final ratio of chloroform/methanol/water (8:4:3, by volume) which was followed by sonication for 4 min. Samples were then centrifuged at a speed of 3000 rpm for 2-3 min. The lower organic layer, which contained the lipids, was then drawn through a Pasteur pipette using a double pipetting technique, into a vial. The extraction process was repeated four times to ensure that all lipids were extracted. Upon removal of the organic layer, the pipettes were each rinsed with 1.5 mL ice-cold chloroform. The extracts were then concentrated to near dryness by roto-evaporation in a water bath heated between 40 - 45°C and transferred to sample vials. Vials were then flushed with nitrogen gas and sealed. Samples were kept at -20°C until analysis.

Derivatization of free sterols was performed by silylation with *N, O*-bis-trimethylsilyl trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) to form their

corresponding trimethylsilyl (TMS)-ethers. Samples were evaporated until dryness under a stream of nitrogen. 100µL of BSTFA containing 1% TMCS was added to the lipid extract and heated at 85ºC for 15 minutes. Samples were then cooled to room temperature and excess reagent was evaporated under nitrogen gas. 500 µL of hexane/dichloromethane (1:1, by volume) was added followed by addition of 100 µL of 100 mg/L of 5α-androstanol as internal standard and then stored at -20˚ until analysis by GC-MS and GC-FID.

3.2.3 Instrumentation

For quantification of sterols, a Varian CP-3800 GC/FID with a CP-8400 autosampler was used. This instrument was located at the Lipid Lab at the Ocean Sciences Centre of Memorial University. The chromatograph has an injector that is temperature and pressure controlled. Detection of sterols was performed using a DB-5 column. Analysis was run in splitless mode, with helium as the carrier gas at a pressure of 14.0 psi. Ten µL of sample was injected, at an injector temperature of 290°C. The initial temperature of the oven was set at 80°C and held for 1.0 minutes. Oven temperature was then increased at a rate of 50.0 °C/min until it reached 200°C. The rate was then decreased to 4.0°C/min to reach a final temperature of 305°C, and held there for a period of 5.00 minutes. The total run time per sample was 34.65 minutes. A detector temperature of 315°C was used.

For identification purposes, sterols were analyzed using an Agilent 6890 N Gas Chromatograph coupled to an Agilent 5973 Mass Spectrometer. Use of this instrument was provided by the Centre for Chemical Analysis, Research and Training (C-CART) at

Memorial University. Separation of sterols was achieved using an Agilent DB-5ms (95% dimethyl-, 5% diphenyl-polysiloxane) GC column (30 m, 0.25 μm thickness, and 0.25 mm i. d.). All analyses were run in splitless mode, using helium gas as the carrier gas at a pressure of 14 psi. One μL samples were injected into the inlet at a temperature of 290°C. The temperature of the column was held at 80°C for 1 min, followed by ramping to 200°C at a rate of 50°C/min. The temperature was finally increased to 305°C at a rate of 4°C/min and held for 5 min, for a total analysis run time of 34.65 min. The quadrupole MS ion source was operated in electron ionization (EI) mode at 70 eV. The ion source temperature was 200˚C and the interface temperature was 180 ˚C. The scan parameters were set to detect m/z values between 50-550, with a scan rate of 2.94 scans/s.

Although relative retention times of common phytosterols can typically be found in the literature, difference in GC instruments, operating parameters, and standards can complicate the chromatography. Mass spectrometry can be used to determine the molecular weight of each sterol, or its derivative, and the fragmentation patterns of the ionized species used to elucidate the analyte structure. Positive identification of specific phytosterols was confirmed by comparison of individual fragmentation patterns to those found in spectral databases as well as interpretation of the resulting mass spectra.

The mass spectra of sterol TMS-ethers show characteristic fragmentation patterns that facilitate identification. The most common fragment ions that are observed are listed in Table 3.2.

Table 3.2: Characteristic phytosterol TMS-ether fragmentation.

m/z	Fragmentation
M^+	Molecular ion
$[M-15]^+$	Loss of methyl ($-CH_3$)
$[M-90]^+$	Loss of trimethylsilanol ($-(CH_3)_3$-Si-OH)
$[M-105]^+$	Loss of methyl and trimethylsilanol
$[M-129]^+$	Loss of $(CH_3)_3$-Si-O^+=CH-CH=CH_2 (fragment of C1-C3 of A ring)

3.2.4 Internal Standard for Quantification

The addition of an internal standard can be used to aid in the sterol identification and quantifications methods. Appropriate selection of an internal standard can eliminate analytical errors due to fluctuations in instrument operating conditions and other experimental variables (Lagarda et al., 2006). The internal standard must be chemically similar to phytosterols and thus be quantitatively extracted and derivatized with the phytosterols. In this procedure, a carefully measured quantity of an internal standard is introduced into each standard and sample, and the ratio of the analyte to internal standard peak areas serves as the analytical variable (Skoog et al., 1998). This method eliminates errors introduced by sample injection, matrix effects, and random instrument fluctuations (Skoog et al., 1998).

The internal standards most commonly used for sterol determinations have been cholesterol, cholestane, and 5β-cholestan-3α-ol (epicoprostanol) (Lagarda et al., 2006). However, in this analysis, cholesterol or cholestane could not be adequately separated from

other sterols present, so 5α-androstanol was employed as the internal standard. Its structure closely resembles that of sterols and it is absent from plant and microbial oil sterol samples.

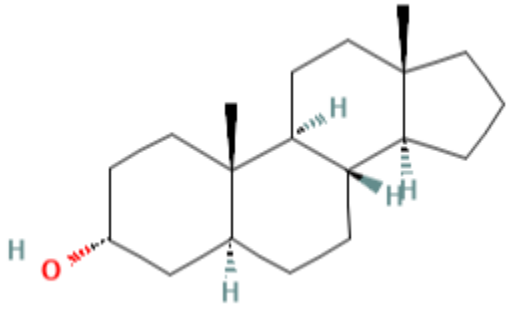

Figure 3.1: Chemical structure of 5α-androstanol used as internal standard.

Concentration curves were obtained from sterol standards of cholesterol, β-sitosterol, campesterol and stigmasterol. Each standard contained 100 µl of 100 ppm androstanol as the internal standard. The ratio of each standard peak area to the internal standard peak area was determined by integration and plotted against the ratios of concentration of standard: internal standard to generate calibration curves. Response factors were obtained for the sterol standards. The response factors for each sterol were calculated and given in Table 3.3 below. For sterols with no standard for comparison, response factors for sterols with similar retention times were used. Response factors can also be compared using the structure of the sterols. Campesterol and β-sitosterol both have saturated side chains and have similar response factors. Stigmasterol has a double bond in the side chain and has a lower response factor than the above-mentioned sterols. Cholesterol has no double bonds

in its side chain, but it also has the smallest side chain length which could lead to a higher response factor.

Table 3.3: Response Factors of Sterols via GC-MS.

Sterol	Response Factor
Cholesterol	1.05
Campesterol	0.701
Stigmasterol	0.663
β-sitosterol	0.713

3.3 Statistical Analysis

Results are presented as the mean ± the standard deviation. Statistical analysis was performed using Minitab Statistical Software (version 18; Minitab Inc., State College, PA, USA). One-way ANOVA (analysis of variance) was used to compare levels of sterols and to test for differences. Significant difference was set at $\alpha = 5\%$ ($p < 0.05$). Tukey's *post hoc* method was used to test for differences amongst samples.

3.4 Results

3.4.1 Microbial Oil

A lipid extract from the microbial oil sample provided by Mara Renewables was derivatized to its TMS-ethers and analyzed by GC-FID. The TIC revealed many peaks, corresponding to the lipid classes present. In the sterol region, the first sterol to elute is typically the cholesterol-TMS-ether. Other sterol-TMS-ethers elute after cholesterol with

respect to retention time. The sterol TMS-ethers identified were cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol, 24-methylenelophenol, stigmasterol, and spinasterol. The peaks were identified by comparing their relative retention times with those of the standards and were confirmed by their corresponding mass spectra. For those sterols without available standards for comparison, their mass spectral and gas chromatographic retention time data were compared to the data for the sterol TMS-ether derivatives in Table 6.2 of Jones et al. (1994).

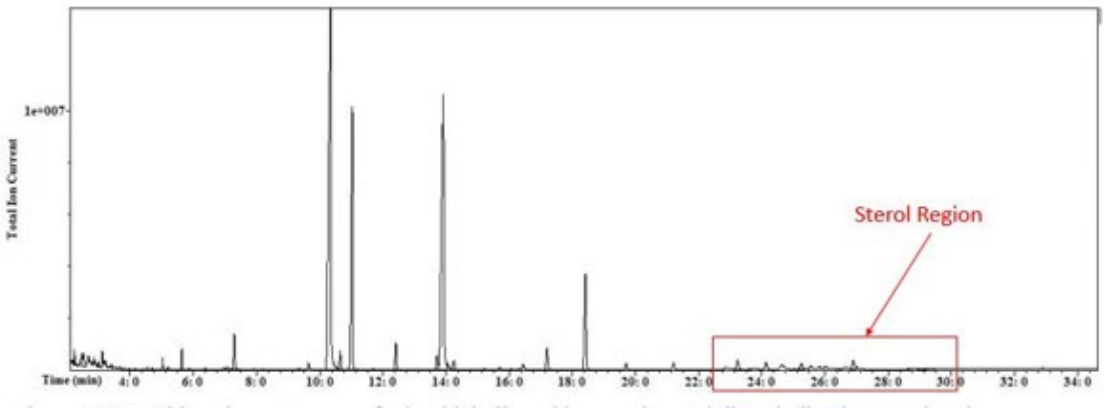

Figure 3.2: Total ion chromatogram of microbial oil used in experimental diets, indicating the sterol region.

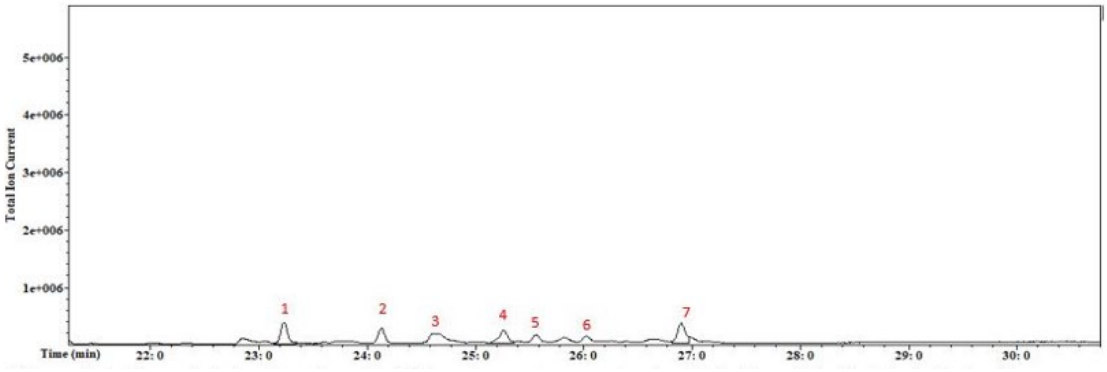

Figure 3.3: Expanded sterol region of total ion chromatogram of microbial oil used in diet trials; indicating sterols identified. Peaks: 1. Cholesterol 2. Lathosterol 3. Brassicasterol 4. 24-Methylenecholesterol 5. 24-methylenelophenol 6. Stigmasterol 7. Spinasterol.

