FISH (*Pangasius pangasius*) OIL EXTRACTION BY DIFFERENT EXTRACTION METHODS: A COMPARATIVE STUDY OF PHYSICO-CHEMICAL PROPERTIES AND FATTY ACID PROFILE

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Roll No.: 0219/01

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Department of Food Processing and Engineering

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Chattogram-4225, Bangladesh

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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List of abbreviations

Words	Abbreviation
hr	Hour
kg	Kilogram
g	Gram
mg	Milligram
mL	Milliliter
MT	Metric ton
rpm	Rotation per minute
BBS	Bangladesh Bureau of Statistics
SE	Soxhlet extraction
WR	Wet rendering
AS	Acid silage
MAE	Microwave assisted extraction
GC-MS	Gas chromatography-mass spectrophotometry
AOAC	Association of official analytical chemists
NQI	Nutritional quality indices
SFAs	Saturated fatty acids
MUFAs	Monounsaturated fatty acids
PUFAs	Polyunsaturated fatty acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
n-3	Omega-3 fatty acids
n-6	Omega-6 fatty acids
DOF	Department of fisheries
FFA	Free fatty acid
FAO	Food and agriculture organization
FLQ	Fish lipid quality
AI	Index of atherogenicity
TI	Index of thrombogenicity

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Abstract

Fish oil from the pangus (*Pangasius pangasius*) is an important source of long-chain PUFAs, which have been found to help humans heart and vascular health, and it has the potential to be a dynamic sector for economic growth and nutritional supplements. However, selection of efficient oil extraction method is a mandate for obtaining superior quality oil. Therefore, four oil extraction methods including; Soxhlet extraction (SE), Wet rendering (WR), Acid silage (AS) and Microwave assisted extraction (MAE) were assessed to extract oils from Pangus fish. Extracted oils were further evaluated for yield percentage, physico-chemical properties, fatty acid profiling and nutritional quality indices (NQI). Based on analysis of variance; MAE method (21.80±0.23) recovered significantly higher amounts of crude oil ($p<0.05$), while containing lower free fatty acids (0.70±0.01%), peroxides (2.08±0.70Meq/kg), and saponification value (287.27±0.96mg/g KOH) than other methods. In addition, microwave assisted oils were less viscous (cP=43.00±0.50) and showed better refractive index (1.45±0.00), and melting point (33.50±0.50). A total of 25 fatty acids comprising SFAs, MUFAs, and PUFAs were identified based on GC-MS analysis. EPA and DHA contents in extracted fish oils varied from (0.036−0.229g/100g) and (0.064−0.421g/100g), regardless of different extraction methods. However, highest recovery of PUFAs, MUFAs and SFAs were observed in SE (19.158±1.710g/100g), MAE (7.997±0.193g/100g), and AS (17.330±1.508g/100g) methods, respectively. In terms of NQI, SE method showed better rations of PUFA/SFA, HH, and LA/ALA while AS method reported better EPA+DHA, n-3/n-6, AI, TI and FLQ indices. In addition, MAE method showed better rations of n-3/n-6 and HPI index while WR method reported better AI index of pangus fish oil. Therefore, in this study considering extractability and health issues MAE could be the effective procedure to obtain high quality fish oil compared to other methods.

Keywords: Pangus fish oil, Extraction methods, Oil quality, Fatty acids, Nutritional quality indices, GC-MS

CHAPTER-1: INTRODUCTION

1.1 Background

Bangladesh is blessed with potential water resources. It is one of the leading fish producing countries in the world with a total production of 43.84 lakh MT in FY 2018-19, of which aquaculture accounted for 56.76 % of total fish production (DOF, 2019). Over the last 12 years, the average growth performance of this sector is almost 8.59 %. Government is trying to sustain this growth performance, which eventually ensures to achieve the projected production target of 45.52 lakh MT of fish by 2020-21 (DOF, 2019). Moreover, exporting fish, shrimp, and other fishery products; Bangladesh earns a significant amount of foreign currencies. Bangladesh currently ranked 3rd in inland open water capture production and 5th in world aquaculture production (FAO, 2018). In 2018-19, this sector contributed significantly in food security via contributing 3.50 % to our national Gross Domestic Product (GDP), and more than one-fourth (25.72 %) to the agricultural GDP. Over 12 % of Bangladesh's 165 million people depend on fishing and aquaculture, either full-time or part-time for their employment and livelihoods (DOF, 2019).

The geological position and congenial environment of Bangladesh, is very favorable for expanding both marine and freshwater aquaculture (Gupta et al., 1999). Since it locates in the delta of three mighty rivers: the Ganges, the Brahmaputra and the Meghna; it provides vast inland water resources in the forms of ponds, beels, haours, baours, canals, rivers, flood plains, reservoirs and impounded brackish water. The country possesses diverse and abundant aquatic resources with a total of 265 freshwater species and 475 marine species and 24 exotic species (DOF, 2009). Notwithstanding, fish and fishery products are the most important sources of essential nutrients required for human consumption (Abdullahi et al., 2001). Besides foodstuff diversity, fisheries products play an important role in ensuring consumption of animal protein through exporting from developing countries (Lestari and Purnamayati, 2020); one of them is native catfish (*pangasius pangasius*) locally known as “Pangus”. In Bangladesh, Pangus fishes are usually found in fresh water, brackish water, big rivers, food plains, estuaries, canals etc. (Rahman, 2005). Bangladesh is the world's second largest producer of pangus fish and the industry has the potential to be a dynamic sector of generating economic earnings and alleviating poverty (Hoque et al., 2021).

People nowadays are becoming more health conscious and thus looking for a healthier diet. Over the past two decades, polyunsaturated fatty acids (PUFAs) have gained much attention among scientists for their therapeutic and nutritional properties. Fish oil, which was previously used for animal feed, is now recognized as the prime source of these PUFAs (Hegde et al., 2016). Today fish oil is highly valued for its positive role in human health and nutrition thanks to the presence of long-chain omega-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA; C22:6) and eicosapentaenoic acid (EPA; C20:5) (Anandganesh et al., 2016). Both of these fatty acids cannot be synthesized by human body and therefore must be obtained from the diet. Omega-3 and omega-6 fatty acids have specific tissue functions, including inhibition of aggregation, anti-inflammation and regulation of cholesterol intake in tissues (Kus-Yamashita et al., 2016). Besides, these fatty acids are also recommended to prevent and treat coronary heart disease, blood platelet aggregation, hypertension, abnormal cholesterol levels, diabetes, arthritis, mental illness, autoimmune disorders, and cancer (von Schacky, 2003; Kim and Mendis, 2006).

Historically, fish oil has been studied for its significant role in human health and hence there has been an increasing demand of fish oil in food and pharmaceutical industries (Rizliya and Mendis, 2014). Fish oil could be supplemented directly to food products to cover the fishy smell (Pike and Jackson, 2010). Some previous studies also reported that fish oil can be used as a food additive in dairy products i.e., yogurt (Zhong et al., 2018), butter (Subroto et al., 2018) and baked goods i.e., cakes (Santhanam et al., 2015). The huge industry of fish processing accommodates diverse extraction and production of health promoting fish oil that can benefit the small fish oil processors and entrepreneurs. However, Sathivel et al., (2003a) also reported a significant demand for high-quality fish oils. The crude oil contains various impurities that require further extraction and purification to achieve quality characteristics suitable for human consumption (Chakraborty and Joseph, 2015; Crexi et al., 2010). Thus, rapid and reliable methods for the quantitative extraction of lipids from aquatic products are very important to preserve their nutrition and quality.

A number of methods including wet rendering, acid hydrolysis, chemical extraction, mechanical pressing, and the use of centrifugal force can be used for the extraction of fish oil. Extraction methods that do not require chemicals during processing are wet rendering and microwave assisted extraction. Wet rendering (WR) involves steaming

of fish muscle, which damages its cellular structure and extract oil from the cooked fish (Nazir et al., 2017). As a result of this process, a large amount of crude fish oil can be obtained. However, for edible purposes, subsequent refining steps are required. However, this method induces oxidation and degradation of heat-labile substances. Other oil extraction methods such as acid silage (AS) and soxhlet extraction (SE) involve separating a substance from its mixture by dividing a solute between two immiscible solvents to extract a solute from one solvent to another. Addition of acids prior to lipid extraction during acid silage methods could be very aggressive and the extracts would be chemically degraded and unsuitable for fatty acid profiling (Johnson and Barnett, 2003; Ghaly et al., 2013). The soxhlet extraction method also involves the use of large amount of hazardous solvents and requires a lot of energy. Besides, fish oils could be oxidized with the relatively high temperature as it takes long time for complete extraction (Ozogul et al., 2018). However, as part of more sustainable production, safer ecological and energy-saving methods have been investigated for fish oil extraction (Marsol-Vall et al., 2020). Among the different emerging green extraction techniques microwave assisted (MAE) extraction is gaining interest for obtaining high quality fish oil. This method reduces energy consumption and also favors safe, robust and controlled processes (Patil et al., 2012).

Furthermore, oil extraction is affected by several factors including the extraction method, temperature, preliminary treatment, particle size and contact time of the material with the solvent (Ghazali and Yasin, 2016). Consequently, to increase the industrial application and utilization of these fats and oils from marine origin, extraction procedures that result in high yields without compromising the quality of the extracted oil are required. Although, several studies on nutritional content of Pangus (*Pangasius pangasius*) fish has been determined by several researchers, such as characterization of chemical and physical properties of fish muscle from head, body, and belly by Ridwan (2010), but research on the effects of different extraction methods on the quality of fish oil have not been done yet. Therefore, this research aimed to investigate the best extraction method by comparing physico-chemical properties, fatty acid profile and nutritional quality indices (NQI) of Pangus (*Pangasius pangasius*) fish oil obtained from different extraction methods including Soxhlet extraction (SE), Wet rendering (WR), Acid silage (AS) and Microwave assisted extraction (MAE).

1.2 Rationale and significance of the study

This study aimed to facilitate the value addition of fish and fishery products through oil exploitation, thus reducing fish wastes and increasing the contribution to the country's GDP. Additionally, the potential of this study is to contribute towards the commercial production of fish oil which will deliver multiple socioeconomic benefits including employment, higher income generation in the fishery sector and the overall livelihood improvement of the local fishermen. Furthermore, this study focused to address SDG's goal number 1 (Eradication of all forms of poverty) and 2 (End hunger, achieve food security, improve nutrition and promote sustainable agriculture) and one of Bangladesh's prominent agenda on manufacturing, low cost marine derived omega-3 and omega-6 enriched fish oil.

