SALMON PROCESSING WASTE: A PROSPECTIVE SOURCE FOR NUTRACEUTICALS

DEEPIKA DAVE
MARINE INSTITUTE, MEMORIAL UNIVERSITY
NOVEMBER 2016

2015-16 HARRIS CENTRE - MMSB WASTE MANAGEMENT APPLIED RESEARCH FUND

THE HARRIS CENTRE Memorial University

Contents

1 Acknowledgements

We would like to express our deepest thanks to Harris Centre for financial support through Waste Management Funds to carry out crucial research on extraction of salmon oil and omega-3 fatty acids from salmon unutilized resources and help to support our large initiative to establish a Salmon Biorefinery Strategy. We also appreciate support from Newfoundland and Labrador Aquaculture Industry Association and Newfoundland Marine Group.

2 Executive summary

This report summarizes a series of small studies on the production of omega-3 concentrates from salmon by-products. Because raw material must be of highest quality for the production of nutraceuticals (i.e. omega-3 capsules), spoilage rates and fatty acid composition changes were examined by applying different handling and pretreatment procedures. Further, different enzymatic methods for concentrating omega-3 fatty acids from oil were compared. The results provided important insight and will be used as part of larger initiatives on the creation of a Biorefinery strategy for the province's aquaculture waste. The knowledge and experience gained by the Bioprocessing team and the fine-tuned analytical methods, especially GC (Gas Chromatography) will be highly valuable for academic and industry use in future projects.

3 Glossary and Acronyms

AG	Acylglyceride
FA	Fatty Acid
FAEE	Fatty Acid Ethyl Ester
FAME	Fatty Acid Methyl Ester
GC	Gas Chromatography
IS	Internal Standard
MUFA	Monounsaturated Fatty Acid
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid
Ω-3	Omega-3

4 Introduction

4.1 Project Background

Fisheries and Aquaculture play a vital role in Newfoundland and Labrador's economy. However currently, the province lacks commercial fish waste management facility. At present, Newfoundland and Labrador has limited capacity to handle this waste in terms of composting, mink feed, rendering and landfilling. The salmon waste has the potential to produce approximately 1600 tons of oil annually in Newfoundland and Labrador. Cumulative recovery of the salmon industry waste for marketable oil extraction is extremely difficult due to the remote location of the salmon processing plants in Atlantic Canada. During salmon processing operations, significant amount of discards including skin, frame and trimmings (gut, fins, tail) were produced which can be used as a great source to generate various value added products such as fish oil, proteins, amino acids, biodiesel and omega-3 fatty acids. Generally, fish contains 2-30% fat and about 50% of the body weight is generated as waste during the fish processing operation. Therefore, fish processing waste could be a great potential source for good quality fish oil that can be used for human consumption (Khoddami et al (2009), Jayasinghe et al (2012), Gbogouri et al (2006), Rubio-Rodriguez et al (2012)). Atlantic salmon contains 2-15% fat content and 57% of the total body fat is present in the inedible portion while skin contains 18% of the total body fat. Fish oils are one of the major sources of long chain polyunsaturated fatty acids including cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) (Khoddami et al (2009), Gbogouri et al (2006), Rubio-Rodriguez et al (2012), Haraldsson et al (2001), Shahidi et al (2007)). The functional and biological properties of omega-3 fatty acids include: prevention of atherosclerosis, protection against arrhythmias, reduced blood pressure, beneficial to diabetic patients, protection against manic-depressive illness, reduced symptoms in asthma patients, protection against chronic obstructive pulmonary diseases, alleviate symptoms of cystic fibrosis, improving survival of cancer patients, reduction in cardiovascular disease and improved learning ability (Shahidi et al (2007), Shahidi (2009), Simonopoulos (2002), Simonopoulos (2002), Kris-Eherton et al (2002)).

Public awareness about health and nutrition is increasing every day. People are more inclined to choose a healthy diet, accessible according to their financial situation. However, because of the busy lifestyle many people opt for pre-cooked or ready to eat food commodities. A major part of the population also has different food allergies which makes their food options more limited. Hence, scientists are trying to develop genetically modified (GM) crops; and food and feed farmers are growing the developed GM crops to increase the availability of various essential and useful nutrient components in different fruits and vegetables which originally never existed. Also, food companies are developing processed food commodities with different food supplements and essential components to accommodate and fulfill the nutritional requirements of general population and people with food allergies (Merrilees et al (2000), Whalley et al (2004), Nestel et al (2004), Siddiqui et al (2004)). The major source of omega-3 fatty acids in human diet are fatty fishes. Supplementation of omega-3 fatty acids in food commodities and availability of encapsulated forms will help in improvement of health conditions without increase in the uptake of food with high oil content. Extraction of omega-3 fatty acids from non-food source such as salmon processing waste will not interfere with the availability of main food sources and mitigate the problems associated with the salmon waste disposal and environmental pollution. In addition, the project will create a significant impact on the economics of aquaculture industries in the province.

4.2 Rationale

The omega-3 fatty acids encapsulation is a growing market world-wide. In most part of the world the omega-3 fatty acids are consumed in the form of fish. To make it available to vegetarian people, population with no access to marine products and people with allergies, consumption of encapsulated omega-3 might be the only option. Development of an economically viable and environmentally friendly method will increase the availability, acceptability and feasibility of fish waste derived omega-3 fatty acid products to a wide spectrum of people. Atlantic marine products and Newfoundland Health Foods Corp. are the commercial producers of omega-3 fatty acids in Newfoundland whose main source is from "Seal". However, Seal farming is still an issue of debate in many parts of the world. The implemented project has taken constructive step towards *better utilization of salmon aquaculture waste for omega-3 fatty acids production from salmon oil as a part of improving waste management practices in the province*. This project was carried out using environmentally friendly enzymatic extraction methods as an *efficient alternative to the existing* chemical extraction methods which produces a lot of chemical waste and consumes lot of energy.

4.3 Objectives

(a) Characterization of salmon waste (b) pretreatment and handling of the salmon waste (c) enzymatic extraction and characterization of salmon oil and (d) enzymatic concentration and concentration of omega-3 fatty acids.

4.4 Research Methodology and Approach

4.4.1 Raw materials and sample preparation

Fresh salmon frames (a total of three samples) were obtained from two local aquaculture sites. The frames were placed on ice immediately after processing and shipped to the Bioprocessing facility without delay. The next day, the frames were processed through a Baader deboner to separate the meat from the bones. The resulting salmon mince was vacuum packed in 1 kg bags and frozen at -80°C until use.

4.4.2 Proximate analysis

Proximate analysis was conducted separately for individual fractions of fish head, frame and guts. Protein analysis was conducted using Kjeldahl method (AOAC 954.01). Lipid content was determined using the Soxhlet method (AOAC 948.15) and ash and moisture were determined according to standard dry ashing and moisture procedures (AOAC 938.08 and 930.15).

4.4.3 Water activity, salinity and colour

Water activity was measured using AquaLab water activity meter (series 3/ 3TE, Decagon devices, Washington, USA) and colour was measured using ColorTec-PCM colorimeter (New Jersey, USA) using L, A and B scale. Salinity was measured using salinity meter.