Table 3.4: Sterols identified in microbial oil used in diet trials.

STEROL	COMMON NAME	FORMULA	MW(+TMS)
Cholest-5-en-3β-ol	Cholesterol	$C_{27}H_{46}O$	458
5α-Cholest-7-en-3β-ol	Lathosterol	$C_{27}H_{46}O$	458
24-methylcholest-5,22E-dien-3β-ol	Brassicasterol	$C_{28}H_{46}O$	470
24-methylcholesta-5,24(28)-dien-3b-ol	24-Methylenecholesterol	$C_{29}H_{48}O$	484
24-methyl-5α-cholest-7,24-dien-3β-ol	24-Methylenelophenol	$C_{28}H_{46}O$	470
24-ethylcholest-5,22E-dien-3β-ol	Stigmasterol	$C_{29}H_{50}O$	486
24-ethyl-5α-cholest-7,22E-dien-3β-ol	Spinasterol	$C_{29}H_{48}O$	484

3.4.2 Experimental Diets

Four experimental diets were analyzed for phytosterol content, using their TMS-ether derivatives. The control diet contained fish (herring) oil (FO). One diet contained fish oil

and 10% canola oil (FO/VO), as a secondary control. Two diets contained microbial oil at 5% (Low MO) or 10% (High MO), with canola oil as the other lipid source.

In the control (FO) diet, four phytosterols were identified. They were cholesterol, campesterol, 23,24-dimethylcholest-5-en-3β-ol, and 24-ethyl-5α-cholest-7-en-3β-ol. 23,24-dimethylcholest-5-en-3β-ol is a 29 carbon, Δ5 sterol which is commonly found in diatoms. 24-ethyl-5α-cholest-7-en-3β-ol is formed in the biohydrogenation pathways of β-sitosterol, a common plant oil phytosterol. These same phytosterols were also identified as components in the FO/VO control diet with the addition of stigmasterol and brassicasterol. Stigmasterol and brassicasterol are commonly identified in vegetable oils. They are likely the result of the canola oil component of the FO/VO diet.

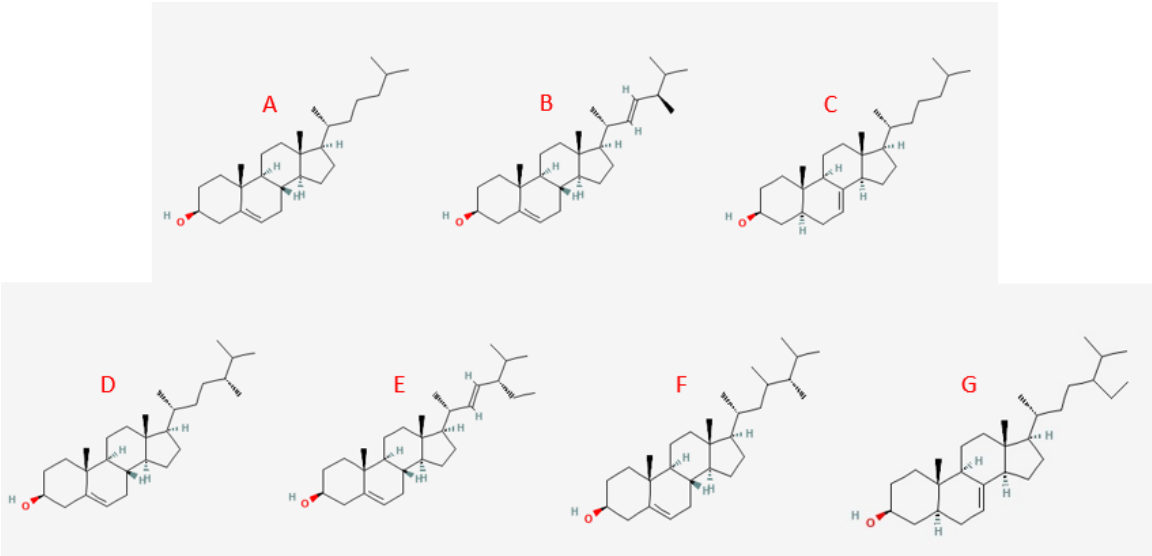

Figure 3.4: Structures of sterols identified in experimental diets: A – cholesterol, B – brassicasterol, C – lathosterol, D – campesterol, E – stigmasterol, F - 23,24-dimethylcholest-5-en-3β-ol, and G - 24-ethyl-5α-cholest-7-en-3β-ol.

The same four phytosterols as were present in the control were identified in the diets containing low (5%) microbial oil and high (10%) microbial oil as well as stigmasterol. Additionally, brassicasterol was detected in both the low MO diet and the high MO diet. In the high MO diet, one other phytosterol, lathosterol, was also detected. This sterol was one of the phytosterols detected in the original microbial oil analysis.

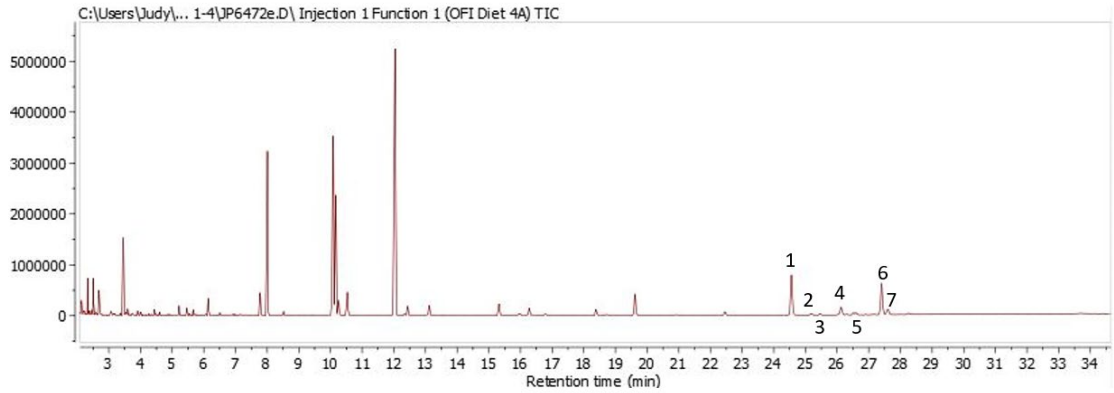

Figure 3.5: Total ion chromatogram of experimental diet 4 (High MO), indicating sterol-TMS ethers identified. Peaks: 1. Cholesterol 2. Brassicasterol 3. Lathosterol 4. Campesterol 5. Stigmasterol 6. 23,24-dimethylcholest-5-en-3β-ol 7. 24-ethyl-5α-cholest-7-en-3β-ol.

Statistical analysis indicated that there were no significant differences between the amounts of free sterols present in each diet. Total sterol amounts ranged from 815 μg/g for the low microbial oil diet to 1024 μg/g in the high microbial oil diet. The low sterol content of the low MO diet is likely due to the relatively low amount of cholesterol found in the low microbial oil diet. The diet containing the high inclusion level of microbial oil had the highest total sterol content, which is consistent with the higher amount of cholesterol in this diet. Iatroscan determined total sterol content was also reported by Wei et al. (2021) with a range of 3090 μg/g for the control (FO) diet to 5230 μg/g for the low microbial oil diet, with an average across all diets of 4148 μg/g. There was no significant difference in the sterol content between the fish oil and microbial oil diets.

The ratios of phytosterol to cholesterol can be seen in Table 3.5. The ratio increases across all treatments, with the high microbial oil diet having the highest phytosterol:

cholesterol ratio. This correlates with the amounts of phytosterols added to the diets, which also increased as the inclusion level of microbial oil increased.

Table 3.5: Sterol composition of lipid extract from experimental diets (µg/g) quantified by GC/FID[1].

Sterol	**Control Diet (FO)**	**Control Diet (FO/VO)**	**Low Microbial Oil (Low MO)**	**High Microbial Oil (High MO)**
Cholesterol	736 ± 229	551 ± 167	304 ± 151	349.8 ± 58.7
Brassicasterol	-	84.0 ± 13	56.9 ± 27	23.9 ± 13.8
Lathosterol	-	-	-	57.6 ± 9.1
Campesterol	58.9 ± 21	163 ± 53	129 ± 44	210.9 ± 58.7
Stigmasterol	-	102 ± 33	63.9 ± 22	38.0 ± 16.3
23,24-dimethylcholest-5-en-3β-ol	209 ± 87	427 ± 139	166 ± 58	247.7 ± 73.6
24-ethyl-5α-cholest-7-en-3β-ol	76.9 ± 28	71.2 ± 17	94.9 ± 19	96.1 ± 20.9
Total Sterol	1081 ± 91	1398 ± 70	815 ± 54	1024 ± 72
Phytosterol:Cholesterol Ratio	0.47	1.53	1.68	1.93

[1] Data expressed as ug/g. Values are means ± standard deviation (n=3).

3.4.3 Muscle Tissue Extracts

Salmon from week 16 of the microbial oil feeding trials were sampled and lipids extracted. Lipid extracts were analyzed for derivatized sterols using GC-MS and GC-FID. The sterols identified in the muscle tissue extracts were cholesterol, cholestanol, campesterol, stigmasterol, and dinosterol.

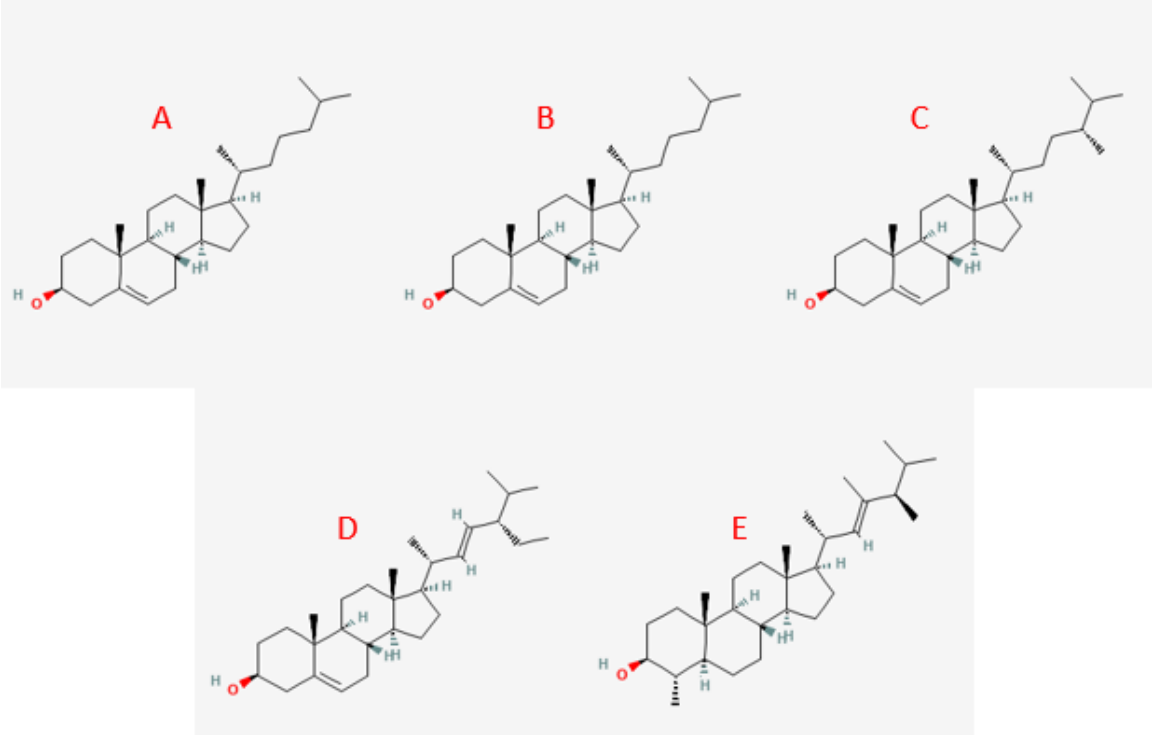

Figure 3.6: Structures of sterols identified in salmon muscle tissue: A – cholesterol, B – cholestanol, C – campesterol, D – stigmasterol, and E – dinosterol.