1.3 Objectives of the study

i. To study the effects of different extraction methods on the yield of fish oils
ii. To characterize the effects of extraction methods on the physico-chemical properties of fish oils
iii. To investigate and compare the fatty acid contents in extracted fish oils
iv. To determine the effects of extraction methods on nutritional quality indices (NQI) of fish oils
v. To generate knowledge and experiencing the best extraction method to extract fish oil

CHAPTER-2: REVIEW OF LITERATURE

2.1 Characteristics and distribution of *pangasius pangasius*

Pangus (*Pangasius pangasius*) is a catfish species of the family Pangasiidae under the order Siluriformes. This fish is valued for its deliciousness and high protein, mineral and fat content in the meat (Islam et al., 2012). It is widespread in Asian countries such as- Bangladesh, India, Pakistan, Myanmar, Indonesia, Vietnam and Thailand (Gupta, 2016). The main habitats of *Pangasius pangasius* are large rivers and estuaries. However, it is also found in irrigation canals, haors, baors, beels, natural lowlands and even ponds, especially during the rainy season (Talwar and Jhingran, 1991; Rahman, 2005).

Pangus fish have an elongated and laterally compressed body with no scales. The tail is constricted behind the adipose fin but somewhat prolonged before the caudal peduncle; the head and abdomen are flat. The top of the head is slightly granular; the occipital process is employed to reach the dorsal fin's basal bone; and the snout is rather pronounced. The eyes are located in the front half of the head, partially on the lower surface. The mouth is sub-terminal; the top jaw is longer than the lower jaw; and the gap between the two jaws is considerable. To reach the opposite side of the front edge of the eye, the cleft of the mouth is used (Talwar and Jhingran, 1991; Day, 1888).

There are four groups of teeth on the palate; the palatal teeth are located in the crescent row, the plates on the palate are separate or almost merge with the teeth on the palate. barbel - two pairs; the maxillary pair reaches the base of the pectoral fin and the mandibular pair is half the length of the head. First dorsal fin with a moderately strong back heavily serrated along the inner edge and finely serrated along the outer edge. The dorsal fat fin is short, free at the back, starting almost opposite the center of the anal fin. The back of the pectoral fin is serrated, strong, of the same length as the dorsal. The anal fin is large and well developed. The caudal fin is deeply bifurcated; the upper lobe is slightly longer. The color of the body is silvery, darkest on the back and with a purple sheen on the sides; the cheeks and the lower surface of the head are golden; caudal fins are light yellow (Talwar and Jhingran, 1991; Day, 1888).

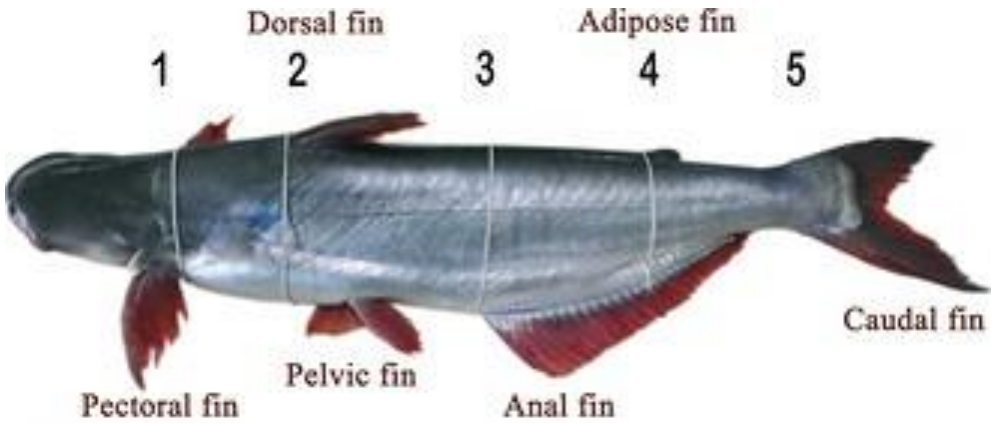

Figure 1: Morphometric traits of *Pangasius pangasius* adopted from Sahu et al., (2013)

2.2 Fish oil

The oil extracted from the tissues of oily fish is known as fish oil. Usually marine lean fish contains 0.1-1% lipid content whereas fatty fish varies from 2-30% depending on the type of species, diet, geographic, environmental, reproductive and seasonal variations (Kim and Mendis, 2006; Macrae et al., 1993). Fish oils are liquid at room temperature but generally solidify below 15-10°C (Pike and Jackson, 2010). The fatty acid composition of the oils comprises saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Certain PUFAs such as omega-3 and omega-6 fatty acids which are known as essential fatty acids (EFA) are the obligatory nutrients for mammals as human body cannot synthesize them (Macrae et al., 1993).

The composition of fish oil differs from other oils because it contains high proportions (5-30% of fatty acids) of the two omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are predominantly found in many marine species including cold water fishes high in unsaturated fatty acids (Kim and Mendis, 2006). Fish lipids are mainly composed of triglycerides and phospholipids (PL). Unsaponifiable components such as hydrocarbons, fatty alcohols, waxes and ethers found in oils affect the properties of these oils (FAO, 1986).

2.3 Pangus fish oil

Pangus fish oil contains considerable amount of EPA and DHA which could be used as an alternative source of the beneficial fatty acids. Usually the head, meat, and bones of *Pangasius pangasius* are used in oil production. Crude oil undergoes several refining processes including neutralization, bleaching and winterization to remove crystallized fats, followed by deodorization to remove odor-causing contaminants (Kasmiran, 2018). The fatty acid profile of *Pangasius pangasius* includes a wide range of SFAs, MUFAs and PUFAs.

Based on identified fatty acids *Pangasius* species represent the dominant proportion of PUFAs (38.02%), followed by SFAs (31.14%) and MUFAs (23.89%). This indicates that fillets of *Pangasius* sp. are high in unsaturated fatty acids (USFAs), which constitute approximately 66.53% of all identified fatty acids (Sokamte et al., 2020).

2.4 Historical background of fish oil

Cod liver oil was used for medicinal purposes dates back to early 1840 (Bockisch, 1998). The source of this oil was mainly attributed to the English, Norwegian and Newfoundland fisheries. Heavy demands for oils by the pharmaceutical industries predisposed the gradual development of techniques related to raw material selection, processing, and refining of fish oils.

At the beginning of the 20^{th} century, chemists discovered that the most important health-promoting properties of fish oil were the presence of vitamins A and D (Bockisch. 1998). The creation of this concept has led to pioneering research into fish that are more commercially viable, especially in sea fishing. After realizing that Atlantic halibut has a higher content of vitamins A and D than fish oil, the cod liver oil industry has been flooded with other species of fish such as tuna, sable, lingcod, sea bass and Pacific coast sea shark (Rice and Ismail, 2016).

With the increasing demands and public awareness of the value of vitamins A and D, fishermen and traders have developed more sophisticated fishing gears and liver processing techniques to ensure a longer shelf life and mass production of fish oil. With the realization of the health benefits associated with omega-3 fatty acids, Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) has opened up new

dimensions for fish oil. Now-a-days, focus has been shifted towards more on fish oils extracted from fatty fishes such as mackerel and herring rather than fish liver oils as oils are tend to accumulate within the fatty layers of flesh (Pike, 2015).

2.5 Basic chemistry and classification of fish oil

Lipids can be divided into two main groups which include neutral (non-polar) and polar lipids. Neutral lipids include monoacylglycerols (MAGs), diacylglycerols (DAGs), triacylglycerols (TAGs), and sterols (Deepika et al., 2014). Polar lipids are mainly composed of free fatty acids (FFA), phospholipids (PL) and sphingolipids as shown in Figure 2. Fish tissues are primarily consists of triacylglycerols, which exist in hydrophobic aggregates and contain fatty acids with varying chain lengths and degrees of unsaturation (Fahy et al., 2011).

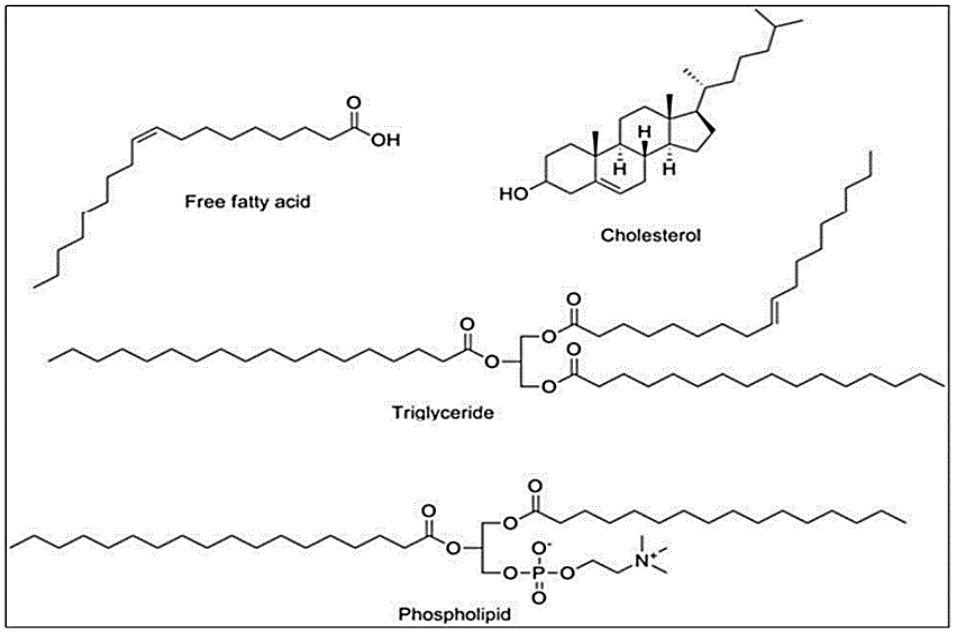

Figure 2: Examples of polar and non- polar lipids adopted from Bettelheim et al., (2009)

The classification of polyunsaturated fatty acids (PUFAs) is based on the position of the double bond, from the carboxyl or the methyl end (Figure 3). Typically, counting in the notation "Omega" or "n" is done by determining the position of the double bond from the methyl group.