4.4.4 Total plate count and yeast and mould count

A homogenate of the freeze-dried salmon by-products mixture was prepared by combining 99ml of sterile diluent and 11 g of sample, which was homogenised for 2 min. Further serial dilutions of $10^{-2}$to 10^{-7} were prepared "using sterile peptone water dilution blanks and homogenized sample from the stomacher bag.

For total yeast and mold count, about 15 to 20 ml of the agar () was poured in the plates and were dried overnight at room temperature and stored at 5-10 ºC for future use. 0.1 ml aliquots of each dilution were transferred to the prepared OGYE plates and distributed evenly on the agar surface using a sterile bent

glass rod. The plates were incubated in dark, for 5 days, after which colonies were count and reported as yeast and mold CFU/g. All the dilutions were tested in duplicates.

In case of total plate count (TPC), for the analysis of total number of bacteria, 1 ml of the aliquot of each dilution was first poured into Petri plates, which was replicated. 12 to 15 ml of plate count agar (freshly prepared, sterilized using autoclave, and cooled to 44-46 ºC) was poured into the petriplates. The sample and agar were mixed and agar was allowed to solidify, after which the petriplates were inverted and incubated at 35 ºC for 48 hours. The colonies were counted and expressed as CFU/g.

4.4.5 Chemicals and Enzymes

All chemicals were ACS grade or higher and were purchased from Sigma-Aldrich (St. Luis, MO, USA) or VWR International (Radnor, PA, USA). Alcalase was purchased from Sigma Aldrich (St. Luis, MO, USA), Sea-B Zyme L200 was purchase from Specialty Enzymes (Chino, CA, USA) and the lipase used for concentration of omega-3 fatty acids war purchased from Amano Enzymes (Chipping Norton, U.K.).

4.4.6 Oil extraction

The required raw material was gently thawed in the fridge without removing it from the vacuum bag. Oil was extracted from the salmon mince by enzymatic hydrolysis. Extraction parameters were selected based on provided optimal conditions by manufacturer. In order to compare oil extraction efficiencies, two different enzymes including Alcalase (Sigma-Aldrich) and Sea-B-Zyme (Specialty Enzymes, California) used.

500 g of thawed, ground fish frames was transferred into 1 L Erlenmeyer flask. 2.5 g (0.5% v/w) of Enzyme (Alcalase or Sea-B-Zyme) was mixed with 100 ml distilled water poured in a 200 ml measuring cylinder maintained at 30°C. An additional 100 ml of distilled water, maintained at 30 °C was used for recuperating the enzyme from the measuring cylinder and added into the ground fish sample. The flasks were flushed with N_2 and loosely sealed. The mixture was digested in an incubator shaker (Thermo- Scientific Max Q 6000, Marietta, Ohio, USA) at 55°C and 200 rpm for 2 hours. The hydrolysate was centrifuged at 4000 rpm for 20 mins, for separation of the digested mass into different fractions based on varying density. The oil fraction was removed and transferred to a clean 50 ml centrifuge tube. The oil was covered with N_2 and stored at -80°C until use.

4.4.7 Concentration of Ω-3 fatty acids

4.4.7.1 *Enzymatic Hydrolysis Method 1 (Okada and Morrissey, 2008)*

20 ml of 0.1 M Phosphate buffer, pH 9.17 and 2 g of oil was placed in an Erlenmeyer flask together with 350 Units of enzyme/g oil (*Pseudomonas sp,* Amano AK) and incubated for 1 h. Following hydrolysis, deionized water (10-fold) potassium hydroxide and 0.1 % methanolic phenolphthalein solution were added to the flask. The KOH was added for neutralization of the FFA released by hydrolysis and the indicator solution was added to monitor pH. An aliquot of $CaCl_2$ was added to the mixture to break down emulsions and obtain a high oil yield. The final PUFA enriched oil was recovered after centrifugation at 6000 rpm for 20 min.

4.4.7.2 *Enzymatic Hydrolysis Method 2 (Mohanarangan, 2012)*

4 g of oil, 6 ml of Phosphate buffer (pH 9.17) and 200 U/g oil of lipase were mixed in an Erlenmeyer flask, flushed with Nitrogen and incubated for 2 hours. The hydrolysis was stopped by adding 2 ml of methanol. 1 ml of 1 % KOH was added to neutralize the FFA released during hydrolysis. The mixture was transferred to a separatory funnel and thoroughly mixed with 100 ml hexane and 50 ml distilled water. The lower

aqueous phase was discarded. The hexane phase was washed again with 50 ml of distilled water. The hexane phase was then separated and evaporated. The recovered oil was subjected to fatty acid analysis according to AOAC991.39.

4.4.7.3 *Enzymatic concentration by Alcoholysis*

Ω-3 fatty acids were concentrated in salmon oil by an ethanol-catalyzed, enzymatic process. Two different enzymes were investigated Enzyme (*P. fluorescens*, Amano PS and *Pseudomonas sp,* Amano AK). For each enzyme, several process parameters were investigated and optimized according to a factorial design (Table 1) using low/high limits for incubation time (12 h, 24 h), enzyme concentration (10 %, 15 %), and molar ratio of EtOH (1:2, 1:6) and process temperature (20 °C, 50 °C). Minitab software was used for the design of experimental matrix followed by statistical analysis. Omega-3 enriched salmon oil analysis was performed by Gas Chromatography after silylation (no derivatization of the sample). Hence only fatty acids were detected that were produced from acylglycerides during alcoholysis. The composition of the FAEE gave an indication of the lipase performance.

2 g of oil were transferred to a clean 25 ml Erlenmeyer flask (previously rinsed with hexane and dried). The appropriate amounts of enzyme and Ethanol were added. The flask was flushed with N_2, capped tightly and incubated in a water bath shaker at 200 rpm and 20°C for the desired incubation time. The reaction was stopped by filtering out the enzyme using a 2 µm syringe filter.

100 µl of sample were transferred to a clean glass tube containing internal standard. 100 µl of tetrahydrofuran and 200 µl BSTFA were added and the tube incubated at 95°C for 15 min. After the sample cooled to room temperature, 5 ml of hexane. 1-2 µl of this solution was injected into GC.

During calculations the ethanol dilution factor was taken into account. The GC results were further normalized against the signal of the Internal Standard (IS).

Table 1: Full Factorial Design for Ω-3 concentration by ethanolysis

StdOrder	RunOrder	CenterPt	Blocks	time	temperature	ethanol	enzyme conc
16	1	1	1	24	50	6	15
1	2	1	1	12	20	2	10
10	3	1	1	24	20	2	15
2	4	1	1	24	20	2	10
12	5	1	1	24	50	2	15
15	6	1	1	12	50	6	15
17	7	0	1	18	35	4	12.5
14	8	1	1	24	20	6	15
5	9	1	1	12	20	6	10
13	10	1	1	12	20	6	15
4	11	1	1	24	50	2	10
3	12	1	1	12	50	2	10
8	13	1	1	24	50	6	10
7	14	1	1	12	50	6	10
18	15	0	1	18	35	4	12.5
19	16	0	1	18	35	4	12.5
6	17	1	1	24	20	6	10
9	18	1	1	12	20	2	15
11	19	1	1	12	50	2	15

4.4.8 Fatty Acid Analysis (based on AOAC 991.39 method)

Fatty Acid analysis was performed by Gas Chromatography according to AOAC 991.39.