Cholesterol content was significantly higher in the diets containing the microbial oil than in the diet containing fish oil and canola oil, but not higher than the fish oil control diet (Table 3.6). The low microbial oil diet had cholestanol, a derivative of cholesterol, present

in all samples, and was highest in the control (FO) samples. Campesterol, which was also identified in the diet samples, was present in all samples, and the highest amount was found in the Low MO sample. Similarly, stigmasterol was present in all samples, with the highest amount being found in the Low MO sample. Dinosterol, a high molecular weight sterol, was present in all samples as well, with a higher amount found in the Low MO sample. 24-methylpavlovol was not present in the control (FO) diet but was present in trace amounts in the diets containing microbial oil. Given the high standard deviations reported in these results, statistically significant differences were not found in tissue sterol concentrations, other than for cholesterol, due to analyses being undertaken on wet tissues. Many additional significant differences were found when sterol data were compared as proportions of total sterols (Figure 3.8). Brassicasterol, lathosterol, 23, 24-dimethylcholest-5-en-3β-ol, or 24-ethyl-5α-cholest-7-en-3β-ol, which were identified in the experimental diets, were not present in the muscle tissue samples. Lathosterol was detected in only one of the microbial oil diets (High MO) at 5.6% of total sterols and was not present in any of the muscle tissues. Campesterol was present in all diets at an average of 13.4% of total sterols and all muscle tissues with an average of 7.2%, but there were no significant differences among the diets. Stigmasterol was present in most diets and all muscle tissue at low (<8%) levels but there were also no significant differences between treatments. Dinosterol was detected at even lower levels (≤7%) in muscle tissue only, but mean values were not significantly different.

Total sterol amounts ranged from 77 μg/g in the muscle tissue from fish fed the control (FO/VO) diet to 165 μg/g in the muscle tissue from fish fed the low microbial oil diet. The low amount of sterols in the control (FO/VO) diet can be attributed to the low amount of

cholesterol in the control diet. The muscle samples with the highest total sterol content found were from fish fed the low microbial oil diet, which was the opposite of what was seen in the diet samples. There was no significant difference in the total sterol content among the muscle tissue from fish fed the four experimental diets versus muscle from fish fed the control diets.

The ratio of phytosterol to cholesterol showed a decreasing trend across the treatments. This is the opposite effect that was seen in the diets analysis. Although there is no significant difference between the ratios, the decrease could indicate that the increasing amount of cholesterol being added with the increased inclusion of microbial oil is reducing phytosterol absorption.

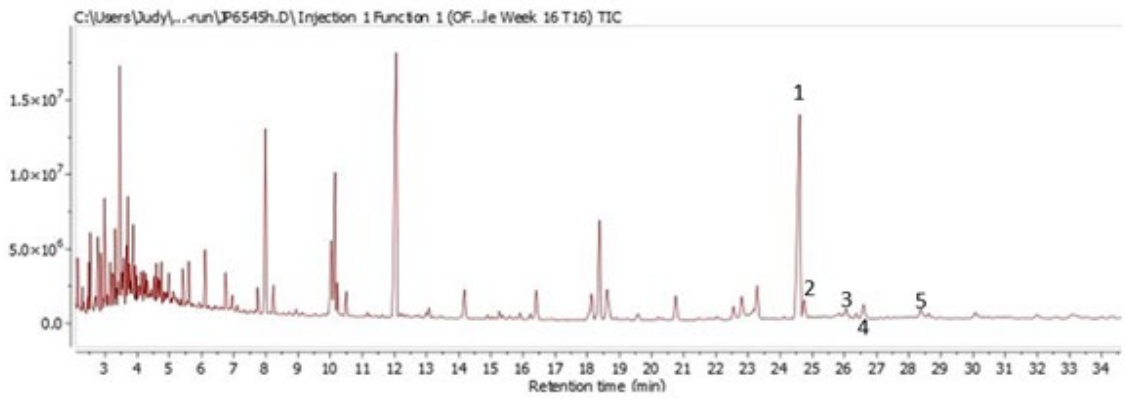

Figure 3.7: Total ion chromatogram of lipids extracted from salmon muscle tissue from the High MO experimental diet, indicating sterol-TMS ethers identified. Peaks: 1. Cholesterol 2. Cholestanol 3. Campesterol 4. Stigmasterol 5. Dinosterol.

Table 3.6: Sterol composition of lipid extract from salmon muscle tissue (μg/g) quantified by GC/FID[1].

Sterol	Control Diet (FO)	Control Diet (FO/VO)	Low Microbial Oil (Low MO)	High Microbial Oil (High MO)
Cholesterol	89.9 ± 16.6[a]	53.9 ± 3.6[b]	119 ± 2.8[a]	117 ± 4.4[a]
Cholestanol	18.3 ± 9.2	3.8 ± 2.1	9.8 ± 7.8	5.0 ± 3.9
Campesterol	7.8 ± 6.1	7.5 ± 6.0	12.8 ± 5.5	7.3 ± 5.5
Stigmasterol	6.9 ± 5.4	6.8 ± 5.2	11.9 ± 4.9	6.4 ± 4.6
Dinosterol	7.7 ± 6.5	5.4 ± 3.9	11.5 ± 5.7	6.4 ± 5.1
Total Sterol	131 ± 9.8	77 ± 5.6	165 ± 5.3	142 ± 4.7
Phytosterol:Cholesterol Ratio	0.45	0.43	0.39	0.21

[1] Data expressed as ug/g. Values are means ± standard deviation (n=6). Means with different superscripts indicate significant differences.

3.5 Discussion

Six sterols were detected and identified in the microbial oil analysis. These were cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol, 24-methylenelophenol, stigmasterol, and spinasterol. A limited amount of data is available on the sterols in thraustochytrids. Lewis et al. (2001) analyzed a thraustochytrids strain and found 20 sterols, 13 of which they identified. Five of those were the same ones detected in this research; cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol and stigmasterol.

The composition of identified sterols found in the microbial oil was comparable to the sterols recovered in Thraustochytrids by Lewis et al. (2001) and by Volkman (2003). As expected, the cholesterol-TMS-ether was eluted first. Three other sterols; lathosterol, brassicasterol and stigmasterol, were also expected to be present, as these were also identified by Lewis et al. (2001). Spinasterol and 24-methylenelophenol are both isomers of stigmasterol, with both having a double bond at Δ7 instead of Δ5, and 24-methylenelophenol having the side chain double bond at C_{24}. 24-Methylenecholesterol is a 28 carbon, 3β sterol derived from cholesterol. It is a sterol typically found in diatoms, but it also found in microalgae in lesser amounts (Volkman, 2003).

By comparing the phytosterol profile of the microbial oil to the sterols found in the experimental diets, four of the sterols (cholesterol, brassicasterol, lathosterol, and stigmasterol) identified in the microbial oil were also detected in the diets. Brassicasterol, a common plant phytosterol, was not detected in the fish oil control diet, but was present in both the FO/VO control diet and the MO diets. The highest brassicasterol content was detected in the High MO diet. The Low MO diet did not show significant levels of brassicasterol as compared to the control FO/VO diet. Campesterol was present in all of the diets, including both controls, but was absent in the microbial oil itself. The campesterol is likely derived from the wheat that is an ingredient in all diets. A 29 carbon sterol, 23,24-dimethylcholest-5-en-3β-ol, was present in both controls and all experimental diets. This is a Δ^5 sterol commonly found in diatoms. Since it was present in all diets, it cannot be attributed to the inclusion of microbial oil. 24-ethyl-5α-cholest-7-en-3β-ol was also found in all diets including the control. This sterol is closely related to β-sitosterol, a common

plant sterol, and could also be attributed to the presence of wheat in the diets. Although there was no statistical difference in the sterol levels amongst the diets, it is interesting to note that lathosterol, a sterol detected in the microbial oil, was only present in the experimental diet containing the highest level of microbial oil. This indicates that it is likely transferred to the feed from the added microbial oil. The presence of stigmasterol was also seen in the diets containing vegetable oil, and those with both vegetable oil and microbial oil, although it is unclear if the canola oil or the microbial oil is the contributing agent as it is a component of both.

The ratio of phytosterols to cholesterol increased across the experimental diets, with the inclusion of canola oil and microbial oil. A similar trend was seen by Sissener et al. (2018) where the phytosterol: cholesterol ratio increased with an increase in the amounts of phytosterols added to the feeds. They also showed an increase in total sterols in the diets with an increase in the amount of phytosterols added.

The amounts of free sterols detected by GC (Table 3.5) are very different than the totals given by Wei et al. (2021). Sterol numbers reported by Wei et al. are much higher than the total sterol values obtained in this study, in some cases nearly three times as much. Considering that Iatroscan values are composed of free sterols as opposed to total sterols, we would expect them to be lower as conjugated sterols are not included. This is in disagreement with literature values of Iatroscan sterols versus total sterols where they were similar (Miller et al, 2007).

In the muscle tissues, five sterols were identified: cholesterol, cholestanol, campesterol, stigmasterol, and dinosterol. Cholesterol was again present in all dietary treatments, with the highest amounts being found in the diets containing microbial oil, however not at significantly higher levels than in fish fed the control (FO) diet. Cholesterol amounts were significantly higher than with the diet containing fish oil and vegetable oil, indicating the cholesterol can be obtained from microbial oil and incorporated by the fish. There were no significant differences in the amounts of the other sterols as compared to the control diets. Cholestanol was present in muscle tissue of salmon fed all diets and is a reduced form of cholesterol. Campesterol, not surprisingly, was also present in all muscle tissue samples since it was identified in all diets, and can be attributed to their wheat content. Stigmasterol was present in all diets, with no significant difference in amounts among the treatments. Dinosterol was present in all diets and is common in dinoflagellates. The total sterol concentration averaged 0.13 mg/g across all treatments. Miller et al. (2007) conducted a study in which fish oil was replaced with thraustochytrid *Schizochytrium* sp. oil in Atlantic salmon diets and found an average sterol content of 0.26 mg/g, with no significant difference among the treatments used in the study (Miller et al., 2007). In the Camelina study discussed in Chapter 2, the average total sterol content was similar to our study at 0.16 mg/g, again with no significant difference among trials.