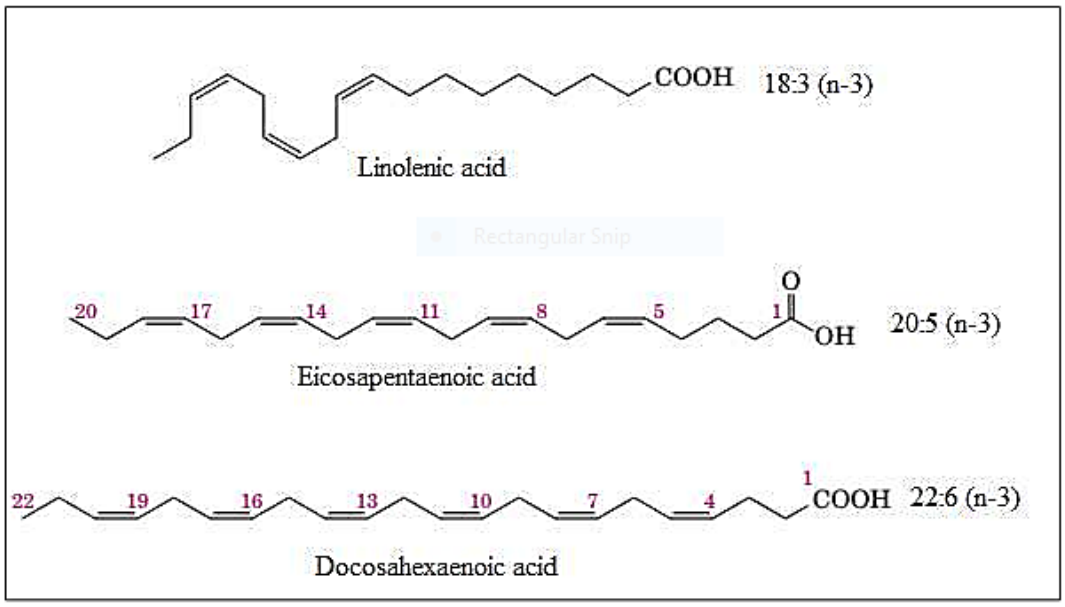

Figure 3: Some members of n-3 fatty acids family adopted from Bettelheim et al., (2009)

Most PUFAs with life expectancy are sub-grouped as n-6 (arachidonic acid) and n-3 (eicosapentaenoic acid). The first double bond of n-3 fatty acids starts at the third carbon atom from the methyl end. Examples of fatty acids of this family include eicosapentaenoic acid (EPA, 20: 5), docosapentaenoic acid (DPA, 22: 5), docosahexaenoic acid (DHA, 22: 6 n-3), and α -linolenic acid (ALA, 18: 3). These molecules are fragile and tend to bend in the area of double bonding due to the presence of non-conjugate (disrupted methylene) suspension of cis-type double-bond (Sahena et al., 2009). Ideally, pre-n-3 and n-6 fatty acids are ALA (alpha-linolenic acid) and LA (linoleic acid), respectively (shown in figure 4) and both EPA and DHA are represented as triglycerides and phospholipids.

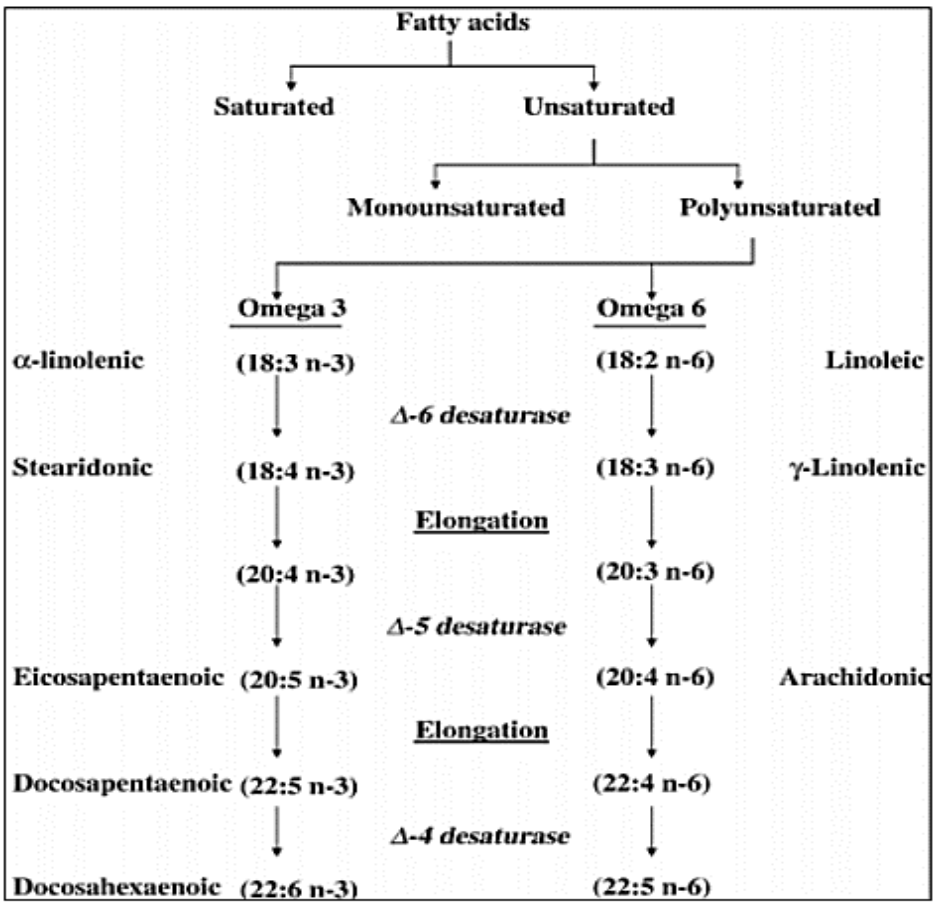

Figure 4: Metabolic pathway of omega-6 and omega-3 fatty acid synthesis adopted from Siddiqui et al., (2007)

According to Sahena et al., (2009), single-celled algae and phytoplankton that accumulate in fish are the source of these fatty acids. The composition and concentration of fatty acids in the most studied marine species varies greatly according to the species, reproductive status, age, size, sex, season, and geographic location (Pigott and Tucker, 1990). In fish, PUFAs are primarily responsible for cell structure formation, function, growth, and development (Cejas et al., 2004).

2.6 Health benefits of fish oil

As a result of the epidemiological shift from infectious to non-communicable diseases over the past few decades, cardiovascular disease (CVD) is considered as an important cause of death and morbidity in many developing countries, including Bangladesh (Al Mamun et al., 2016). Thus, rapid promotion of the health benefits of fish oil to the population of developing countries could be of great importance.

The consumption of fish oil has been linked to improve human health to fight against many diseases. Lack of essential fatty acids (EFAs) in human diets, leads to improper growth and dysfunction, as well as systematic abnormalities and muscle function. These include- impaired trans-epidermal water barrier function, squamous dermatitis, electrophysiological abnormalities of the heart and retina, cell-mediated immunity, platelet aggregation, and reproduction (Macrae et al., 1993).

EPA is believed to be of particular benefit to cardiovascular health; support heart health by improving blood circulation, lowering homocysteine levels and improving the immune function. DHA is being extensively studied for its effect on improving memory and cognition, as well as its role in infant brain development.

Most of the recommendations for omega-3 fatty acids relate to a daily intake of 0.25g to 0.5g (Pike and Jackson, 2010). Of all the known nutritional factors, long-chain omega-3 fatty acids may provide the greatest protection against coronary artery disease. Increasing the consumption of omega-3 fatty acids by a person with coronary heart disease by about 1g per day can prevent the occurrence of these diseases (GOED, 2014). It has also been suggested that consuming 0.25 to 1.8g of omega-3 fatty acids such as EPA and DHA per day in the form of oily fish or supplements would be enough to get the desired benefits, such as reduced platelet aggregation, reduced blood plasma triglycerides levels (Gogus and Smith, 2010).

The physiological effects attributed to omega-3 fatty acids include lowering blood pressure; reduction of arthritis, psoriasis, asthma; decrease in blood viscosity; reduction of plasma triglycerides and reduction of tumors. Sahena et al., (2009) described the potential of PUFAs in therapy, food and nutrition. Some of the structural and functional benefits to human health associated with both omega-3 and omega-6 PUFAs include the regulation of architecture, dynamics, phase transitions and membrane permeability. Some of the membrane-associated proteins, such as ATPase, histocompatibility complexes, and transport membranes, are also regulated by PUFA.

In addition, the activity of certain genes attributed to the coding of sodium channel proteins, fatty acid synthase and nitric oxide synthase are also regulated by PUFAs. This in turn has overall effects on cellular biochemistry, cellular response to stimuli, and transport processes that ultimately contribute to adapting to common cold,

improve immune responses, and prevent cardiovascular disease. Lagarde (2008) also reported that DHA is an abundant component of brain phospholipids and therefore essential for brain development and function.

2.7 Factors affecting oil production from fish

Several factors affect the amount of fats or oils to be extracted from fish. Some of these factors include fish species, gender, environmental conditions, and season. Due to morphology and physiology, it is clear that different species have different amounts of oil stored in different parts of the body. Typically, species of fish that store their lipids within the liver are said to be lean, while those that store their lipids in fat cells that are distributed in other parts of the body tissues are known as fatty fish. Examples of lean fish include bottom-dwelling fish such as cod, pollock and hake, while fatty fish include pelagic fish such as herring, mackerel and sprat. Some species, such as barracuda, mullet, and shark are referred to as semi-fatty species because they store their lipids within limited parts of the body or in smaller amounts than typical oily fish (Turchini et al., 2022).

Feeding is another important factor that plays a key role in determining the amount of oil within the same species. Some of the physiological and behavioral changes in fish that significantly affect fish food consumption include migratory swimming and sexual changes during spawning (Love, 1970). During these periods of migration and spawning, the fish often starve, focusing on migration and spawning. At this point, these fish species are heavily dependent on stored energy such as lipids. Migration coupled with spawning often uses both stored lipids and proteins, which affects the overall biological condition of the fish (Stansby and Hall, 1967). Fish species often have the least amount of fat during these physiological (spawning) and behavioral (migration) changes.

In addition, plankton feeders such as herring often experience fluctuations in oil content due to other environmental factors that affect plankton productivity. Size is another important factor influencing the fat content in fish. Watanabe (1971) noted that the fat content of fish species varies according to their size, while the larger one contains 1% fatter than the smaller ones.

2.8 Fish oil extraction methods

The conventional method of fish oil extraction involves cooking, pressing and centrifugation although it is associated with several challenges such as relatively low oil yield and thermal degradation (Pigott, 1967). Despite the above challenges this is the most preferred method of oil extraction since it is cheaper, easier, and quicker and does not require highly qualified personnel to perform. In addition, this method is also recommended for the type of oil used for both human consumption and animal feed because it does not use organic chemicals (Liyanage, 1999).

The second method is solvent extraction which is commonly used in the isolation of lipids from food samples including fish (Sahena et al., 2009). This method is based on the principle that lipids are soluble in organic solvents (di-ethyl ether and n-hexane) and insoluble in water hence can easily be isolated from water-soluble compounds such as carbohydrates, minerals, and proteins. The efficiency of this method of extraction depends on the polarity of both sample and the solvent whereby polar lipids such as phospholipids and glycolipids are more soluble in polar solvents such as alcohol compared to non-polar solvents such as hexane. A good example of a technique that uses the principle behind solvent extraction is soxhlet extraction (McClements, 2004).