4.4.8.1 *Preparation of glass tubes*

All test tubes used for GC analysis were thoroughly cleaned with soap and water and rinsed with distilled water. After drying the tubes in the drying oven at 100 °C, the tubes were rinsed with hexane and dried again. The tubes were capped and stored at room temperature until use.

4.4.8.2 *Preparation of sample tubes containing Internal Standard 23:0*

For those samples requiring an internal standard, test tubes were prepared as follows: 25 mg of 23:0 methyl ester internal standard (IS) was accurately weighed into a 25 ml volumetric flask and diluted to volume with Hexane. 1 ml portions of this solution was pipetted into 25 previously cleaned and hexane-rinsed test tubes. The solvent was evaporated in a gentle stream of N_2. The dry tubes were capped and stored at -80°C until use.

4.4.8.3 *Sample preparation for GC analysis (based on AOAC991.39)*

25 mg (+- 0.1 g) of oil was accurately weighed into a glass tube containing the Internal Standard. 1.5 ml 0.5 M methanolic NaOH was added. The tube was flushed with N_2, capped, mixed, and heated for 5 min at 100°C. After cooling, 2 ml Boron Trifluoride (BF3) in Methanol were added to the mixture. The tube was flushed with N_2, capped, mixed, and heated for 30 min at 100°C. After cooling to 30-40°C, 1 ml hexane was added. The tube was flushed with N_2 and agitated vigorously for 30 s while still warm. Immediately,

5 ml of saturated NaCl solution was added. The tube was flushed with N_2, capped, mixed, agitate thoroughly. The tube was then cooled to room temperature without agitation to allow for phase separation. The hexane phase was transferred to a clean glass tube. The aqueous phase was mixed with 1 ml of hexane, capped and mixed thoroughly. The mixture was given time to settle into two phases again. The hexane fraction was removed and added to the glass tube containing the first hexane fraction. The aqueous fraction was discarded. The hexane fraction was evaporated to a volume of about 1 ml in a stream of dry N_2. 1-2 µl of this mixture was injected into the GC.

4.4.8.4 *Gas Chromatography*

The fatty acid analysis of fish oil and biodiesel after transesterification was carried out using Trace 1300 gas chromatograph with flame ionization detector (FID) (Thermo Fisher, Canada). The column used for the analysis was TR FAME 30 m x 0.32 mm, ID 0.25 µm, P/N 260M143P, S/N 1088052B06. The inlet temperature was maintained at 255°C under spilt mode. The spilt flow of the carrier gas was maintained at 100 ml/min and purge flow was 2 ml/min. The inlet pressure was maintained at 81.7 kPa. The flame ionization detector was maintained at 270°C and the ignition threshold was 1 pA and the data collection rate was 10 Hz. The gas settings included an air flow at 350 ml/min, makeup gas flow at 35 ml/min and hydrogen flow at 35 ml/min. The GC oven temperature was initially maintained at 130°C for 3 min and it was ramped to 250°C at the rate of 10°C/min and kept under hold for 15 min. The final run time was 30 min.

4.4.8.5 *Analysis of oil and Ω-3 concentrates*

Fatty acid composition and determination of the conversion rates was done by quantitative Gas Chromatography against an internal standard of 23:0 fatty acids. Conversion rates, yields and fatty acid compositions were calculated as described in L.M. Valverde et al., (2013):

The masses of fatty acids were calculated as follows:

$$fatty\ acid\ [mg] = 1.0\ \frac{f_x\ \times\ area_x}{area_{IS}}$$

Where 1.0 is the concentration in *mg* of internal standard in the sample, f_x is the fatty acid response factor, $area_x$ the fatty acid chromatographic area and $area_{IS}$ the area of the internal standard peak. The response factors were close to 1 for all the fatty acids with a molecular weight close to that of the internal standard and therefore $f_x = 1$ was used for all fatty acids except for DHA for which factor 1.08 was calculated.

The conversion X was expressed as weight ratio:

$$Conversion\ X = \frac{fatty\ acid\ ethyl\ esters\ [mg]}{total\ fatty\ acids\ [mg]}$$

Where *fatty acid ethyl esters* are the sum of all fatty acid amounts obtained after lipase-catalyzed alcoholysis. Fatty acid ethyl esters were quantified by Gas Chromatography without derivatization of the sample. Hence only fatty acids were detected that were produced from acylglycerides during alcoholysis. *Total fatty acids* are the sum of all fatty acid amounts detected in the oil before alcoholysis. Quantification was performed by gas chromatography with derivatization. Hence all fatty acids, including acylglycerides were esterified and detected.

Calculation of remaining acylglycerides:

$$Acylglycerides\ [mg] = total\ fatty\ acids\ [mg] - fatty\ acid\ ethyl\ esters\ [mg]$$

During sample preparation for GC, no esterification was performed, just silylation. This means that acylglycerols were not detected as they are not volatile enough.

5 Results

5.1 Milestone 1 and 2: Characterization of the salmon processing waste and Extraction of oil

5.1.1 Sample processing and oil extraction

The composition of the salmon processing waste varies based on the source, location, farming practices, fish feed and processing steps as well. To maintain the consistency in the raw material available for the project, qualitative and quantitative information was documented including quality of the raw material focus on the proximate analysis of the source material to assess the fat content of the raw material. A sample of the received salmon frames was analyzed for proximate composition. The results are shown in Table 2. The results are all in the expected range for salmon mince.

Table 2: Summary of proximate analysis of waste streams

	Reference values Dec 2012	Reference values Feb 2013	Sample values Nov 2015
Moisture [%]	57.07	57.30	56.53
Lipids [%]	23.73	21.57	25.58
Protein [%]	14.52	18.20	14.21
Ash [%]	4.65	2.64	3.65

**All values are based on wet weight*

Sample preparation and enzymatic oil extraction was performed according to the procedure as described in chapter 4.4.6. Two enzymes were tested in parallel: Alcalase and Sea-B-Zyme. From 500 g salmon mince, 80 ml and 66.5 ml (16% v/w and 13.3% v/w) of oil were recovered using Alcalase and Sea-B-Zyme, respectively. Due to the significantly higher yield achieved with Alcalase, the oil obtained from that reaction was used for further experiments.

5.1.2 Fatty acid analysis

Fatty acid analysis was performed according to the method described in section 4.4.8. The results indicated low levels of EPA and DHA close to the detection limit. The results of the fatty acid analysis were compared to previous work carried out by our research team and other authors. Table 3 summarizes the typical lipid class profiles found in farmed and wild salmon and show the profile of the current salmon sample in comparison.

Fatty acid profiles of both oil extracts were created using derivatization of the oil into esters and subsequent gas chromatography. The chromatographic method was modified for optimal peak resolution. Peak identification was achieved with 27 FAME analytical standard and menhaden oil analytical standard. An internal standard (23:0) was run with every sample for quality control. The analytic results showed

very small levels of Ω-3 fatty acids and medium amounts of Ω-6. In fact, the levels were so low that concentration experiments were not possible.