Figure 3.8 shows the % of the total sterol content for each sterol detected in both the diet samples and the muscle tissues from fish fed the experimental diets. Although there were significant differences in the proportions of cholesterol in the diets, there were no significant differences in the cholesterol in the muscle tissues. The FO/VO and two MO

tissues had cholesterol proportions which were all significantly higher than in the diets. Cholestanol and dinosterol were only present in the tissue samples and were not present in the diets. Brassicasterol, 23,24-dimethylcholest-5-en-3β-ol, and 24-ethyl-5α-cholest-7-en-3β-ol were detected in the diets, but not in the muscle tissues. While campesterol and stigmasterol were present in both diets and tissue samples, there were no significant differences between the treatments.

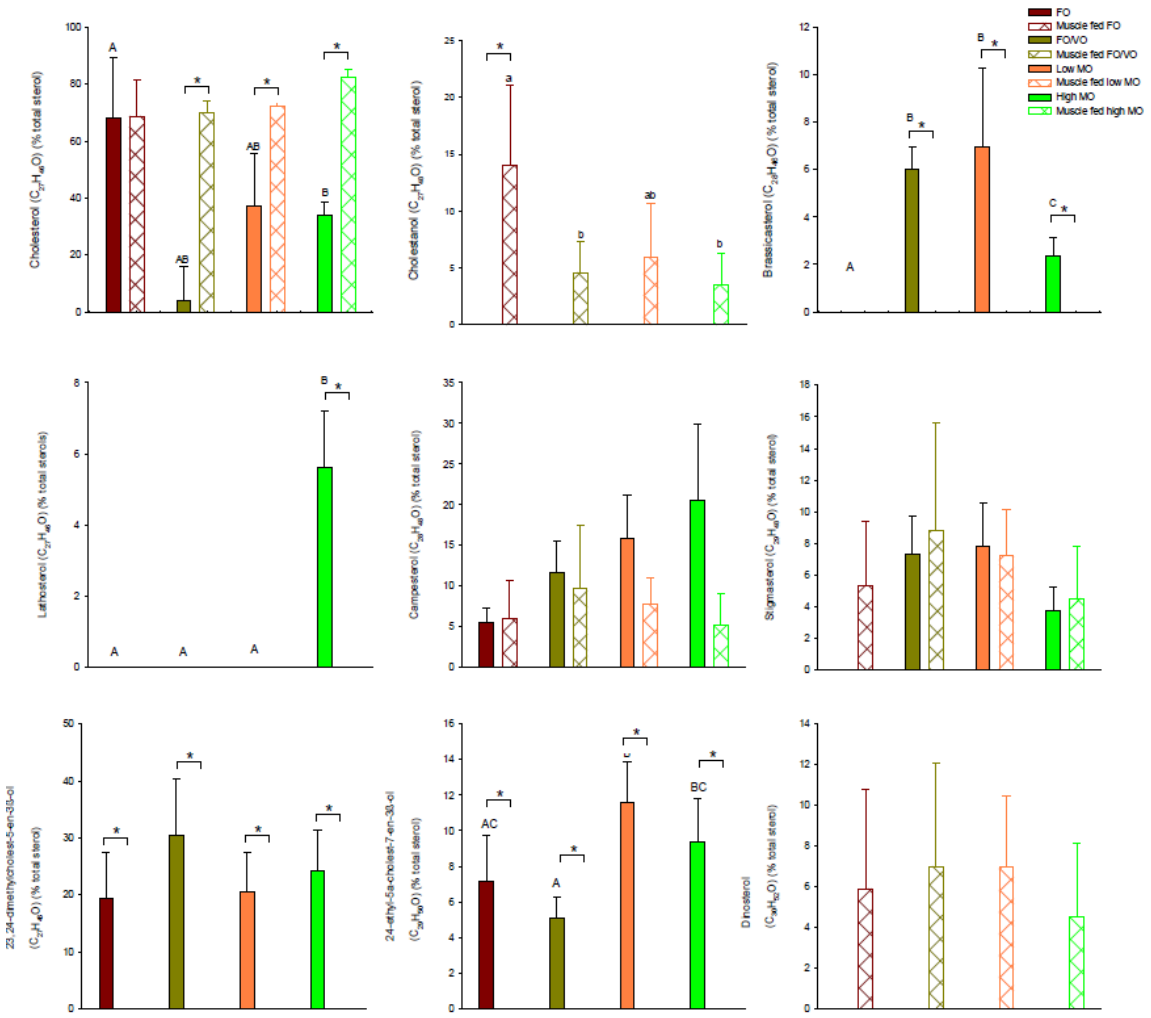

Figure 3.8: Identified sterols (in % of free sterols) found in experimental diets and corresponding muscle tissue from fish fed the control and experimental microbial oil diets. Upper case letters indicate significant differences in diet proportions; lower case letters in muscle proportions. Asterisks indicate significant differences between diet and muscle.

Research by Sissener et al. (2018) looked at the retention of sterols compared to the dietary sterols that were added to each experimental diet. They found that the retention of cholesterol was clearly dependent on the level of dietary cholesterol, with decreasing retention as dietary levels increased (Sissener et al., 2018). They also looked at phytosterol

retention levels. Campesterol was the predominant sterol in all samples, and they found that the retention of campesterol correlated negatively with both dietary cholesterol and dietary phytosterol content. Retention of brassicasterol correlated negatively with dietary phytosterol content but not with dietary cholesterol (Sissener et al., 2018). Brassicasterol was not detected in the muscle tissue samples from the microbial oil trials; however campesterol was detected in all tissue samples. It does not appear that phytosterol retention increased or decreased in any of the samples from the microbial oil trial, as there was no significant difference among the treatments. Sissener et al. (2018) determined that dietary phytosterols did not seem to affect cholesterol absorption or tissue cholesterol levels, and also did not affect tissue phytosterol levels.

Miller et al. (2007) replaced fish oil with *Schizochytrium* sp. oil in diets for Atlantic salmon parr. They found no significant differences in the lipid composition of the fish fed the four diet treatments, with a sterol content in white muscle samples at an average of 1.7 % of the total for all treatments (Miller et al., 2007). The inclusion of thraustochytrids oil in the diets significantly increased the amount of DHA in the salmon muscle. Based on both this finding and the growth results, they concluded that thraustochytrid oil can be used to replace fish oil in Atlantic salmon diets without being detrimental to the growth of parr (Miller et al., 2007).

3.6 Conclusion

This study identified the sterols present in microbial oil from *Schizochytrium* sp. and looked at which of these sterols were transferred to the experimental diets, and in turn the muscle tissue of fish fed these diets. Six sterols were identified in the microbial oil, four of which were detected in the diets, and two of these were subsequently found in the muscle tissue. There were also two different sterols that were found in the muscle tissue that were not in the diets or the microbial oil. This indicates that sterols in microbial oil can be transferred to the fish by consumption, but not all sterols were found to do so. Also, phytosterols can be metabolized to other sterols by the fish, as was seen with cholesterol and cholestanol.

Work by Wei et al. (2021) looked at this same experimental trial in terms of growth performance and potential as a replacement for fish oil in farmed Atlantic salmon diets. Based on the positive results, it was concluded that dietary microbial oil from *Schizochytrium* sp. can be used to replace fish oil in the diet of farmed Atlantic salmon without negatively impacting fish growth and the fatty acid composition of the tissues (Wei et al., 2021). Therefore, microbial oils, such as that from *Schizochytrium* sp., might provide an alternative source of lipids and sterols for aquaculture fish feed.

Increasing levels of plant proteins and lipids in fish feed may reduce the levels of dietary cholesterol available to the fish, as plant sources are generally lower in cholesterol and additionally phytosterols may impact cholesterol uptake. Since fish have the ability to synthesize cholesterol, the presence of dietary cholesterol has not been previously widely studied. However, if fish fed diets derived from plant sources are seen to be more

susceptible to diseases due to decreased lipid digestibility and cholesterol levels, replacing fish oil could have detrimental effects on the fish. Cholesterol does occur in microalgae, and there are examples where it is the major sterol present (Volkman, 2003), thus microbial oil may be desirable for inclusion in fish feed as opposed to plant based sources.

Chapter 4: Conclusions and Recommendations

4.1 Conclusions

In this thesis, gas chromatographic techniques including GC-MS and GC-FID were used to identify and quantify sterols in various samples. The sterol profiles of Camelina seed oil, *Pavlova* oil, and a microbial oil from *Schizochytrium* sp. were determined. Lipid extracts from feed samples and muscle tissue samples from experimental feeding trials were investigated. Total lipids were extracted from samples using a modified Folch procedure followed by derivatization with BSTFA to obtain TMS-ethers.

The most abundant phytosterols in Camelina oil were brassicasterol, campesterol, stigmasterol, and β-sitosterol, in order of elution. In the experimental diets containing camelina oil, brassicasterol, campesterol, stigmasterol, and β-sitosterol were also identified, however in the muscle tissue from salmon fed these diets, only campesterol and β-sitosterol were detected. Cholesterol was also detected in the camelina oil, the experimental diets, and the fish tissue samples. Free sterol content increased in the fish fed the diet containing 100% substitution with camelina oil. A sample of oil from *Pavlova* sp. was also analyzed and 9 sterols were detected: cholesterol, campesterol, stigmasterol, stigmastanol, spinasterol, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, 24-methylpavlovol, and 24-ethylpavlovol. Both camelina oil and Pavlova oil have potential to be used as replacements for fish oil in fish feeds and are worthy of further study.

In the microbial oil experiment, cholesterol, lathosterol, brassicasterol, 24-methylenecholesterol, 24-methylenelophenol, stigmasterol, and spinasterol were the sterols identified. In the experimental diets containing microbial oil, cholesterol, brassicasterol, lathosterol and stigmasterol were found (all of which are present in the microbial oil), with the addition of campesterol, and two other sterols (23,24-dimethylcholest-5-en-3β-ol and 24-ethyl-5α-cholest-7-en-3β-ol). In the muscle tissue from salmon fed the diets containing microbial oil, cholesterol, cholestanol, campesterol, stigmasterol, dinosterol and 24-methylpavlovol were detected.

Overall, it is apparent that certain phytosterols are transferable from plant based or microbial based oils, to diet formulations, and ultimately to the fish tissue. Other studies related to this experiment have demonstrated that the microbial oil can be used to replace fish oil in diets for Atlantic salmon without negatively impacting fish growth performance (Wei et al, 2021). It has also been established that Camelina oil can be used as a replacement for fish oil in diets for farmed Atlantic salmon with no difference in weight gain, growth rate or sensory quality to the fish (Hixson et al, 2014).

Gas chromatography has been the technique of choice for the analysis of sterols and related compounds for a number of years (Lagarda et al, 2006) and this study supports that. GC-MS was effective in detecting sterols present in samples and mass spectrometry aided in the identification of sterols when standards were not available. GC-FID analysis along with GC-MS data allow for quantification of results. Sterols can be routinely analyzed by GC-FID based on known sterol retention times and FID responses versus those of standards, while GC-MS is useful for confirmation of peak identities.