Enzymatic hydrolysis is the third potential method of fish oil extraction which has received attention in the recent work of Deepika et al., (2014). Many enzymes have been clinically tried but the most promising one is the enzyme alcalase. Usually, this process of oil extraction is carried out under mild condition and yields relatively high oil content than other methods (Deepika et al., 2014).

In recent years, the microwave assisted extraction method has attracted the interest of researchers, as it allows rapid extraction of solutes from solid matrices using microwave energy as the heat source, with extraction efficiency comparable to classical techniques. The breakdown of analyst from the sample matrix to the extractor depends on the temperature and the nature of the extractor. Unlike traditional heating, microwaves heat the entire sample simultaneously without heating the crucible; thus, the solution reaches the boiling point very quickly, leading to a very short extraction time. MAE appears to be a viable alternative to conventional extraction techniques for various solid matrices, both spiked and containing native

compounds. In addition, MAE offers substantial reductions in solvent consumption and increased sample throughput. Optimization of MAE conditions are rather easy, because there are few parameters (matrix moisture, nature of solvent, time, power and temperatures in closed vessels), and it is cheaper than other modern techniques, such as SEF and PLE (Ahmed, 2003).

2.9 Effects of extraction methods on the quality of oils

The most important processing technologies used for fish oil extraction and their effect on the quality were reviewed in this section. It is estimated that the quality of fats or oils are influenced by each action during processing, extraction and purification. However, extraction methods applied in an innovative way will yield superior quality products with higher nutritional content.

Taati et al., (2018) investigated the effect of wet pressing (WP) and enzymatic extraction (EE) on the yield of extracted oil from tuna by-products, as well as the chemical parameters like neutral lipids, fatty acid profiles and acid value**.** The highest yield of oil was reported by the EE method than that of the WP method ($p<0.05$). Besides, significant difference between the levels of acidity, WE, TAG and FFA in the EE and WP treatments were also observed in this study ($p<0.05$). Cholesterol levels in obtained oils did not express significant differences ($p>0.05$) among the extraction techniques. Fatty acids analyzed in both experimental groups, including levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), did not show significant differences between the studied treatments ($p>0.05$).

The impact of various oil extraction procedures on yield, color properties, fatty acid profile and oxidative stability of common Kilka (*Clupeonella cultriventris caspia*) oil were evaluated by Sayyad and Ghomi (2017). Extraction with supercritical fluid (SFE-CO_2) with carbon dioxide showed the highest oil yield (96.94%) followed by wet reduction, ammonia and enzymatic extraction, respectively. Best oxidative stability and color characteristics in terms of acid value (AV), peroxide value (PV), yellowness and redness were also observed in supercritical fluid (SFE) extraction. SFE techniques also have the highest mean total of unsaturated fatty acids (10.33), specifically the omega-3 fatty acids.

Domiszewski et al., (2011) also investigated the effect of conventional cooking and microwave treatment on the quality of striped catfish lipids which results in an approximately 10% variation in the amount of PUFA, including EPA and DHA, whereas significant differences were not observed on the percentages of SFA and MUFA. In spite of substantial influences on the amount of both primary and secondary oxidation products, heat treatment maintained good quality of striped catfish lipids as peroxide values (PV) of all samples were below 3 meq O_2/kg lipids.

Albarin et al., (2018) characterized the lipid fractions obtained from fresh mackerel heads using enzymatic hydrolysis methods. The chemical properties of these fractions differed significantly in terms of protein, lipid, and ash content. Enzymatic extraction accelerates the rate of oil released from mackerel head, with no significant difference ($p<0.05$). When compared to lipids extracted by solvent both enzymatic hydrolysis and lipids of emulsified fraction had the similar PUFA content. The main PUFAs were reported as EPA (6.99 - 7.56%) and DHA (11.26 - 15.86%).

Aryee and Simpson (2009) had studied and analyzed the oils obtained from the skin of Atlantic salmon via solvent extraction using different solvent systems. Both hexane and petroleum ether were suitable solvents for the extraction of oils, though the yield obtained with hexane was significantly higher ($p<0.05$) than the later one. The study further indicated that salmon skin is one of the richest sources of fish oil accounted for 23.32–61.53 % (oil in dwb).

Haq et al., (2017) also evaluated the procedures for the production of edible oils from Atlantic salmon byproducts. Oil extracted by n-hexane, was considered as the best method since the yield was higher amongst SC-CO_2 and pressed oil, respectively. However, SC-CO_2 extracted oils had a more appealing color and viscosity than n-hexane extracted oils. The acid value, peroxide value and free fatty acid value were reported as the lowest in pressed oil followed by SC-CO_2 and n-hexane, respectively.

The efficiency of green extraction methods i.e. ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) over conventional methods (Soxhlet and Bligh and Dyer) on lipid content and fatty acid profiles of six different fish species; red mullet (*Mullus barbatus*), goldband goatfish (*Upeneus moluccensis*), surmullet (*Mullus surmuletus*), European eel (*Anguilla Anguilla*), common pandora (*Pagellus erythrinus*), and brushtooth liardfish (*Saurida undosquamis*) were investigated by

Ozogul et al., (2018). The lipid content of fish species revealed that the Bligh and Dyer method, as well as UAE in general, were more efficient than other methods. The contents of SFA, MUFA and PUFA of fish species ranged from 29.51mg/100 g of fish (Soxhlet); 1400mg/100 g (UAE), 15.52mg/100 g (EAU); 2237.18mg/100 g (Bligh and Dyer) and 14.36% (Soxhlet); 646mg/100 g (Bligh and Dyer) respectively. They came to the conclusion that extraction methods influenced the lipid yield and fatty acid profiles of extracted oil from various fish species.

Nazir et al., (2017) carried out a study to evaluate the effects of different fish oil extracting methods (pre-cooked wet rendering, acid silage and solvent extraction) while extracting oils from tuna's head. They have also assessed the physico-chemical properties and fatty acid profile of extracted fish oil. Obtained result indicated that wet rendering is the most efficient and promising extraction method to use because it yields the highest (12.80%) followed by silage process (6.16%) and solvent extraction method (8.49%). The PUFA content during the wet rendering method was 44.34%, which was statistically similar to the solvent extraction method (44.49%), but was higher than the silage method (32.77%), respectively.

CHAPTER-3: MATERIALS AND METHODS

3.1 Site and period of Study

The study was conducted in Department of Food Processing and Engineering, Faculty of Food Science and Technology and Nutrition and Processing Lab, Faculty of Fisheries of Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh. The study was carried out for a period from January, 2020 to December, 2020.

3.2 Collection of fish sample

The freshly captured experimental native pangus (*Pangasius pangasius*) fishes were sorted and identified. Total 30 fishes (Average weight 0.6 ± 0.15 kg, length 38 ± 2 cm), were obtained from the Fishermen of Sandwip Channel at the estuary of the Meghna River of Halishahor point near the main port city of Chattogram, Bangladesh.

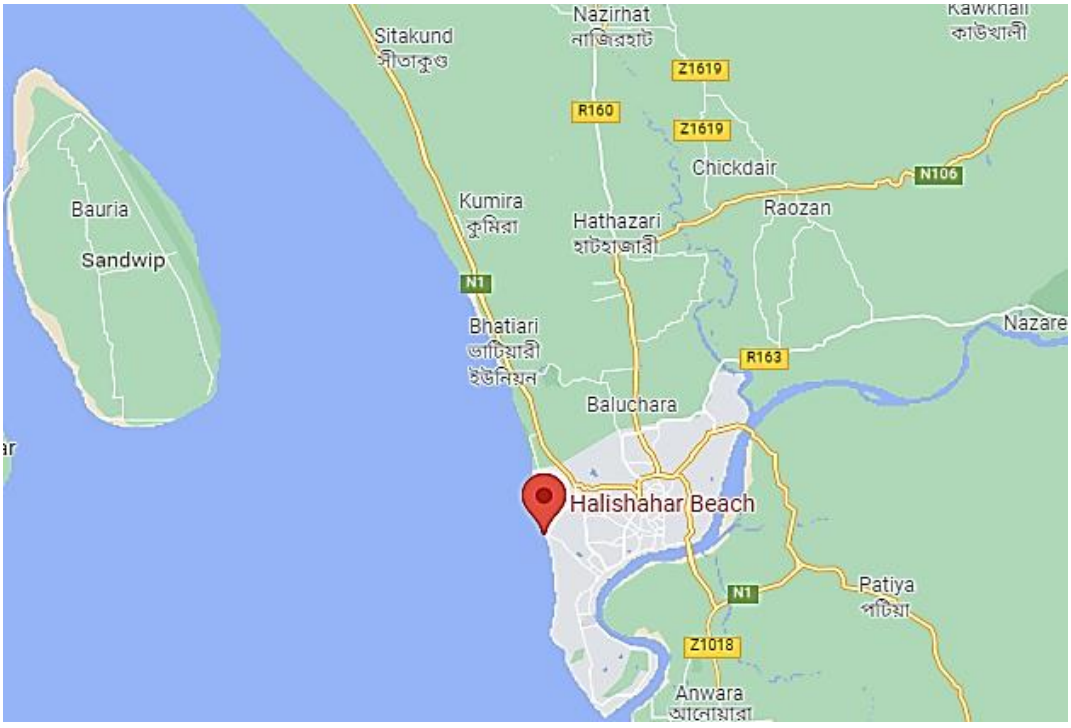

Figure 5: Sampling location in Chattogram, Bangladesh (Retrieved from Google Map)

3.3 Sample preparation

Samples of pangus fishes were beheaded, eviscerated, washed and then transported to the laboratory in ice boxes [2:1 (w/ w), ice to fish]. In the laboratory, fish muscles from the belly flap were taken and cut into very small pieces (1–8 mm in diameter) which were further preserved in airtight polythene bags and stored at refrigerated temperature (-20 °C) for further analysis.

3.4 Reagents and instruments used

Ethanol, Potassium iodide, Sodium thiosulfate, Acetic acid, n-hexane, di-ethyl ether, potassium hydroxide, chloroform were purchased from Sigma-Aldrich Co., St. Louis, Missouri, USA. Soxhlet apparatus (Model: SER 148/3, Velp, Italy), Rotary evaporator (Model: Hei-VAP series, Heidolph, Germany), Electric balance (Model: EK600i, Korea), Microwave oven (Model: ME21K7010DS/AA, Samsung, South Korea). Refractometer (Model: R9500, Reed Instruments, China), Viscometer (Model: DVII-Brookfield, Middleboro, USA) and Gas chromatography-mass spectrometry (GC-MS) (Model: GC-2010 Plus, Shimadzu, Japan) were used in this study. All the chemicals and reagents used for the analysis were of analytical grade.