Table 3: Fatty acid profiles from various oils found in literature

Analytes	Units	Kronch Salmon oil (wild)**	Wild Salmon°	Farmed Salmon°	Wild Salmon°°	Farmed Salmon°°	Farmed Salmon Reference value 2013 ***	Farmed Salmon current project
Fat(GC/FID)	g/100g oil	89						
Saturated		24.2	23.2	19.7			21.0	17.916
Cis-Monounsaturated		38.9	45.6	47.9			33.8	45.425
Cis-Polyunsaturated		36.3	26.2	30.4			36.4	27.487
Ω-3 Polyunsaturated		**29.1**	**24.4**	**21.2**	**12.22**	**33.73**	**25.0**	**4.025**
Ω-6 Polyunsaturated			1.7	9.1	1.28	8.8	11.3	22.311
EPA (20:5 n-3)		8.9	6.5	5.5	4.14	10.79	9.6	0.753
DPA (22:5 n-3)			2.5	2.8	1.2	5.19	4.39	
DHA (22:6 n-3)		11.4	12.5	8.4	6.29	15.69	8.47	2.024
EPA/DHA ratio		0.78	0.52	0.65	0.66	0.69	1.13	0.37
Ω-3 /Ω-6 ratio			**12.50**	2.27	**9.55**	3.83	2.21	0.18
Ω-6/Ω-3 ratio			**0.07**	0.43	**0.10**	0.26	0.45	5.54

Note: Values express the analyte content in the lipid fraction of the fish
**Terra Nova Ω-3 Seal oil capsules; **Kronch Pet Food, nutritional information; °I.J.Jensen et al., 2011; ***Our previous work °°M.C. Hamilton et al.*

A possible explanation of this result is oxidative degradation of Ω-3 fatty acids, which are prone to oxidation due to their reactive double bonds. Since the material was collected fresh and analyzed without delay, it was assumed that oil oxidation was unlikely.

Nevertheless, the oil extraction and fatty acid analysis was repeated with freshly sourced raw material. To minimize oxidation, the salmon waste was again transported to the bioprocessing unit on ice immediately after collection at the processing plant. Oil extraction was performed the next day and during all process steps, the oil was covered with a layer of Nitrogen. After extraction the oil was stored at -80°C until further use.

This time, enzymatic hydrolysis was performed with 1 kg of salmon mince and 0.5% Alcalase. 155 ml or 15.5% of oil were recovered. Fatty acid analysis also indicated low levels of Ω-3 fatty acids in the oil. See Figure 2: This pictures shows the fatty acid profile of the original oil (top chromatogram) compared to the oil obtained after hydrolysis using method 1 (middle chromatogram) and method 2 (bottom chromatogram). No noticeable concentration of EPA/DHA and DPA occurred, however, there was no significant amount of those FA in the original oil.

While feeding regimes as well as details of growing conditions are confidential, these results clearly indicated that the salmon were fed a diet low in marine animal fats and therefore have low levels of EPA and DHA in their oil.

5.2 Milestone 2: Handling and Pretreatment

5.2.1 Drying effect on quality

Fish biomaterial has high water content and lots of enzymes and therefore it is susceptible to rapid spoilage. The digestive enzymes present in the fish lead to autolysis causing solubilization of blood water

containing both protein and oil. Also other enzymes such as lipases can cause rapid deterioration leading to loss in quality and yield of fish oil (Ghaly et al (2010)). Oxygen and microbial spoilage also leads to rapid deterioration of fish tissues and therefore require special handling and pretreatment to minimize spoilage. Handling and pretreatment studies were performed with the RDC Ignite project. In this study, the salmon waste samples were partially dried and the lipid content in the partially dried samples was compared against the undried salmon waste samples. This will result in determining the efficient handling and pretreatment technique before nutraceutical grade salmon oil production. The main aim for this study was to prevent any mode of contamination and preservation of raw material for the production of high quality nutraceutical oil. In this study microbial quality, colour and water activity of the dried salmon processing byproducts were analyzed during the storage period of 35 days at 5 and -30 ºC. This was one of the first studies assessing the quality parameters of the dried salmon by-products. Colour of the salmon by-product was not significantly affected during the low temperature storage and water activity also remained very low; hence, polyethylene transparent bags can be potentially employed as a storage bag for the packing of dried salmon-by-products for a short period of 35 days at low temperature. Microbial count decreased with drying which further depleted with increased storage time. However, total plate count and Oxytetracycline Gentamicin Yeast extract count at -30 ºC were higher than observed at 5 ºC. Hence, further studies are necessary to identify these microorganisms which can survive such low temperatures in salmon by-products.

Table 4: Characteristics of freeze-dried salmon by-products

Sample	Ash content (% dry wt)	Protein content (% dry wt)	Fat content (% dry wt)	Moisture content (% wet basis)	Salinity (%)
Freeze-dried sample	6.481 ± 0.757	29.084 ± 3.979	56.884 ± 1.439	58.634 ± 0.231	0.303 ± 0.012

Table 5: Summarization of colour and water activity of different freeze-dried salmon by-product samples at different temperatures and time of storage

SAMPLE	Days	Colour	Water activity	Temperature
Freeze dried		yellowish	0.053	22.3
Freeze-dried stored at -30 ºC	*14*	yellowish	0.071	21.3
	28	yellowish	0.051	21.2
	35	yellowish	0.049	21.9
Freeze-dried stored at 5 ºC	*14*	yellowish	0.065	21.9
	28	yellowish	0.052	20.6
	35	yellowish	0.062	21.3

5.2.2 Effect of storage at freezing temperatures

In case of application of salmon by-products as a source of oil for omega-3/6 fatty acids and biodiesel production, storage of by-products under optimum conditions is very necessary. Storage at low temperatures and freezing are common methods of preservation of biological materials. However, lipid peroxidation during freezing has been observed by Apgar and Hultin (1982) in fish muscle microsomes.

There are various studies reporting effect of storage parameters on quality of fish products, however very few on salmon- by-products (Wu and Bechtel, 2008). According to our, literature study there are no studies reporting the effect of storage at freezing temperatures on the oil yield and composition of salmon oil extracted from frames. Hence, the objective of this study was to identify and quantify various fatty acids in salmon frame oil, harvested at various periods, employing enzymatic extraction. Among the samples harvested at different time periods, DHA/ EPA ratio (4gm/100gm of oil), total omega-3 content PUFA content were not suggestively different; however, mono-unsaturated fatty acids decreased and saturated fatty acids increased with increased storage period (Figure 1). Oil yield increased with storage period, however further studies are required to optimize storage period as a conditioning factor for obtaining higher oil yield. It can be concluded that, storage at very low temperatures can maintain the quality of salmon fish frames for oil extraction for applications in derivation of omega-3 fatty acids.