4.2 Future Work

Although this work gave some insight into the sterol content of alternative lipid sources, several improvements could be made to obtain better results. To obtain better quality, more reliable data in future research, the number of analyses should be increased. This would allow for better comparison of experimental data to literature information. It would also allow for better quantification of results. Sample clean up, such as solid phase extraction (SPE), could be useful to isolate the sterol fraction from the lipid extract. Optimization of the SPE would help achieve ideal separation and would likely lead to better identification of sterols. Phytosterol content of other organs and fish tissues could be examined as well, as sterols may be present in other tissues, particularly the liver.

Future research in this area could be conducted to further investigate the potential of phytosterols for use in aquaculture feeds. Other types of plant based, or microbial based oils could be tested. Finally, although gas chromatography is the generally accepted method for sterol analysis, other methods could be investigated to look at their ability to detect and identify sterols. MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight) could be used for sterol analysis without requiring extensive sample preparation or clean up. DESI-MS (desorption electrospray ionization mass spectrometry) could allow for rapid structural analysis of sterols and is a powerful and sensitive technique.

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Appendix A

Mass spectrum of sterols identified in camelina experiment

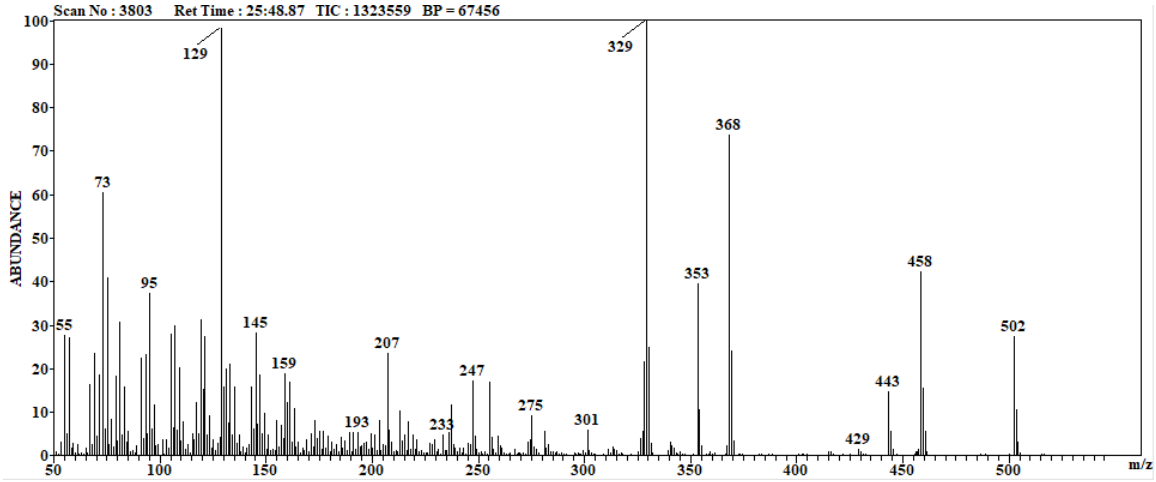

Figure A1: Mass spectrum of cholesterol.

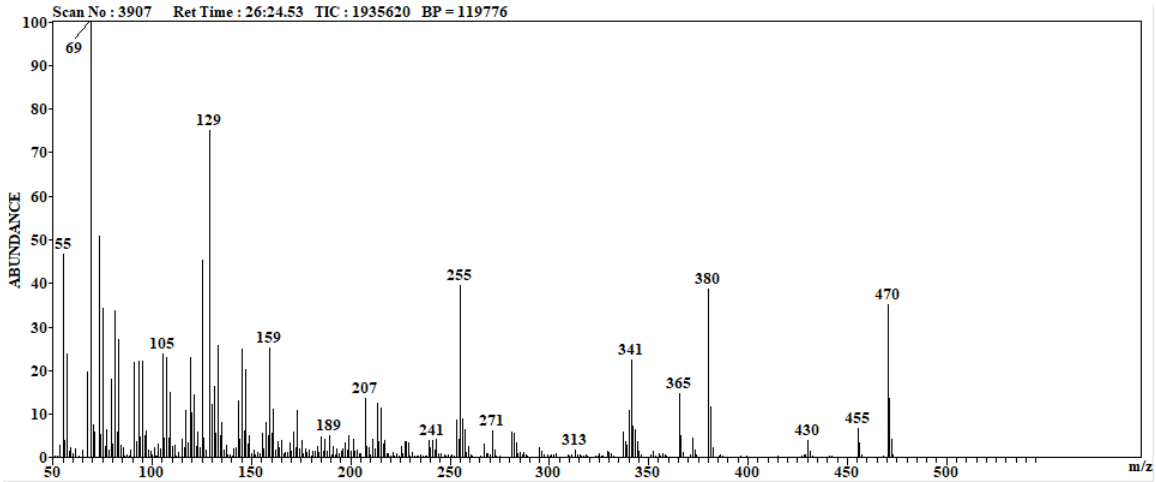

Figure A2: Mass spectrum of brassicasterol.

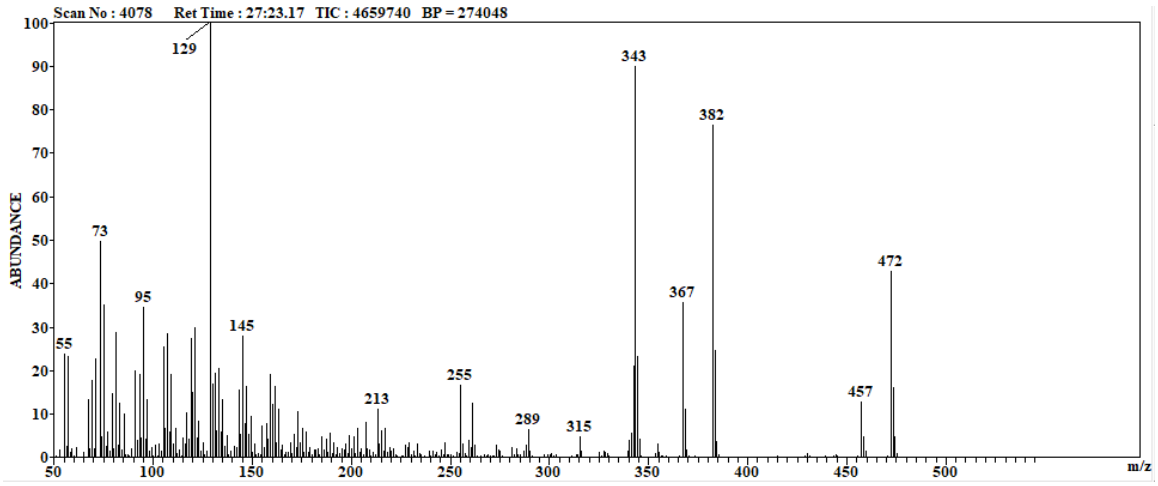

Figure A3: Mass spectrum of campesterol.

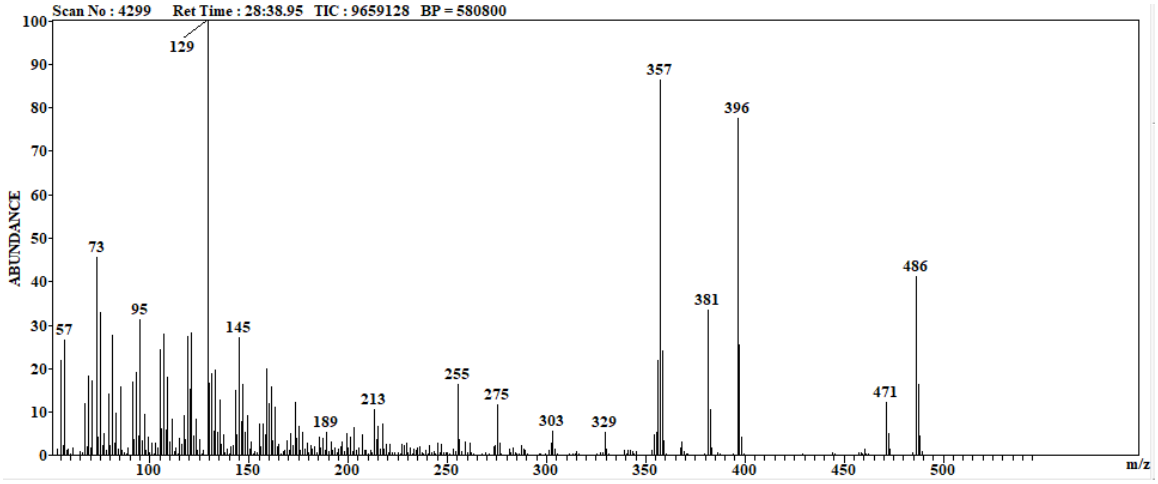

Figure A4: Mass spectrum of stigmasterol.

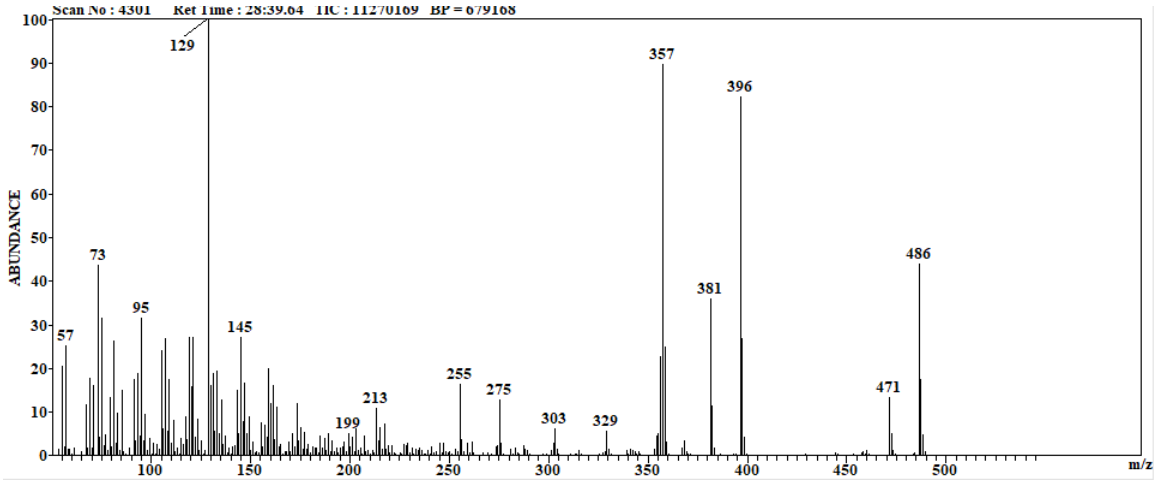

Figure A5: Mass spectrum of β-sitosterol.