3.5. Fish oil extraction methods

3.5.1 Soxhlet extraction (SE)

Soxhlet extraction process was done according to AOAC method (AOAC, 2005). 10g of solid samples were weighed into cotton-coated porous thimble, which were then placed into the central chamber of the Soxhlet apparatus. A 250 mL clean, oven-dried, round-bottomed flask was weighed and then connected to the Soxhlet siphon and condenser. 80 mL of diethyl ether (40–60°C) were added to the flask and refluxed for 3 h. The heating flow rate was maintained low enough to prevent the solvent escaping from top of the condenser during refluxing. The solvent was then distilled off and the extracted oil was collected and measured (Nazir et al., 2017).

3.5.2 Wet rendering (WR)

The wet rendering extraction technique was carried out according to the method described by Rubio-Rodrı´guez et al., (2012) with slight modifications. Fish sample (100g) was mixed with 150 mL of water in a 1000 mL glass bottle and steamed at 105

°C for 30 min. During the cooking process, the sample was stirred in every 15 min. Then the cooked samples were transferred to a cloth bag and pressed manually. Obtained liquid was filtrated off by using separatory funnel. Ultimately, the oil phase was centrifuged at 10,000 rpm for 10 min and the crude fish oil was skimmed off.

3.5.3 Acid silage (AS)

In acid silage extraction technique fish samples were soaked with 3% formic acid and kept at room temperature for 4−7 days. Produced liquid and cake was separated by filtration followed by centrifugation at 10,000 rpm for 10 min. Residual cake was pressed again to produce oil-water mixture and again centrifuged at 10,000 rpm for 10 min and thus the crude oil was collected and stored for further analysis (Nazir et al., 2017).

3.5.4 Microwave assisted extraction (MAE)

Fish muscles were spread on the rotary plate of a domestic microwave oven. The sample was heated at high power level (600 W, 2450 MHz for 3-4 min) according to Moreno et al., (2003). Then the sample was removed from the plate and the oil was extracted by squeezing, pressing manually through a cloth mesh and filtrating. Extracted oil was stored at -20 °C until further use.

3.6 Yield determination

Crude oil fractions from three consecutive replicates were pooled together, and the yield was calculated as the percentage of oil extracted from fish muscle. Yield was calculated as follows:

$$\text{Yield (\%)} = \frac{Extracted\ fish\ oil\ (g)}{Weight\ of\ sample\ taken\ (g)} \times 100$$

3.7 Determination of physical properties of the extracted fish oil

3.7.1 Refractive index

A few drops of sample were transferred to a refractometer's glass slide. Water at 30°C was circulated around the slide to keep the temperature uniform. The dark areas observable through the eyepiece of the refractometer were adjusted to coincide with

the cruciform intersection. At no parallax error, the pointer on the scale pointed to the refractive index (AOCS, 1997).

3.7.2 Density

The bottle type method was used. A density bottle was weighed and 10 mL of oil was transferred into the bottle. The density bottle was re-weighed again with its present content. The density was then calculated using:

$$\text{Density (g/mL)} = \frac{mass}{volume}$$

3.7.3 Melting point

Melting point measures the temperature at which the oil starts to melt. This value serves as an indicator of the types of fatty acids present in triglyceride. 10 mL of oil was poured into a beaker and was placed in a freezer to freeze before inserting thermometer into the beaker. The oil was heated and the temperature at which the oil started to melt was recorded (Ndidiamaka and Ifeanyi, 2018).

3.7.4 Viscosity

Viscosity of oils were measured using a viscometer, with a small sample adapter, spindle− 62, which permits the use of only 10 mL of oil in each analysis. Temperature was controlled using a water bath at 30±2°C (Ahmed et al., 2017).

3.8 Determination of chemical properties of the extracted fish oils

3.8.1 Free fatty acid (FFA) and Acid value (AV)

FFA and AV were determined according to AOCS Official method, Ca 5a-40 (AOCS, 1997).

Procedure

A well-mixed 5g oil sample was accurately weighed into a 250 mL Erlenmeyer flask and 75 mL of hot neutralized 95% ethanol and 2 mL of 1% phenolphthalein indicator solution were added to the oil sample. The hot neutralized 95% ethanol was prepared by heating 75 mL of 95% ethanol with 2 mL of 1% phenolphthalein indicator solution to incipient boiling. 0.25N sodium hydroxide (NaOH) was added to neutralize the

ethanol. The oil samples were then titrated against 0.25N sodium hydroxide (NaOH) until the appearance of the first permanent pink colour of the same intensity as that of the neutralized ethanol before the addition of sample. A constant pink color persisted for at least 30s during the titration.

Calculation

Percentage for FFA was expressed as oleic acid and the acid value was calculated as the following equation:

$$\text{FFA (\%) as oleic acid} = \frac{mL\ of\ alkali \times N \times 28.2}{w}$$

Where, N = Normality of NaOH solution,

W= Weight of oil (g)

$$\text{Acid value (mg KOH/g)} = \text{FFA (\%)} \times 1.99$$

3.8.2 Saponification value (SV) and Saponification equivalent (SE)

The saponification value (SV) of the fish oil was determined according to the procedure described in AOCS method (AOCS, 1997).

Procedure

Oil sample (1g) was weighed into a volumetric flask. Then 15 mL of 1N alcoholic potassium hydroxide (KOH) was pipetted and allowed to drain for about 1 min into the mixture. A condenser was connected to the flask and the mixture sample was allowed to boil gently but steadily for 45 min until oil droplets gets disappeared and left to cool down to room temperature. 1 mL Phenolphthalein indicator was then added and the solution was titrated against 0.5N Hydrochloric acid (HCl) until a pink end point was reached. A blank titration was also carried out simultaneously without the addition of oil sample.

Calculation

The SV was calculated using the following equation:

$$\text{Saponification value (SV)} = \frac{56.1 \times (a-b) \times N}{W}$$

Where, a = Volume of HCl used in the blank, (mL)

b = Volume of HCl used in the test, (mL)

N = Normality of HCl

W = Weight of oil sample, (g)

Saponification equivalent (SE) was also determined according to the formula described by Rahman et al., (2018).

$$\text{Saponification equivalent} = \frac{56100}{Saponification\ value\ (SV)\ of\ the\ lipid}$$

3.8.3 Peroxide value (PV)

The Peroxide values (PV) of fish oils were determined according to AOAC method (AOAC, 2005).

Procedure

Oil sample (5g) was weighed into a 250 mL conical flask and mixed with 30 mL of glacial acetic acid and chloroform (3:1) and mixed thoroughly by swirling the flask. Saturated potassium iodide (0.5 mL) was then added and the mixture was stirred occasionally in the dark place for 1 min, then 30 mL distilled water was also added. Saturated potassium iodide solution was prepared by adding 10g potassium iodide to 6 mL boiled distilled water so that un-dissolved potassium iodide crystals were not present during analysis. The mixture was titrated against 0.1N sodium thiosulphate solution with 1 mL of 1.0% soluble starch as indicator until the blue color disappeared. A blank titration was also carried out in the same manner without the addition of fish oil.

Calculation

The peroxide value (milliequivalents peroxide/1000g sample) was calculated as the following equation:

$$\text{Peroxide value} = \frac{(a-b)\times N\times 1000}{Weight\ (g)\ of\ the\ sample}$$

Where, a = Volume (mL) of titrant consumed in blank test

b = Volume (mL) of titrant consumed in the test

N = Normality of sodium thiosulfate solution

3.8.4 Iodine value (IV)

Iodine value was determined according to the method of AOAC as described by AOAC (2002).

Procedure

Fish oil sample (0.1g) was weighed into a conical flask and 20 mL of carbon tetra chloride was added to dissolve the oil. Then 25 mL Hanus solution was added and sealed. It was shaken for about one min, kept sealed and left in a dark room (about 20°C) for 30 min. 10 mL of 15% potassium iodide and 100 mL water were also added, sealed and again shaken for 30 s. The mixture was titrated against 0.1M sodium thiosulfate to obtain iodine value. Likewise, blank test was also performed to obtain blank level.

Calculation

The iodine value was obtained using the following equation:

$$\text{Iodine value (IV)} = \frac{127 \times (a-b) \times N}{10W}$$

where, a = Volume (mL) of 0.1M sodium thiosulfate consumed in the blank test

b = Volume (mL) of 0.1M sodium thiosulfate consumed in the test

N=Normality of sodium thiosulfate

W=Weight of sample

3.8.5 Ester value (EV)

The Ester value is the amount (in mg) of potassium hydroxide required to saponify the esters in 1g of a substance. Once the saponification value and acid vale have been determined, the difference between these two values represents the ester value (Rahman et al., 2018).

$$\text{Ester value (EV)} = \text{Saponification value} - \text{Acid value}$$

3.9 Determination of fatty acid profile of the extracted fish oils

3.9.1 Preparation of fatty acid methyl esters (FAMEs)

For determining fatty acid profile, extracted fish oils were subjected to methylation (O'Fallon et al., 2007). Fatty acid methyl esters (FAMEs) of total lipid were prepared for GC-MS analysis as described by Harynuk et al., (2006). 250mg of oven heated (70-80°C) extracted lipids were taken in a test tube and saponified with methanolic sodium hydroxide solution (1.5 mL). The solution was heated at a sonicator for about 5 min. 2 mL of boron trifluoride (BF_3) was also added to the oil solution. Then, 5 mL of saturated sodium chloride (NaCl) and 1 mL of iso-octane was also added to the test tube. The mixture was homogenized with vigorous shaking and allowed for 10 min to separate the clear- colored FAME solution from a cloudy aqueous layer. Lastly, 1 mL of the organic layer on top was carefully pipetted off and inserted into a new vial for GC-MS analysis.

3.9.2 Identification of FAMEs standard

To determine each type of fatty acid, standards of FAMEs 25 was used. The fatty acids in the oil samples were determined by comparing the retention times of the samples with the FAMEs 25 standard used in this study for each chromatographic peak of individual fatty acids.

3.9.3 Gas chromatography–mass spectrometry (GC-MS) analysis

The fatty acids present in oil samples were measured in GC-MS using a MS detector at a predetermined wavelength. Prior to sample injection, hexane was injected three times to rinse GC-MS machine. A 1 μL sample from the vial containing 1 mL FAMEs solution is injected into GC-MS using a capillary column with CP-Sil 5CB stationary phase with a pre-programmed oven temperature of 60-220°C with a temperature rise rate of 10°C / min. The carrier gas is 12 kPa pressurized Helium with a total rate of 11 mL / min, and a split ratio of 1:50. From the chromatogram, the type and content of fatty acids belonging to SFAs, MUFAs and PUFAs, respectively can be easily measured and identified (Nazir et al., 2017).