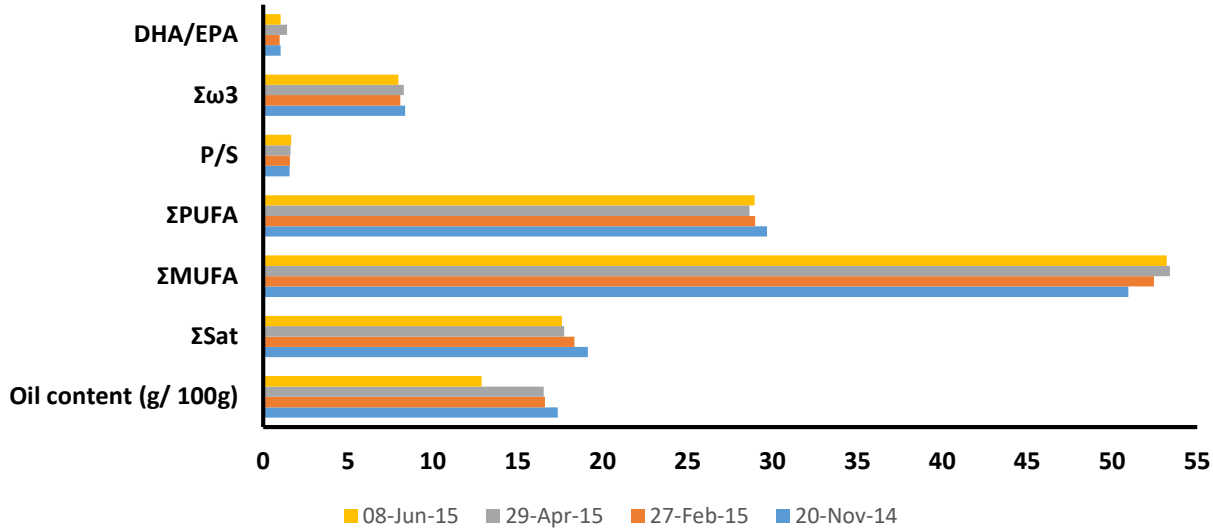

Figure 1: Figure presenting content of major components and ratio of major components obtained through enzymatic extraction from the fish frame samples harvested at different periods of year

5.2.3 Concentration of Ω-3 fatty acids using enzymatic hydrolysis

This research was mainly conducted to investigate the suggested experimental procedure for ease of sample handling, phase separation and oil recovery. However, since it was known that the omega-3 content was at undetectable levels in the extracted oil, a measurable concentration of EPA-DHA and DPA was not expected.

Hydrolysis was performed according to the procedures described in section 4.4.7.1 and 4.4.7.2.

Method 1: Calcium Chloride did not break up the emulsion sufficiently to achieve good phase separation and oil recovery. Further, the reaction volume was quite large considering the small amount of oil. Large processing volumes can be limiting factors during scale-up of a process.

Method 2: Using this method, phase separation was better and oil recovery slightly increased. Although the reaction volume was a bit smaller than using method 1, this method employs the solvent hexane to recover the oil. Scaling-up this process would use large amounts of hexane, which is flammable and explosive and may therefore present many practical problems.

The following Figure 2, 3 and 4 show the changes in fatty acid profile before and after hydrolysis. No significant changes in fatty acid profiles was observed.

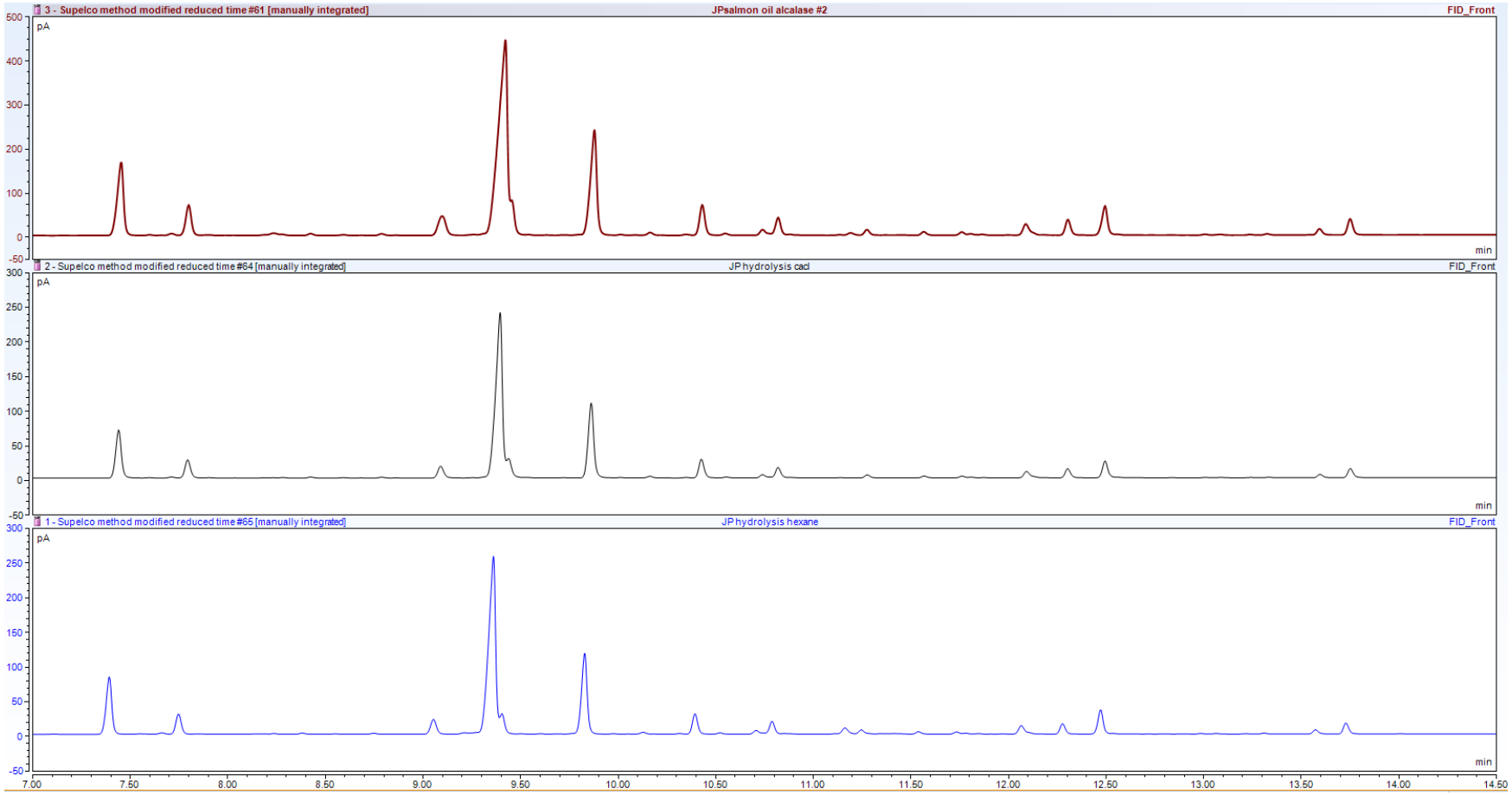

Figure 2: This pictures shows the fatty acid profile of the original oil (top chromatogram) compared to the oil obtained after hydrolysis using method 1 (middle chromatogram) and method 2 (bottom chromatogram). No noticeable concentration of EPA/DHA and DPA occurred, however, there was no significant amount of those FA in the original oil.