Appendix B

Mass spectrum of sterols identified in *Pavlova*

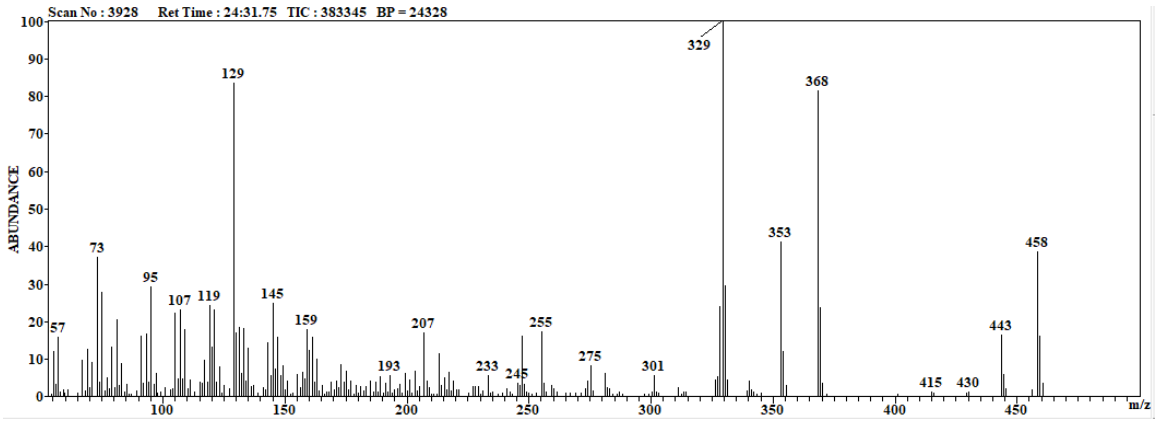

Figure B1: Mass spectrum of cholesterol.

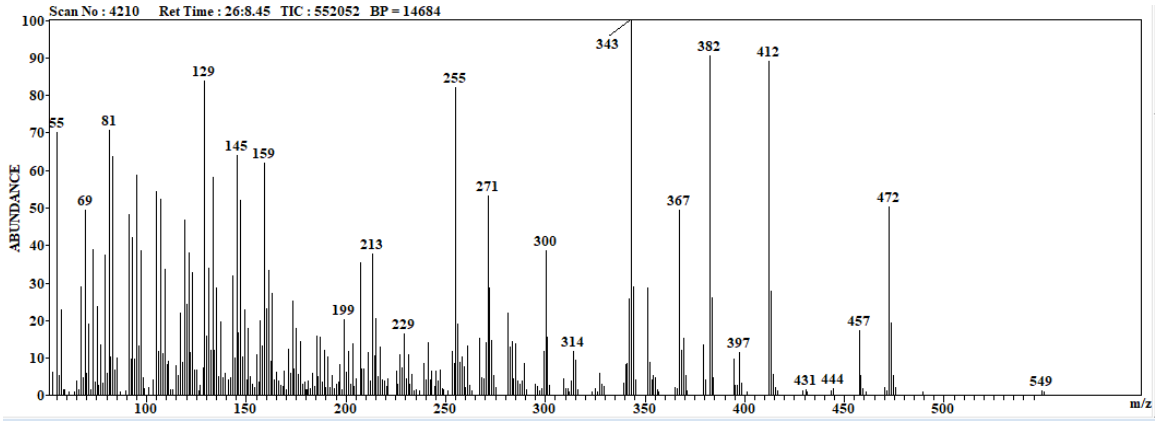

Figure B2: Mass spectrum of campesterol.

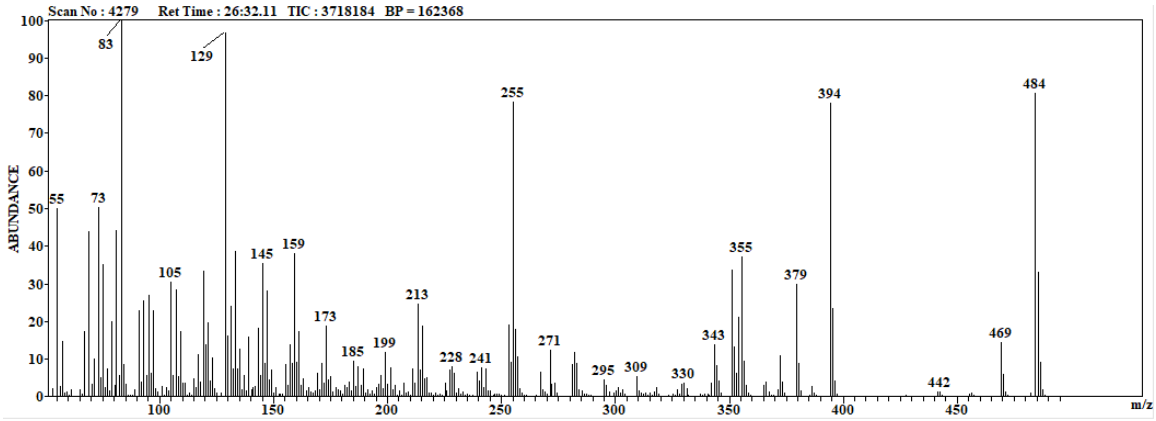

Figure B3: Mass spectrum of stigmasterol.

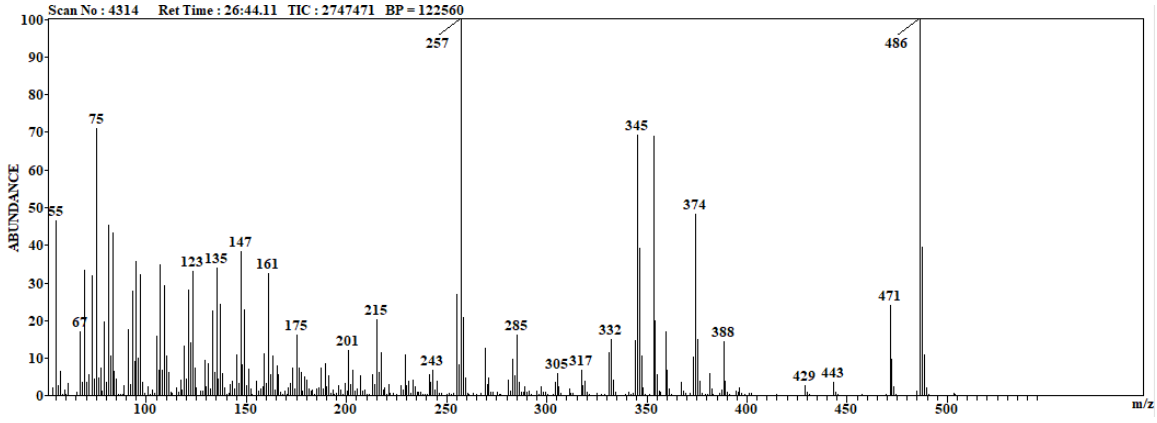

Figure B4: Mass spectrum of stigmastanol.

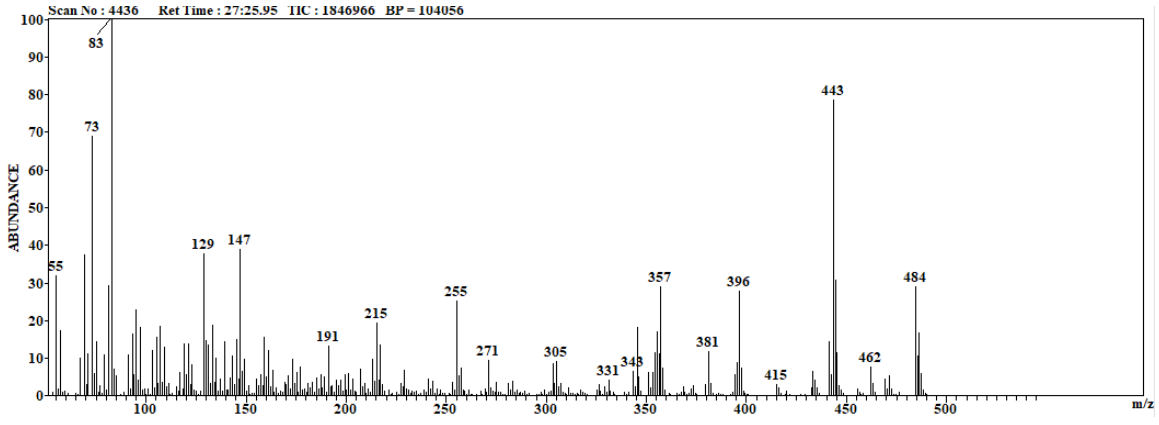

Figure B5: Mass spectrum of spinasterol.

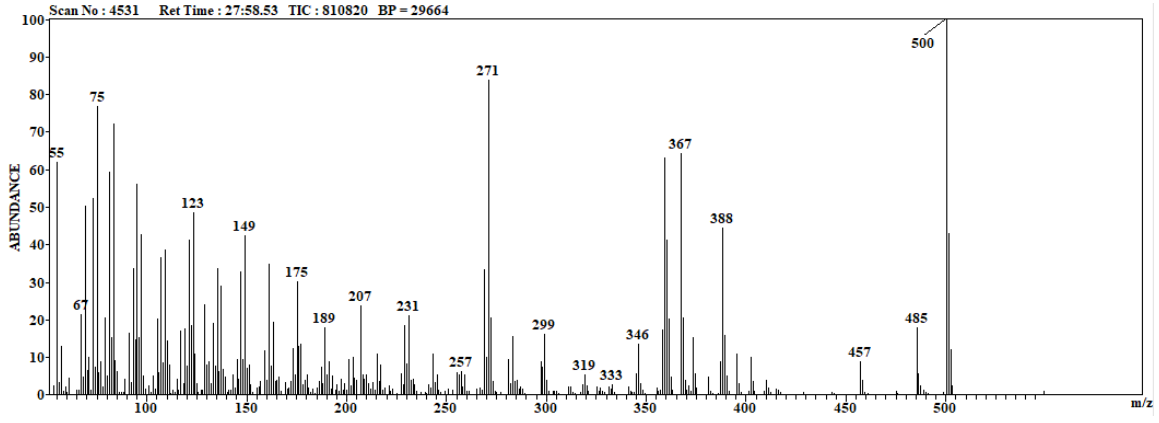

Figure B6: Mass spectrum of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol.

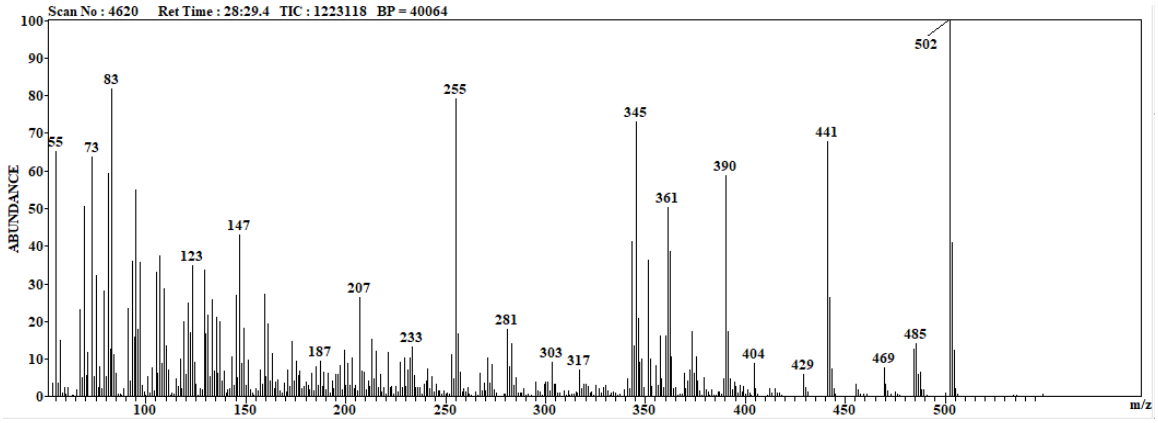

Figure B7: Mass spectrum of 4α-methyl-24-ethyl-5α-cholestan-3β-ol.