3.10 Determination of nutritional quality indices (NQI)

Nutritional quality indices (NQI) of oils derived from various extraction methods were calculated according to the equations summarized by Chen and Liu (2020).

i. Polyunsaturated fatty acid/saturated fatty acid ratio (PUFA/SFA) = $\frac{\Sigma PUFA}{\Sigma SFA}$

ii. Index of atherogenicity (AI) = $\frac{[\mathrm{C12{:}0} + (4 \times \mathrm{C14{:}0}) + \mathrm{C16{:}0}]}{\Sigma UFA}$

iii. Index of thrombogenicity (TI) = $\frac{(C14{:}0 + C16{:}0 + C18{:}0)}{[(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma n-6\ PUFA) + (3 \times \Sigma n-3\ PUFA) + (n-3/n-6)]}$

iv. Hypo-hypercholesterolemic ratio (HI) = $\frac{(cis{-}C18{:}1 + \Sigma PUFA)}{C12{:}0 + C14{:}0 + C16{:}0)}$

v. Health-promoting index (HPI) = $\frac{\Sigma UFA}{[C12{:}0 + (4 \times C14{:}0) + C16{:}0]}$

vi. Sum of EPA and DHA (EPA + DHA) = C22:6 n-3 + C20:5 n-3

vii. Fish lipid quality (FLQ) = $\frac{100 \times (C22{:}6\ n{-}3 + C20{:}5\ n{-}3)}{\Sigma FA}$

viii. Linoleic acid /α-linolenic acid ratio (LA/ALA) = $\frac{C18{:}2\ n{-}6}{C18{:}3\ n{-}3}$

3.11 Statistical analysis

Each analysis was carried out in triplicates. Obtained data were stored in Microsoft Excel 2010 and the significant differences were determined by one-way analysis of variance (ANOVA) followed by Fisher's LSD test using Minitab Statistical Software (Version: 19.1.1 0; Minitab, Ltd. United Kingdom). The significance level was measured at the level of $p<0.05$.

CHAPTER-4: RESULTS

4.1 Yields of fish oil

The results of the yield percentage with different extraction methods are presented in Table 1. Significant differences were observed among the different extraction methods ($p<0.05$). However, the highest yield was reported in MAE method (21.80%) followed by WR (19.24%), SE (13.50%) and AS extraction (10.23%) method, respectively.

Table 1: Yields percentage of fish oil

Extraction methods	Yield (%)
SE	13.503 ± 0.048^{c}
WR	19.247 ± 0.661^{b}
AS	10.233 ± 0.352^{d}
MAE	21.800 ± 0.233^{a}

* Results are expressed in wet weight basis as means ± standard deviations of three replicates. Different superscripted lower-case letters in the same column within each fraction indicate significant differences between mean values (one-way ANOVA followed by Fisher's LSD, $p<0.05$). SE = Soxhlet extraction, WR = Wet rendering, AS = Acid silage, MAE = Microwave assisted extraction

4.2 Physical properties of fish oils

Physical properties (density (g/mL), refractive index, viscosity (cP) and melting point (°C) values) of pangus fish oil were observed. The viscosity ranged from 43.00 − 52.00 with significant differences ($p<0.05$) among extraction methods. Furthermore, there were no significant differences ($p>0.05$) in density (0.909 to 0.913), refractive index (1.45 to 1.46), and melting points (32.50 to 34.23) among the oil samples obtained from four extraction procedures (Table 2).

Table 2: Physical properties of oils from different extraction methods

Physical properties	SE	WR	AS	MAE
Refractive Index at	1.455 ± 0.003^{a}	1.460 ± 0.002^{a}	1.459 ± 0.002^{a}	1.457 ± 0.004^{a}

30°C				
Density (g/mL)	0.909±0.002^{bc}	0.912±0.001^{ab}	0.913±0.001^{a}	0.909±0.001^{c}
Melting Point (°C)	32.50±0.50^{b}	34.23±0.25^{a}	33.47±0.50^{a}	33.50±0.50^{a}
Viscosity, cP at 30°C	46.33±1.33^{c}	48.00±0.50^{b}	52.00±0.40^{a}	43.00±0.50^{d}

* Results are expressed in wet weight basis as means ± standard deviations of three replicates. Different superscripted lower-case letters in the same row within each fraction indicate significant differences between mean values (one-way ANOVA followed by Fisher's LSD, $p<0.05$). SE = Soxhlet extraction, WR = Wet rendering, AS = Acid silage, MAE = Microwave assisted extraction

4.3 Chemical properties of fish oils

The values of free fatty acid (FFA), acid value (AV), peroxide value (PV), iodine value (IV), saponification value (SV), saponification equivalent (SE), and ester value (EV) of pangus fish oil obtained by different extraction methods are presented in Table 3. All of these parameters showed significant differences among all of the oil extraction methods ($p<0.05$). Based on differentiating the extraction methods the acid value (AV), free fatty acid (FFA, % oleic acid), saponification value (SV), saponification equivalent (SE), iodine value (IV), peroxide value (PV) and ester value (EV) of the extracted fish oils varied from 1.40 to 1.52 (mg KOH/g), 0.70 to 0.76 (%, as oleic acid), 162.96 to 195.28 (mg/g KOH), 287.27 to 344.24, 49.34 to 61.18 (g/100g), 2.08 to 4.65 (Meq/kg) and 161.49 to 193.88, respectively.

Table 3: Chemical properties of oils from different extraction methods

Chemical properties	**SE**	**WR**	**AS**	**MAE**
Acid Value (mg KOH/g)	1.469±0.006^{b}	1.523±0.008^{a}	1.442±0.008^{c}	1.402±0.008^{d}
Free fatty acid (% oleic acid)	0.738±0.003^{b}	0.766±0.004^{a}	0.725±0.004^{c}	0.704±0.004^{d}
Saponification value (mg/g KOH)	162.964±0.004^{d}	186.697±0.011^{b}	177.555±0.502^{c}	195.286±0.654^{a}

Saponification equivalent	344.248±0.008[a]	300.487±0.017[c]	315.960±0.894[b]	287.272±0.964[d]
Iodine Value (g/100g)	61.187±0.539[a]	49.345±0.514[d]	51.708±0.365[c]	54.439±0.591[b]
Peroxide Value (Meq/kg)	4.146±0.292[b]	3.308±0.437[c]	4.645±0.482[a]	2.081±0.703[d]
Ester Value	161.495±0.003[d]	185.174±0.005[b]	176.113±0.508[c]	193.885±0.647[a]

* Results are expressed in wet weight basis as means ± standard deviations of three replicates. Different superscripted lower-case letters in the same row within each fraction indicate significant differences between mean values (one-way ANOVA followed by Fisher's LSD, $p<0.05$). SE = Soxhlet extraction, WR = Wet rendering, AS = Acid silage, MAE = Microwave assisted extraction

4.4 Fatty acid profile of extracted fish oils

Fatty acid composition of the extracted pangus fish oils are depicted in Table 4. A total of 25 fatty acids (SFA+MUFA+PUFA; 13+5+7) were identified and the concentration was calculated based on the retention time displayed in chromatograms (enlisted in Appendix E). Oil extracted by using the AS method had significantly higher ($p<0.05$) levels of saturated fatty acids (17.330±1.508mg/ 100g sample) compared to other methods; MAE (13.57±2.50mg/ 100g sample), WR (10.634±0.714mg/ 100g sample) and SE (8.530±1.005mg/ 100g sample), respectively. However, Myristic acid was reported to be the major component of saturated fatty acid (SFA) in both SE (4.040±0.484mg/ 100g sample) and WR (4.085±0.903mg/ 100g sample) extraction method while Palmitic acid was found to be the major SFA in both AS (7.478±0.689mg/ 100g sample) and MAE (6.401±1.369mg/ 100g sample) methods.

Monounsaturated fatty acids (MUFAs) were found to be the most abundant in oils extracted by using MAE (7.997±0.193mg/ 100g sample) method followed by AS (4.860±0.725mg/ 100g sample), SE (2.752±0.392mg/ 100g sample) and WR (1.554±0.153 mg/ 100g sample) extraction method, respectively. Meanwhile, fish oil extracted by using SE (19.158±1.710mg/ 100g sample) method had the highest polyunsaturated fatty acids (PUFAs) followed by WR (13.236±0.789mg/ 100g

sample), MAE (12.137±1.216mg/ 100g sample) and AS (8.600±0.573mg/ 100g sample) extraction method, respectively. However, significantly higher Eicosapentaenoic acid (C20:5n-3) content was observed in both MAE and WR extraction methods while significantly higher Docosahexaenoic acid (C22:6n-3) content was observed in AS extraction method (Table 4).

Table 4: Fatty acid profile of oils from different extraction methods (mg/100g fish)

Fatty acid	SE	WR	AS	MAE
Saturated Fatty Acid (SFA)				
Caprylic acid (C8:0)	0.108±0.003^{b}	0.047± 0.005^{d}	0.469±0.016^{a}	0.071±0.014^{c}
Capric acid (C10:0)	0.196±0.002^{a}	0.028±0.015^{d}	0.108±0.004^{b}	0.055±0.012^{c}
Lauric acid (C12:0)	0.621±0.186^{a}	0.891± 0.120^{a}	0.795± 0.184^{a}	0.573± 0.189^{a}
Tridecyclic acid (C13:0)	0.114±0.022^{a}	0.111± 0.025^{a}	0.053±0.017^{b}	0.063±0.005^{b}
Myristic acid (C14:0)	4.040± 0.484^{a}	4.085± 0.903^{a}	2.046± 0.592^{b}	1.524± 0.660^{b}
Palmitic acid (C16:0)	0.753±0.188^{b}	1.637± 0.543^{b}	7.478± 0.689^{a}	6.401± 1.369^{a}
Margaric acid (C17:0)	0.027± 0.012^{b}	0.119± 0.005^{a}	0.027±0.004^{b}	0.013±0.003^{c}
Stearic acid (C18:0)	0.089±0.019^{c}	0.182± 0.047^{b}	0.616± 0.035^{a}	0.029±0.004^{d}
Arachidic acid (C20:0)	0.097±0.006^{a}	0.072±0.009^{b}	0.011±0.004^{c}	0.016±0.009^{c}
Heneicosylic acid (C21:0)	2.135± 0.709^{a}	2.352± 0.187^{a}	0.785±0.081^{b}	2.450± 0.384^{a}
Behenic acid (C22:0)	0.200± 0.073^{c}	0.736± 0.012^{c}	2.496± 0.488^{a}	1.685± 0.365^{b}
Tricosanoic acid (C23:0)	0.057±0.015^{c}	0.159± 0.040^{bc}	1.021± 0.129^{a}	0.227± 0.059^{b}
Lignoceric (C24:0)	0.093±0.008^{d}	0.217±0.063^{c}	1.425±0.104^{a}	0.464±0.040^{b}
ΣSFA	8.530± 1.005^{c}	10.634±0.714^{bc}	17.330±1.508^{a}	13.57± 2.50^{b}
Mono Unsaturated Fatty Acid (MUFA)				