5.2.4 Concentration of Ω-3 fatty acids using enzymatic alcoholysis

Conversion of acylglycerides into Fatty acid ethyl esters was observed at a temperature if 20-50 C, an Ethanol to Oil molar ratio of 2-5 and enzyme concentrations ranging from 10-15 %. The incubation time was varied between 12 and 24 hours. Table 6 shows the factorial design and the results measured in total peak are detected with GC.

Figure 3 demonstrates the changes in oil composition achieved by enzymatic alcoholysis. While practically no volatile components are present in the original oil (see top chromatogram), alcoholysis produced considerable amounts of FAEE as recorded in peaks seen left of the internal standard, which eluted at ~12 min.

While the total amount of FAEE produced in the process varied from 9.14 to 53.81, the profile obtained was very similar as can be seen by the peak distribution and relative peak height in the two lower chromatograms.

Statistical analysis revealed that the conversion rates was mostly dependent on reaction temperature while the influence of incubation time, enzyme concentration and ethanol/oil ratio was not significant. Figure 4, Figure 5 and Figure 6 show the results of the statistical analysis. Since only one of the factors investigated showed significant effects on the reaction, no interaction plots could be created.

Table 6: Factorial design for alcoholysis experiments

StdOrder	RunOrder	CenterPt	Blocks	time	temperature	ethanol	enzyme conc	Response: Total Peak area
16	1	1	1	24	50	6	15	20.88
1	2	1	1	12	20	2	10	20.19
10	3	1	1	24	20	2	15	26.22
2	4	1	1	24	20	2	10	28.12
12	5	1	1	24	50	2	15	16.62
15	6	1	1	12	50	6	15	17.27
17	7	0	1	18	35	4	12.5	20.33
14	8	1	1	24	20	6	15	53.81
5	9	1	1	12	20	6	10	25.52
13	10	1	1	12	20	6	15	38.98
4	11	1	1	24	50	2	10	27.81
3	12	1	1	12	50	2	10	21.33
8	13	1	1	24	50	6	10	16.16
7	14	1	1	12	50	6	10	9.16
18	15	0	1	18	35	4	12.5	41.35
19	16	0	1	18	35	4	12.5	24.25
6	17	1	1	24	20	6	10	48.38
9	18	1	1	12	20	2	15	20.52
11	19	1	1	12	50	2	15	27.59

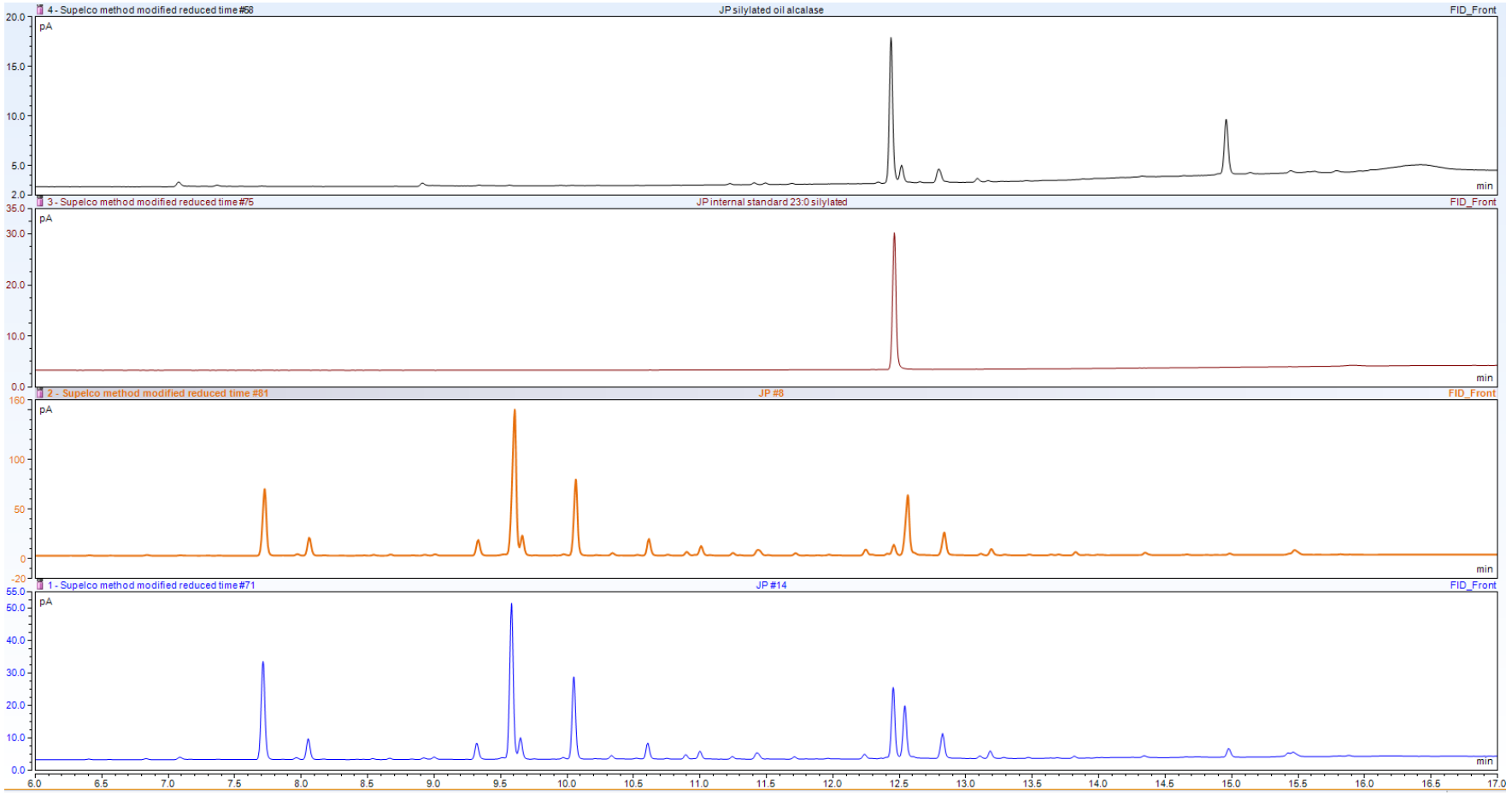

Figure 3: This pictures shows the resulting chromatograms of alcoholysis in direct comparison. The top picture shows the silylated oil extracted using Alcalase. No peaks are detected under 15 min apart from the internal standard peak. The peaks beyond 15 minutes are original components of the oil like Acylgrycerides. This indicated that the oil does not contain any significant amounts of FFA or esters before alcoholysis. The chromatogram second from the top is the internal standard alone. The bottom two chromatograms show examples of the silylated samples after alcoholysis. Clearly, AG were converted into FAEE enzymatically. Sample 8 resulted in successful conversion of oil into FAEE (total peak area: 58.33), while conversion rate in sample 14 (bottom) was much smaller (total peak area: 9.17). Nevertheless, the FA distribution is comparable in both samples.