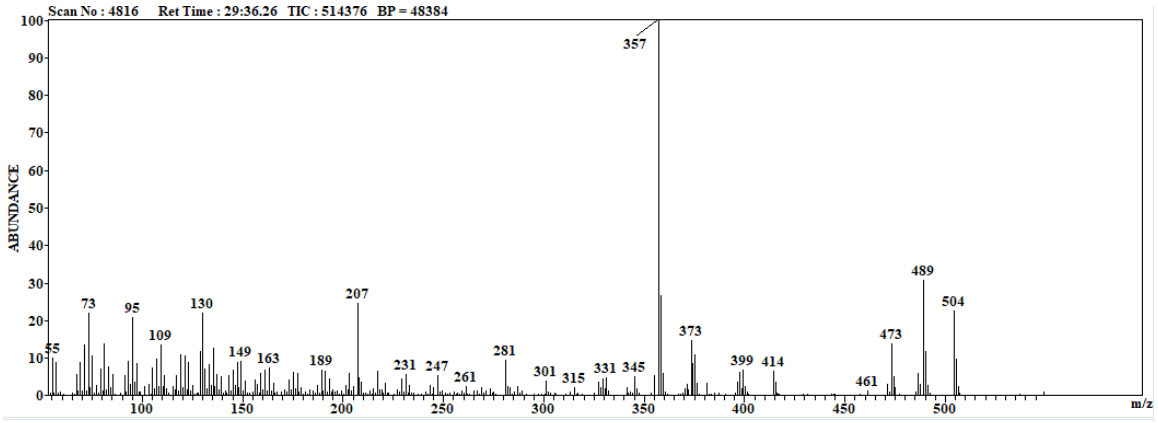

Figure B8: Mass spectrum of 24-methylpavlovol.

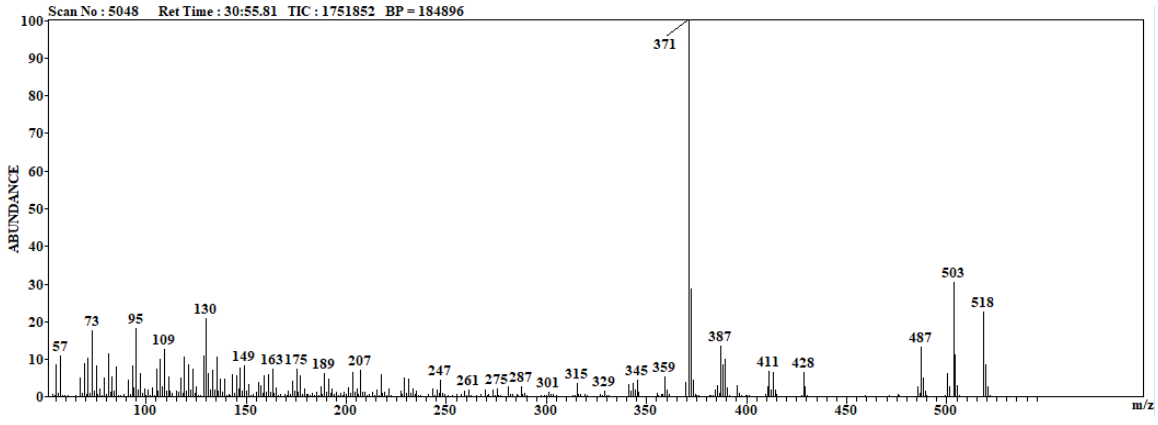

Figure B9: Mass spectrum of 24-ethylpavlovol.

Appendix C

Mass spectrum of sterols identified in microbial oil from *Schizochytrium*

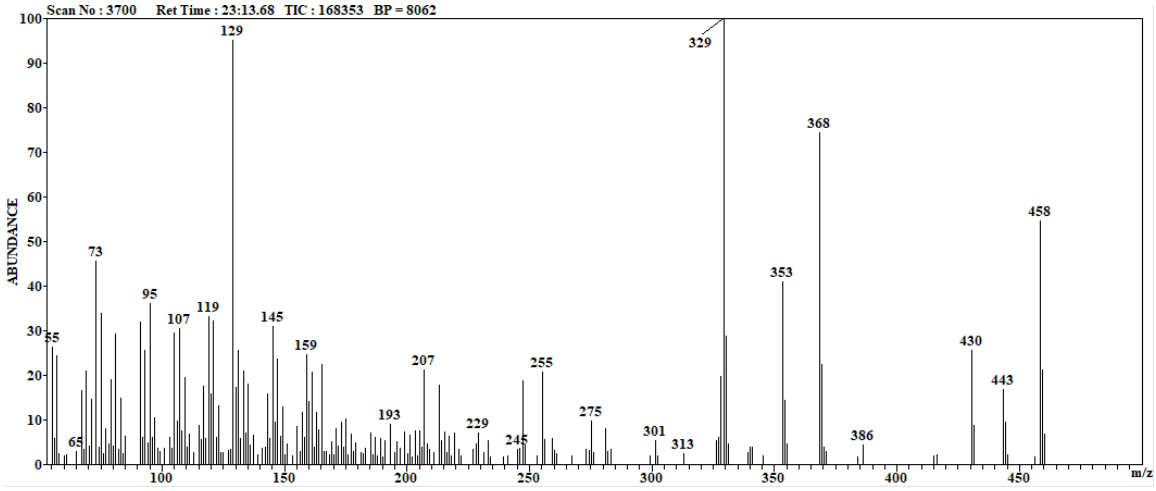

Figure C1: Mass spectrum of cholesterol.

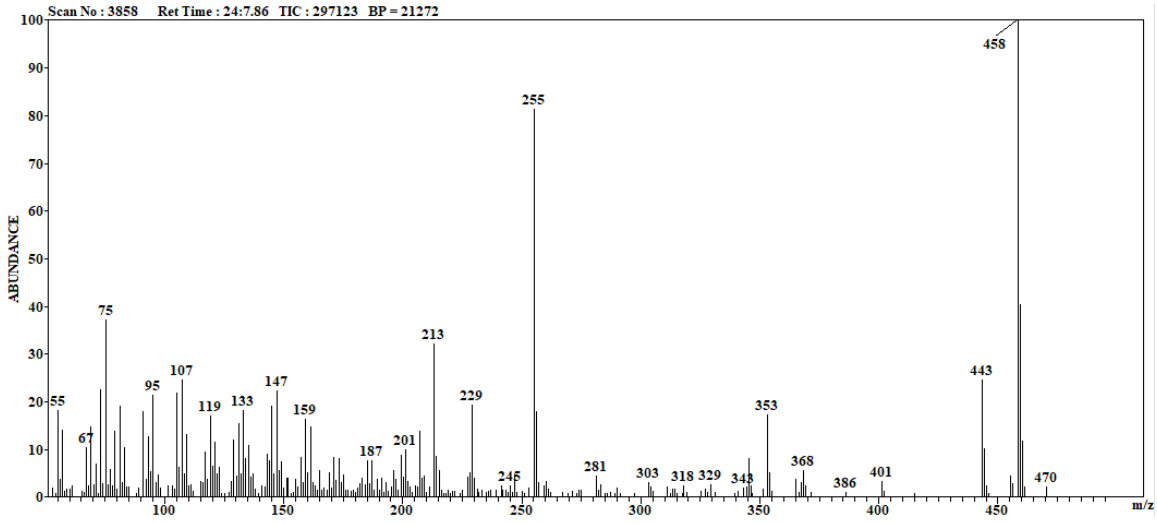

Figure C2: Mass spectrum of lathosterol.

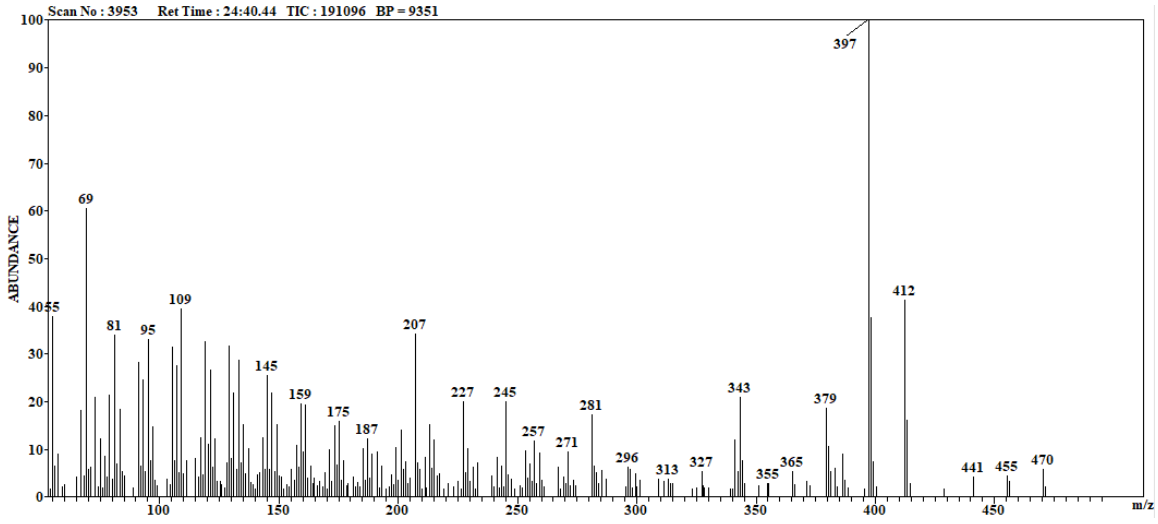

Figure C3: Mass spectrum of brassicasterol.

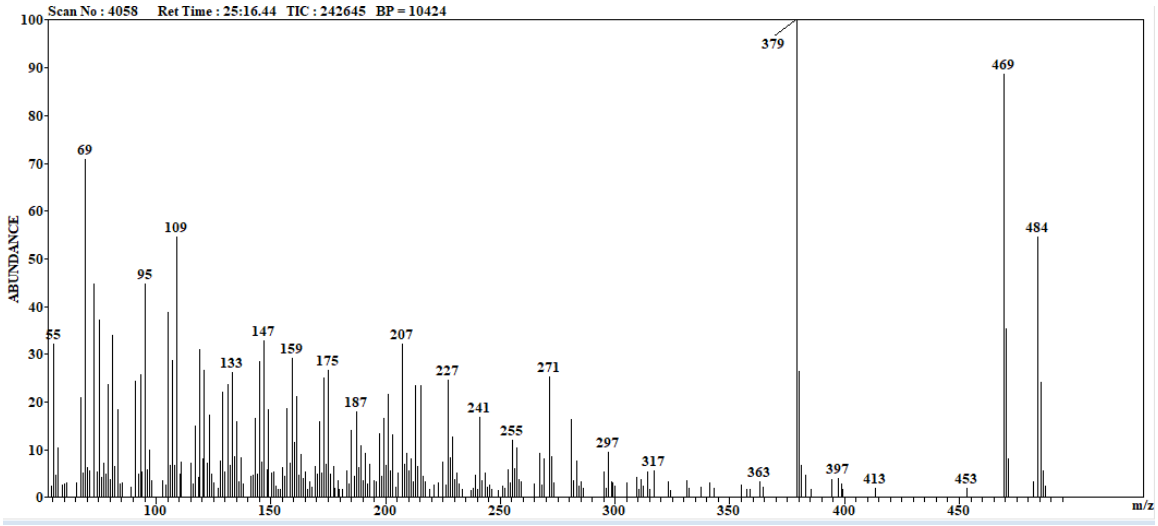

Figure C4: Mass spectrum of 24-methylenecholesterol.