Palmitoleic acid (C16:1)	0.358±0.145[a]	0.397±0.077[a]	0.191±0.004[b]	0.028±0.006[c]
Oleic acid(C18:1n-9)	0.915± 0.205[b]	0.431±0.069[c]	0.079±0.008[d]	3.341±0.114[a]
Eicosenoic acid (C20:1n-9)	0.394±0.020[c]	0.305± 0.072[c]	1.049± 0.279[b]	2.782± 0.489[a]
Erucic acid (C22:1n-9)	0.718± 0.173[a]	0.396±0.017[b]	0.870± 0.226[a]	0.879±0.148[a]
Nervonic acid (C24:1n-9)	0.367±0.058[c]	0.023± 0.007[d]	2.670± 0.268[a]	0.967± 0.191[b]
ΣMUFA	2.752± 0.392[c]	1.554±0.153[d]	4.860± 0.725[b]	7.997± 0.193[a]
Poly Unsaturated Fatty Acid (PUFA)				
Linoleic acid (C18:2n-6)	17.500±2.01[a]	10.603±0.816[b]	5.014±0.571[d]	8.290±0.893[c]
α-Linolenic acid (C18:3n-3)	0.069±0.002[d]	1.091± 0.022[b]	1.487±0.010[a]	0.656± 0.0362[c]
Arachidonic acid (C20:4n-6)	0.028±0.007[c]	0.278± 0.016[a]	0.071±0.001[b]	0.005±0.003[d]
Eicosapentaenoic acid (C20:5n-3)	0.036±0.004[c]	0.214±0.017[a]	0.098±0.007[b]	0.229±0.005[a]
Eicosatrienoic acid (C20:3n-3)	0.927± 0.187[a]	0.328± 0.039[b]	0.149±0.008[b]	0.186± 0.018[b]
Docosahexaenoic acid (C22:6n-3)	0.064± 0.007[c]	0.103± 0.008[b]	0.421± 0.026[a]	0.064±0.009[c]
Docosapentaenoic acid (C22:5n-3)	0.536± 0.122[c]	0.618± 0.089[bc]	1.359± 0.155[b]	2.707± 0.768[a]
ΣPUFA	19.158±1.710[a]	13.236±0.789[b]	8.600± 0.573[c]	12.137±1.216[b]

*Results are expressed in wet weight basis as means ± standard deviations of three replicates. Different superscripted lower-case letters in the same row within each fraction indicate significant differences between mean values (one-way ANOVA followed by Fisher's LSD, $p<0.05$). SE = Soxhlet extraction, WR = Wet rendering, AS = Acid silage, MAE = Microwave assisted extraction

4.5 Nutritional quality indices (NQI) of extracted oils

The nutritional quality indices of pangus fish oil extracted by different extraction methods are summarized in Table 5. Significant differences ($p<0.05$) were observed in PUFA/SFA and FLQ indices regardless of different extraction methods. The ration of PUFA to SFA were at the highest level in SE (2.252±0.094) followed by WR (1.248±0.104), MAE (0.905±0.094) and AS (0.498±0.044) extraction methods, respectively. Significantly lower ($p>0.05$) ratio of n-3/n-6 were observed in SE (0.010±0.002) and WR (0.129±0.009) methods while no significant differences ($p>0.05$) were observed in both AS and MAE methods. However, AS extraction method yielded better EPA+DHA, AI, TI and FLQ indices while better HA and LA/ALA rations were observed in SE method (Table 5).

Table 5: Nutritional quality indices (NQI) of oils from different extraction methods

NQI	SE	WR	AS	MAE
PUFA/SFA	2.252±0.094^{a}	1.248± 0.104^{b}	0.498±0.044^{d}	0.905±0.094^{c}
EPA+DHA	0.099±0.011^{c}	0.317±0.025^{b}	0.519±0.028^{a}	0.293±0.014^{b}
n-3/n-6	0.010±0.002^{c}	0.129±0.009^{b}	0.398±0.044^{a}	0.398±0.044^{a}
AI	0.807±0.065^{b}	1.275± 0.201^{a}	1.223±0.156^{a}	0.668±0.123^{b}
TI	0.459±0.026^{b}	0.559± 0.085^{b}	0.892±0.097^{a}	0.511±0.238^{b}
HH	3.703±0.182^{a}	2.048± 0.188^{b}	0.845±0.076^{c}	1.865±0.302^{b}
HPI	1.252±0.088^{ab}	0.799±0.138^{c}	0.827±0.112^{bc}	1.627±0.411^{a}
LA/ALA	250.6± 31.8^{a}	9.717± 0.722^{b}	3.370±0.372^{b}	12.69± 1.76^{b}
FLQ	0.329±0.066^{d}	1.249±0.129^{b}	1.686±0.049^{a}	0.954±0.093^{c}

* Results are expressed in wet weight basis as means ± standard deviations of three replicates. Different superscripted lower-case letters in the same row within each fraction indicate significant differences between mean values (one-way ANOVA followed by Fisher's LSD, $p<0.05$). SE = Soxhlet extraction, WR = Wet rendering, AS = Acid silage, MAE = Microwave assisted extraction. AI = Index of atherogenicity, TI = Index of thrombogenicity, HH = Hypo and Hypercholesterolemia ratio, HPI = Health promoting index, FLQ = Fish lipid quality

CHAPTER-5: DISCUSSION

5.1 Effects of different extraction methods on the yield of fish oil

Oil yields owing to different extraction methods depend on whether the fish is cooked prior to extraction, the contact temperature, and whether there is contact with certain solvents (Chantachum et al., 2000; Aryee and Simpson, 2009; Rubio-Rodríguez et al., 2012). However, in this study the oil yields obtained from SE, WR and AS extraction methods were much lower than the MAE method. The highest yield from the MAE method is attributed to coagulation of protein, which releases both bound water and oil. The oil is further separated by pressing, resulting in an improved extraction (Taghvaei et al., 2014). The WR method also involves coagulation of fish protein, so oil and solid materials gets separated and skimmed off (Chantachum et al., 2000). In contrast, the lower extraction efficiency in SE method might be attributed to the higher internal mass transfer resistance after initial recovery of most accessible oils; which slows down the extraction rate (Rubio-Rodríguez et al., 2012). Meanwhile, the AS method gives the lowest yield, since some of the fat remained emulsified as a stable skim fraction due to the action of acids or natural enzymes that cause the fats to bind tightly within the protein matrix (Taati et al., 2018). However, the results are in consistent with the results published by Nazir et al., (2017) and Afolabi et al., (2018) on the yields of oils from tuna (*Thunnus albacares*) head and eel (*Monopterus albus*) fish. Therefore, MAE based on extraction yield might be the most efficient method to obtain fish oil compared to the other three methods.

5.2 Effects of different extraction methods on the physical properties of oils

The refractive index of an oil or fat is somewhat dependent on its degree of unsaturation, and a higher refractive index indicates the presence of more unsaturated materials. It also measures the changes in unsaturation due to different extraction methods. The refractive index of oils varies according to molecular weight, chain length of fatty acids, degree of unsaturation and degree of conjugation (Andhale et al., 2017). However, in this study no significant differences ($p>0.05$) were observed in refractive index regardless of different extraction methods. In addition, obtained values are within the standard limits (1.40 to 1.47) as recommended for oils by Adeniyi and Bawa (2006) and Abdulkadir et al., (2010).

Density is an important factor affecting oil absorption capacity and mass transfer rate owing to different extraction methods (Yilmaz, 2011). The density of fish oil varies according to the degree of heat treatment applied while extracting the oils. In this study, higher temperature in MAE followed by SE method resulted in a slight decrease in density as the oil occupies more volume through the diffusion of molecules. However, regardless of the extraction methods; obtained density values are lower than that of water (1.000g/mL) and compatible with other edible oils such as canola oil (0.913g/ml), olive oil (0.908g/ml) as reported by Sahasrabudhe et al., (2017).

The melting point characterizes the physical properties of oils and fats, such as hardness and thermal behavior. Obtained melting points of the extracted fish oils (34.23 − 32.50°C) using different extraction methods were below the melting points of cottonseed oil (42.8°C), sheep tallow (42°C), and palm oil (35°C) while higher than that of Sunflower oil (-17°C), soybean oil (-16°C) and olive oil (-6°C) as described by Nassu and Gonçalves, (1999) and Engineering ToolBox, (2008). According to Ulfah et al., (2016) the melting point is influenced by the degree of unsaturation, chain length and the number of double bonds. However, significantly lower melting point in SE method is reported due to reduced level of saturation which is also in agreement with the result published by Lestari and Purnamayati, (2020).

The viscosity of oils or fats can be affected by the presence of impurities, including free fatty acids, proteins, pigments, moisture, volatile compounds, and the degree of unsaturation (Wiedermann, 1981; Zahir et al., 2017; Suseno et al., 2015). In this study, the viscosity of microwave-assisted fish oil was significantly lower than the SE, WR and AS methods ($p<0.05$). The MAE method uses microwave energy at increasing temperature, which reduces the intermolecular attractions between molecules, thereby reducing the density and subsequently the oil becomes less viscous. However, the viscosity of the resulting fish oils is in consistent with that of sardine oil (51.70 cP) as reported by Suseno et al., (2015). In contrast, higher viscosity generally indicates lower purity of the oil (Suseno et al., 2015). Meanwhile, the selectivity of the AS extraction method might be poor and thus extract all classes of lipids and other molecules, which could be the possible reason behind the increased viscosity in the resulting oil. Also, highly viscous oils or fats require more refining to lower their viscosity level (Farag and Basuny, 2009).

5.3 Effects of different extraction methods on the chemical properties of oils

The acid value is an important quality parameter related to the presence of free fatty acids and other non-lipid acidic compounds such as acetic acid. The acidity of the oil depends on several factors, such as- the oil composition, extraction procedure and the freshness of the raw materials (Rubio-Rodríguez et al., 2012). According to the data obtained from Table 3, the highest acid value was reported in the WR extraction method (1.52 mg KOH/g), followed by SE (1.47 mg KOH/g), AS (1.44 mg KOH/g) and MAE (1.40 mg KOH/g) method, respectively. The higher acidity in WR extraction method might be attributed to the hydrolysis of triglycerides during heating and air exposure of the fish muscles. In contrast, the lower acid value in MAE might be due to the shortened extraction time owing to microwave heating. Lower acid value usually indicates the purity and suitability of the cooking oil, while a higher value is associated with rancidity (Das et al., 2016). Besides, the acid value of oils for edible purposes should not exceed 4 mg KOH/g and the values obtained in this study are within the recommended limits (Sasongko et al., 2017).