Figure 4: Normal Plot of the Standardized effects, showing that only the temperature had a significant effect on FA conversion

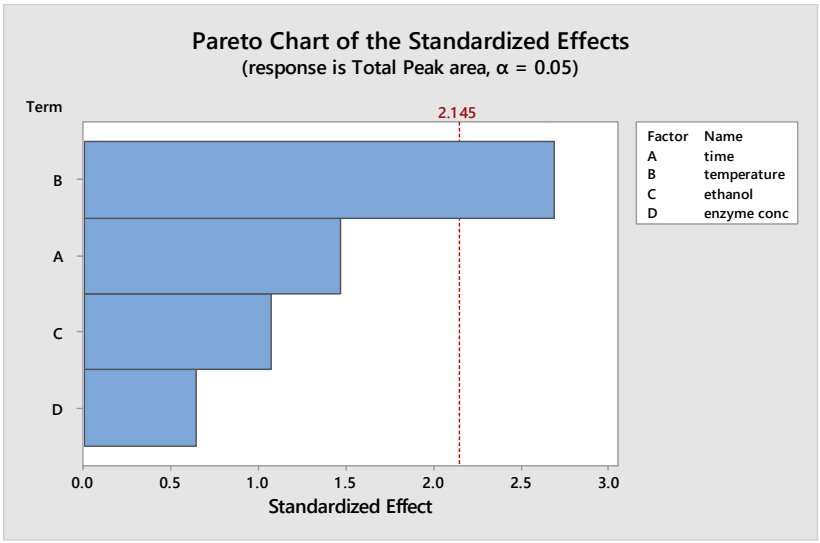

Figure 5: Pareto chart of standardized effects, showing that only the temperature had a significant effect on FA conversion

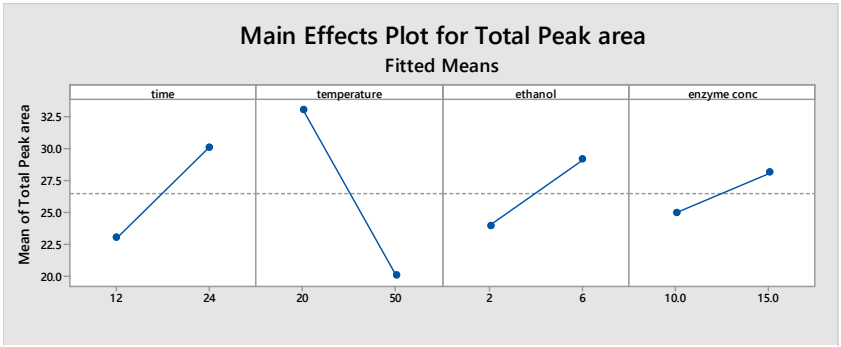

Figure 6: Main effects plot of fitted means

5.3 Other Results

5.3.1 Fatty acid composition of Salmon raised in aquaculture

Salmon feed consists of a mixture of food sources. In the early days of aquaculture, fish meal and marine oils were used, resulting in a feed close to the natural food sources of salmon. In the late 90's, the price of fish meal and marine oils soared as the aquaculture industry experienced fast growth. More and more farmers switched their feeding regime to include a mix of marine and plant-based oil and protein sources. Similar to humans, salmon are not able to synthesize Ω-3 fatty acids or the pigment Astaxanthin. Rather, it is taken up with the feed and stored in the fatty and muscle tissue. Hence, if the feed is low in Ω-3 fatty acids, the salmon fillet will also have very little Ω-3 fatty acids in their oil. If the feed does not contain pigment, the fillet will be white. While the color of the fillet is one of the most important criteria in quality control for salmon products, the Ω-3 content is not. Therefore, producers make sure that the feed contains appropriate amounts of pigment. The levels of Ω-3 have only secondary importance. An Aquaculture enterprise will only add Ω-3 to their feed if they intend to utilize all by-products for production of Ω-3 -rich oils and/or if they want to label their salmon product as "high in Ω-3 Fatty acids".

Salmon oil producers would naturally only use oil from salmon who have received highest quality feed resulting in high Ω-3 contents. Salmon farmers who primarily focus on fast growth would chose feed product that achieves that rather than accepting the higher cost of feed high in Ω-3 fatty acids. While this is no new development in the industry, it was not possible to identify a farmer in Newfoundland at this point who was willing to provide information on their feeding regime or was able to provide a sample of their waste product that was high in omega-3 FA.

In a previous project completed for a Newfoundland aquaculture company, EPA and DHA were present at levels 4-5 times higher than usually found in wild salmon. Again, their feeding regime was confidential. However, in order to perform EPA and DHA concentration experiment as outlined in the proposal for this study, a suitable oil is needed as a starting point.

Analysis of three different samples of salmon from two different sites and two different companies revealed low EPA/DHA/and DPA contents in all of them.

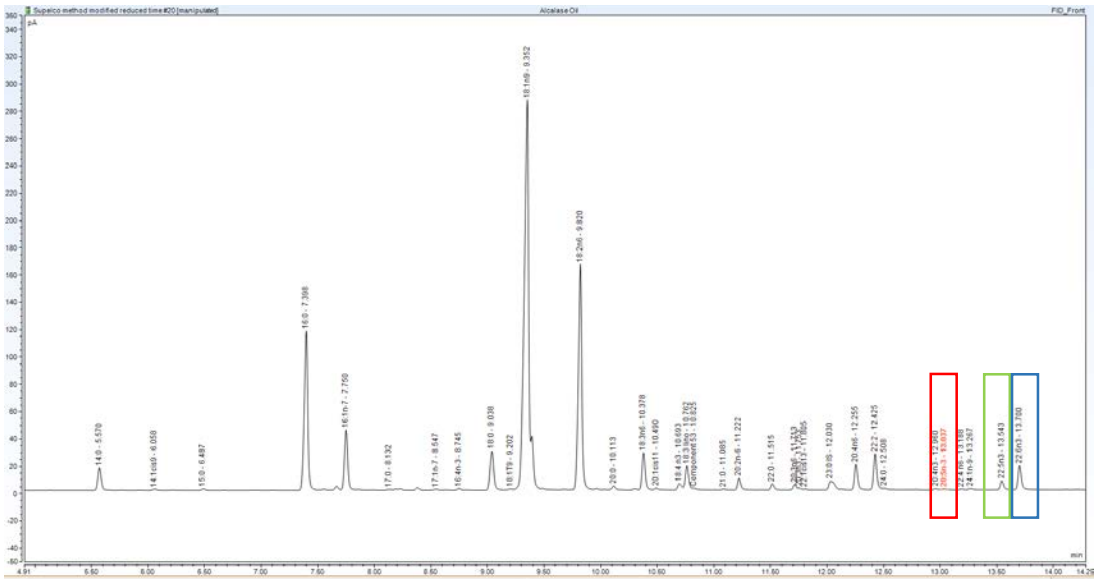

Figure 7: Fatty acid profile of salmon oil obtained from Newfoundland aquaculture site I November 2015. The retention times at which long-chained omega-3 fatty acids are expected are marked in red (EPA), green (DPA) and blue (DHA).