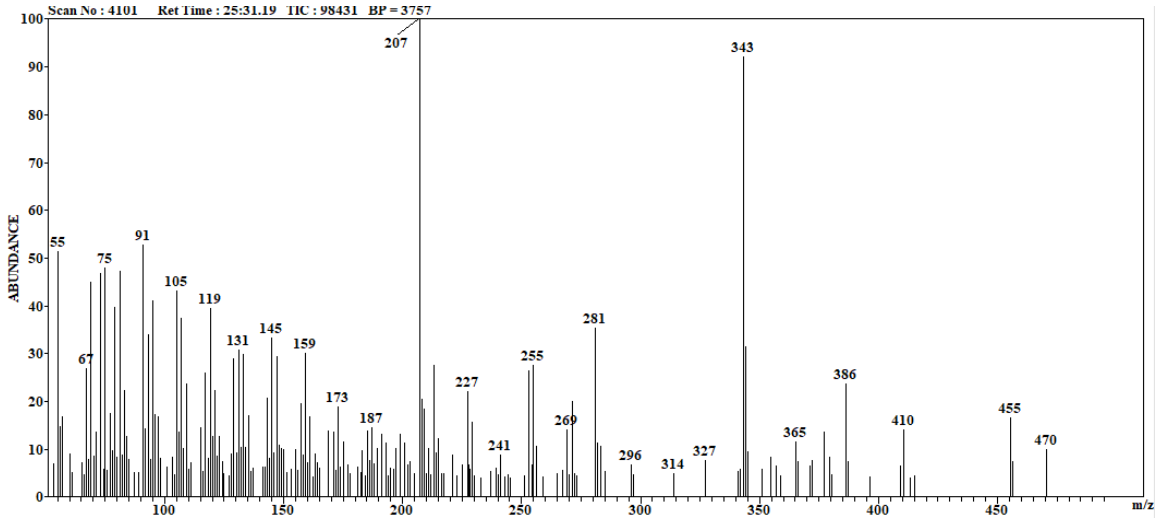

Figure C5: Mass spectrum of 24-methylenelophenol.

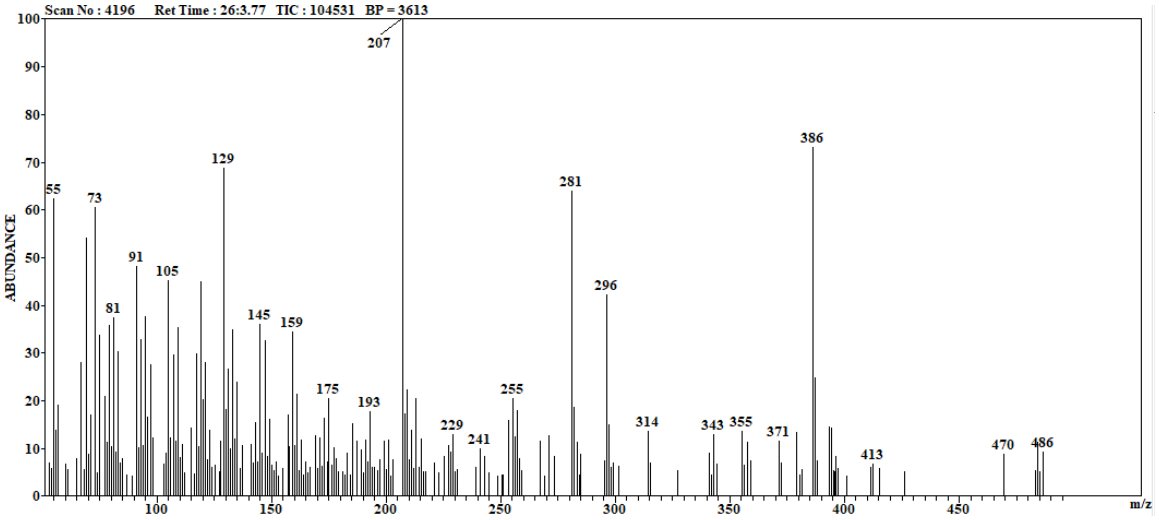

Figure C6: Mass spectrum of stigmasterol.

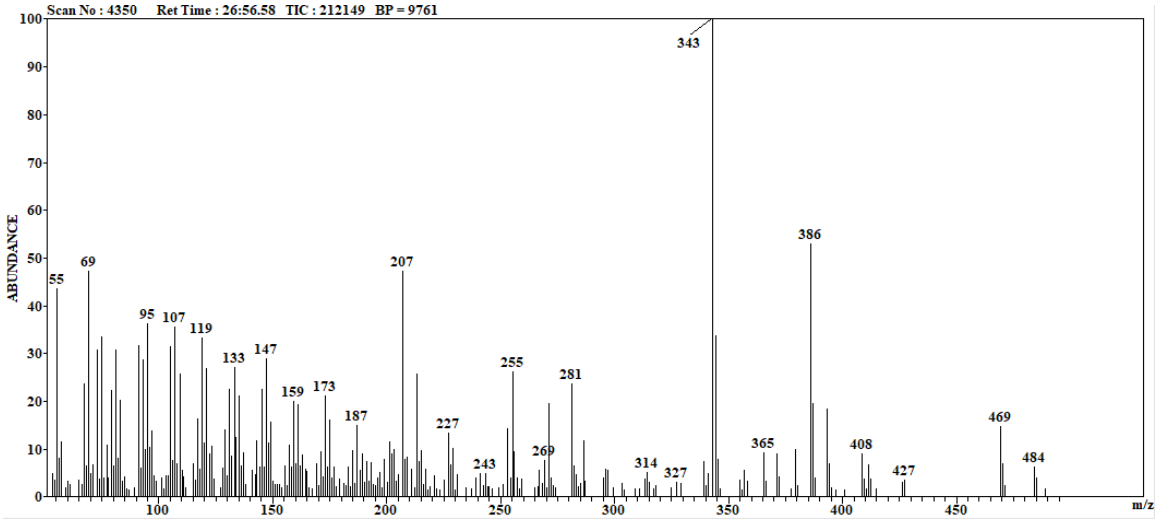

Figure C7: Mass spectrum of spinasterol.

Appendix D

Mass spectrum of additional sterols identified in microbial oil diets.

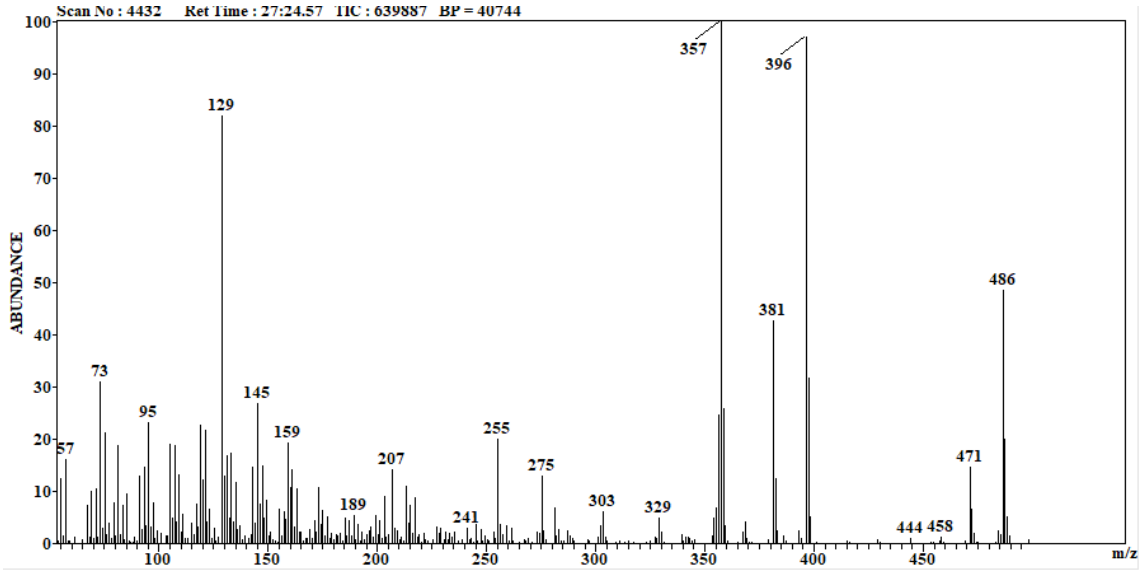

Figure D1: Mass spectrum of 23,24-dimethylcholest-5-en-3β-ol.

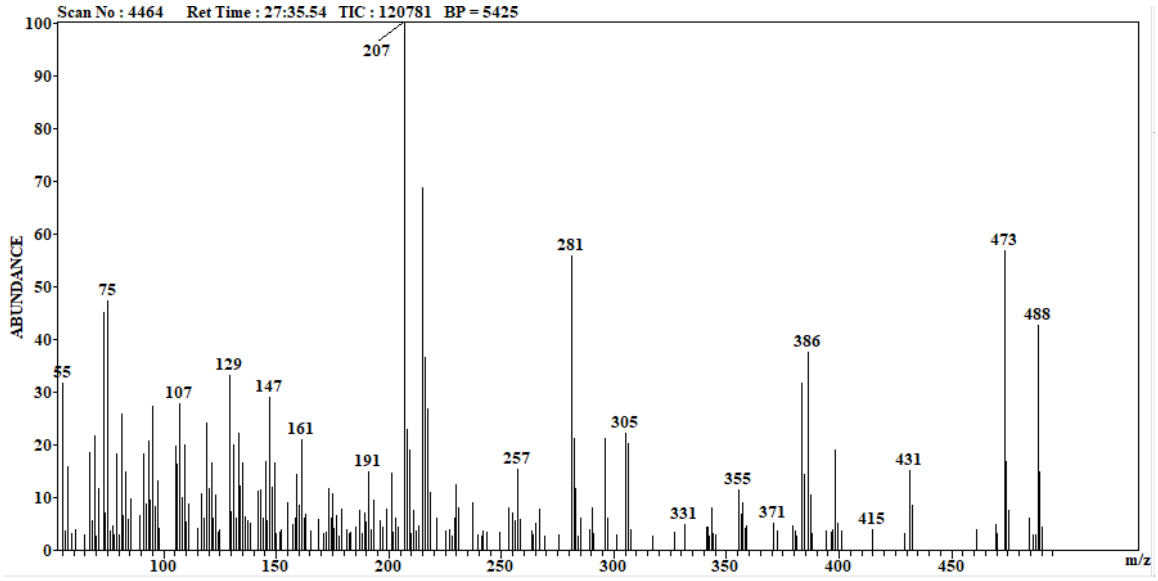

Figure D1: Mass spectrum of 23,24-dimethylcholest-5-en-3β-ol.