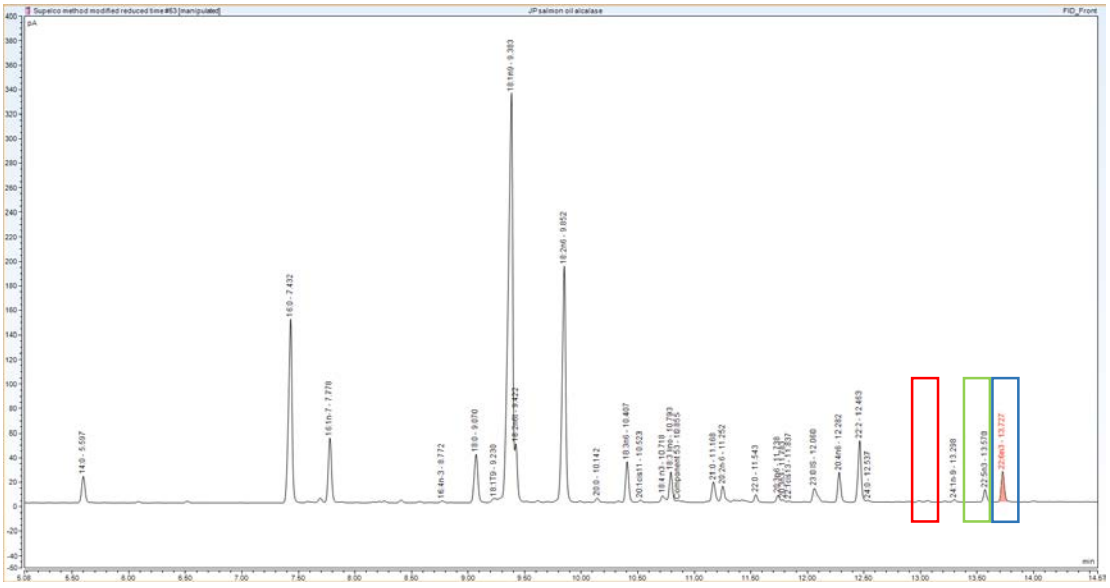

Figure 8: Fatty acid profile of salmon oil obtained from Newfoundland aquaculture site II March 2016. The retention times at which long-chained omega-3 fatty acids are expected are marked in red (EPA), green (DPA) and blue (DHA).

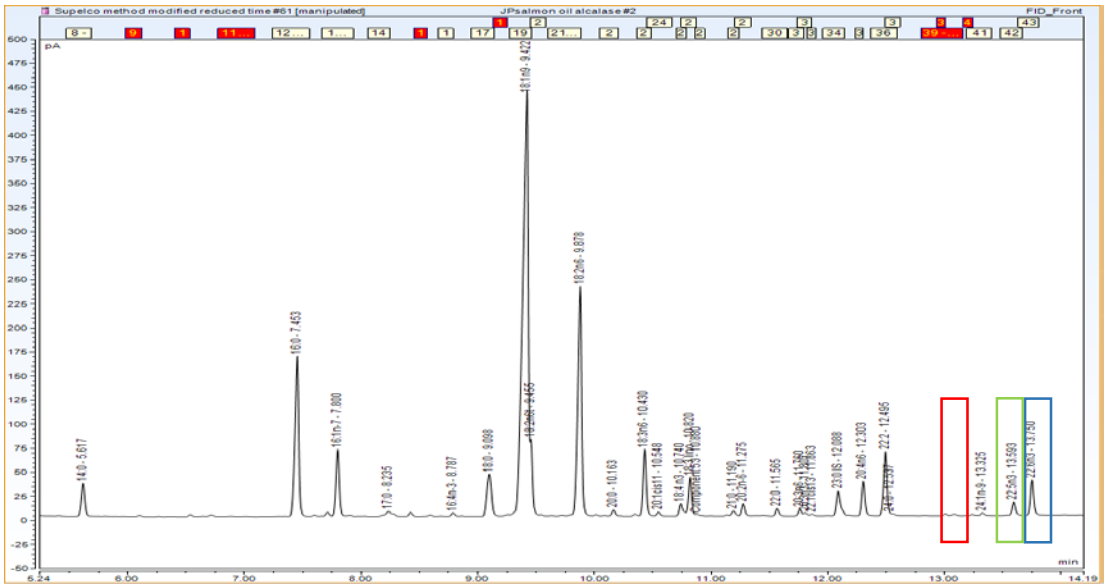

Figure 9: Fatty acid profile of salmon oil obtained from Newfoundland aquaculture site II June 2016. The retention times at which long-chained omega-3 fatty acids are expected are marked in red (EPA), green (DPA) and blue (DHA).

5.3.2 Gas Chromatography method validation

In 2015, the Bioprocessing unit of the Centre for Aquaculture and Seafood processing was able to purchase and install a Gas Chromatograph in their research laboratory. This analyzer allows for highly sensitive and accurate identification and quantification of a multitude of chemical components and is an essential piece of equipment for the analysis of fatty acids and proteins (amino acids). While samples used to be sent off for analysis to the Oceans Science Centre, the current project helped the Bioprocessing unit to establish the methods for fatty acid analysis on their own equipment while comparing their results to those from the OSC. Three CASD technicians were trained on the method, enabling CASD to offer their services for this kind of analysis to future clients.

6 Challenges and Recommendations

The process development for the enzymatic production of an omega-3 concentrate could not be completed. The main challenge was to source a suitable raw material from local aquaculture sites that had omega-3 levels comparable to wild salmon. Hence the experiments performed using several different omega-3 concentration protocols were mainly for the purpose of analyzing and evaluating the processes for potential practicability on larger scale in terms of:

- product handling
- batch size
- chemical cost
- safety concerns
- product recovery

Considering batch size, chemical cost as well as oil recovery, a combination of alcoholysis and molecular distillation seemed most promising. The only reagent needed other than the enzyme is ethanol, which is readily available and cheap. The overall reaction volume is much smaller than for hydrolysis. The enzyme

itself can be immobilized and reused so that the cost is minimized. Further, the oil recovery is easy as ethanol and FAEE can be evaporated during molecular distillation.

Hydrolysis, depending on the method used, requires large amounts of water or solvent, which is not practical to do on large scale. Not only does a large reaction volume reduce oil throughput but the use of large amounts of hexane requires an explosion proof facility and a sophisticated ventilation and solvent recovery system. Further, phase separation is difficult to achieve and formation of emulsions likely, especially when the conversion of TAG to FFA is very successful.

To obtain a better idea of the feasibility of enzymatic concentration of omega-3 oils from Newfoundland-grown Atlantic salmon, further studies are necessary.

7 Conclusion

Overall, based on our observations it can be concluded, that the oil extraction method developed maximizes recovery of oil using a gentle enzymatic method. The impacts of handling and pretreatment of salmon waste were investigated and will gives important direction when evaluating practical processes for waste utilization. Also, significant conversion of oil into omega-3 concentrates was achieved using an enzymatic process. Here, temperature was identified as the most influential parameter and it was concluded that, while promising, additional tests are necessary to optimize the method. This is a pioneer study in case of fatty acids extraction and concentration from fish by-products, which can be used as a reference for future studies and will also be useful for designing and augmentation of experiments in this area of study. Last but not least, this study facilitated the further development of the scientific staff at the Bioprocessing unit of the Centre of Aquaculture and Seafood Development allowing future support of the industry and academic groups in the areas of by-products utilization, edible oil analysis and edible oil refining and polishing.

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