Unsaturated fatty acids with double bonds in their structures react with heat, air or water to form free fatty acids that affect the quality of fish oil (Nazir et al., 2017). FFA value usually indicates the degree of hydrolysis or oxidation. The percentages of free fatty acids in lipids (≤ 1.5%) are recommended for edible purposes (Molla et al., 2007). However, FFA values in all the extracted fish oils are within the acceptable range, indicating no or less lipid degradation occurred during or after extraction procedures. Among the extracted oils, wet rendered fish oil contained the highest FFA (0.76%), followed by SE (0.74%), AS (0.73%) and MAE (0.70%), respectively. Aryee and Simpson (2009) also reported similar FFA content (0.6-1.2%) in salmon oil. However, the lowest FFA in MAE might be attributed to the optimal extraction temperature for a shorter extraction time. On the contrary, oil exposed to heat and air for a longer time resulted in a higher FFA value (Chantachum et al., 2000). However, lower the FFA value better will be the oil quality.

Saponification is the process of breaking down a neutral fat into glycerol and fatty acids through alkaline conditioning. The saponification value measures the amount of fatty acids found in fish oil. A high saponification value indicates that the oil contains fatty acids with lower molecular weights (Low and Ng, 1987). In this study, the highest saponification value was observed in MAE (195.286mgKOH/g) followed by WR (186.697mgKOH/g), AS (177.555mgKOH/g) and SE (162.964mgKOH/g),

respectively. Regardless of SE method, saponification values of all the extracted fish oils are within the standard limit (min. 170mgKOH/g) as reported by Sasongko et al., (2017). However, obtained saponification values have close similarities with Cottonseed oil (175 to 198mgKOH/g) and castor oil (175 to 180mgKOH/g) as described by Rahman et al., (2018).

Fats or oils usually contain unsaturated fatty acids and some saturated fatty acids such as myristic acid, palmitic acid, and some unsaponifiable matter. In this study, the highest saponification equivalent of fish oils was reported in the SE (344.248) method, followed by AS (315.960), WR (300.487) and MAE (287.272) methods, respectively. Obtained results are in agreement with the result published by Rahman et al., (2018) who concluded that oils with higher acidity generally have saponification equivalents of around 290.80. In addition, higher saponification equivalent indicates the presence of significantly higher fatty acids. Thus, findings of saponification equivalent clearly indicate that pangus fish oil contains considerable amount of saturated and unsaturated fatty acids owing to different extraction methods.

The amount of iodine absorbed measures the number of reactive double bonds or unsaturated bonds present in oil (Handajani et al., 2010). This value can be used to determine the extent of oils to be oxidized. The results of this study indicate that iodine value in the SE (61.18g/100g) method is higher than that of MAE (54.44g/100g), AS (51.71g/100g) and WR (49.34g/100g) methods, respectively. However, a higher iodine value indicates higher unsaturation of fats and oils (Knothe, 2002). Thus, higher iodine value in the oils from SE method indicates higher unsaturation and is prone to oxidation. In addition, iodine value also regulates the melting point of fish oil. According to Hasibuan (2012), higher the double-bonded unsaturated fatty acids, more liquid would be the fish oil and vice versa. This trend was also followed by the obtained melting points in this study. However, obtained iodine values in this study were higher than that of the oil from tilapia 9.13g/100g (Nugroho et al., 2014) and lower than the fish oil of fresh mackerel 121.60 g/100g (Ndidiamaka and Ifeanyi, 2018).

The peroxide value determines the extent to which the oil undergoes rancidity during processing, extraction and storage. Besides, it can be used to monitor the quality and stability of fats and oils (Ekwu and Nwagu, 2004). Various factors such as fatty acid composition, presence of light and air affect the formation of hydro peroxides and degradation into secondary oxidation products (Sullivan and Budge, 2010; Ritter,

2012). The smaller the peroxide value, the better the quality of the oil. In this study, fish oils obtained from WR and AS extraction methods contained higher peroxides than SE and MAE methods. Degradation of fish muscles due to prolonged processing and exposure to air releases more free ions, thus wet rendered oils contained more free ions, resulting in higher oxidation rate (Arruda et al., 2007). The higher oxidation rate detected in AS extraction is also predicted by natural enzymes or protein denaturation caused by acids. Denaturation of the protein molecule weakens the unsaturated bonds or links, increasing the likelihood of oxidation. In addition, Gracey et al., (1999) reported that oil with a peroxide value of less than 5 Meq/kg can be considered fresh oil, while oil with a peroxide value of 7.5 Meq/kg is unacceptable for human consumption (Robards et al., 1988; Schnepf et al., 1991). It is known from this study that the peroxide values of all the extracted samples are relatively good as they are within the recommended limits. Similar results were also reported by Nazir et al., (2017).

Esters are naturally occurring components in fats and oils that are responsible for flavor development and pleasant odors. The ester value indicates the amount of alkali consumed to saponify the esters contained in fats or oils. The oils extracted using the MAE (193.88) method represent higher ester values than the WR (185.17), AS (176.11) and SE (161.495) methods, respectively. Microwave heating hydrolyzed the esters found in the extracted oils, causing the ester value to increase. However, the results from this study are just below the ester value of striped catfish (*Pangasius sutchi*) oil previously reported by Islam et al., (2012).

5.4 Effects of different extraction methods on fatty acid profile of fish oils

Oil extraction methods have a significant effect on the fatty acid profile ($P<0.05$) of pangus fish oil. The results showed that the SE method is better in recovering PUFAs than WR, MAE and AS extraction methods. Gentle heating during extraction procedure prevents oxidation and can therefore be used to efficiently recover PUFAs. However, the lowest recovery of PUFAs in the AS extraction method might be attributed to emulsion formation during oil extraction (Hajeb et al., 2015). Previously, researchers reported EPA and DHA as the main PUFAs in marine fish (Ozogul et al., 2011; Ozogul et al., 2012). The highest EPA content was obtained from both MAE (0.229±0.005 mg/100g) and WR extraction method (0.214±0.017 mg/100g) followed by AS (0.098±0.007 mg/100g) and SE (0.036±0.004 mg/100g) methods, respectively.

However, similar results were also observed by Hajeb et al., (2015) who have concluded that higher amounts of EPA can be recovered by using the WR method. In addition, DHA contents in extracted fish oils ranged (0.064±0.007 to 0.421±0.026mg/100g) and did not show significant differences in both SE and MAE methods. Therefore, AS extraction method works best to recover DHA while both WR and MAE methods works best to recover EPA.

In contrast, MAE followed by AS extraction methods are much better in extracting MUFAs. It was also found in previous studies that the MUFA contents (C16:1, C18:1) obtained by the MAE method were higher than other methods (Sathivel et al., 2003b). Furthermore, AS extraction method yields the maximum SFAs while SE method yields the least count. The lowest SFA contents in fish oils were also observed by Ozogul et al., (2018). However, the interactions between different extraction methods to recover fatty acids are in line with previous studies undertaken by Aursand et al., (1994) and Jobling et al., (2002), in which they concluded that abundance of polar and non-polar fats within the fish muscle, degree of saturation and using solvents or not during extraction might be attributed to the variations in fatty acid contents. Moreover, consumption of unsaturated fatty acids (UFAs) is more important than saturated fatty acids (SFAs) for health and wellbeing (Lawrence, 2010). Despite SE yields the maximum PUFAs, it requires longer time and high purity solvents. Besides, contamination with possibly hazardous and flammable organic solvents, emission of toxic compounds during SE procedure might be a cause for concern. Therefore, considering the facts MAE might be effective to maintain health and preserving better nutritional quality.

5.5 Effects of different extraction methods on the NQI of fish oils

The nutritional value of dietary food ingredients is repeatedly evaluated through nutritional quality indices (NQI). It is calculated by several indices of fatty acid composition which provides greater insights regarding the possible health effects of certain fatty acids such as Lauric acid (C12:0), Myristic acid (C14:0) and Palmitic acid (C16:0) has been evidenced to increase the total serum cholesterol which eventually causes coronary heart diseases (Ulbricht and Southgate, 1991; Zong et al., 2016).

The PUFA/SFA ratio is currently one of the main parameters to assess the nutritional quality of seafood and dietary fat (Larsen et al., 2011). The highest PUFA/SFA ratio

in the extracted fish oils was observed in SE method (2.252±0.094) followed by WR (1.248±0.104), MAE (0.905±0.094) and AS (0.498±0.044) extraction methods. However, PUFA/SFA ratio is recommended to be higher than 0.4, so as to reduce the risk of cardiovascular, autoimmune and other chronic diseases (Simopoulos, 2002). A lower PUFA/SFA ratio indicates a higher level of dietary saturated fatty acids, which are considered as the major risk factors for cardiovascular disease (Dieter and Tuttle, 2017). In the current study, this ratio exceeded the minimum recommended for all samples regardless of extraction methods. The greatest increase in PUFA /SFA ratios might be attributed to the maximal absorption of PUFAs during extracting the oils (Karimian-Khosroshahi et al., 2016).

EPA and DHA are long chain n-3 fatty acids, which are precursors of hormones known as eicosanoids that play important roles in biological processes in the body (Gladyshev et al., 2006). A daily intake of approximately 500 – 1000 mg of "EPA+DHA" has been recommended by the American Heart Association to reduce the risk of coronary heart diseases (Huynh and Kitts, 2009). Previously, Hosseini et al., (2014) reported that the cooking procedure reduces the content of "EPA+DHA" in cooked kutum roach (*Rutilus frisii kutum*). However, this study reported that WR and AS extraction methods showed significant differences ($P<0.05$) in "EPA+DHA" content regardless of MAE and SE methods. The recovered "EPA+DHA" contents might be attributed to minimizing physical losses during the extraction of oils from fish muscle.

The n-3/n-6 ratio is considered as a useful indicator for comparing relative nutritional values of fish oils. According to health recommendations, the n-3/n-6 ratio should be lower than 0.67 thereby reducing the incidence of cardiovascular disease, and leading to pro-inflammation, cancer, and obesity (Simopoulos et al., 2002; Osman et al., 2001; Mansara et al., 2015). In addition, the lower n-3/n-6 ratio enables better utilization of n-3 fatty acids in human body (Wood et al., 2008). Results from this study showed that all the extracted oils have a very good n-3/n-6 ratio with SE and WR having a lower ratio than MAE and AS methods. Solvent extracted oils had the lowest n-3/n-6 ratio because of the prompt absorption of linolenic acid and other n-6 fatty acids.

Atherogenicity index (AI) and Thrombogenic index (TI) are two indices proposed by Ulbricht and Southgate, (1991), that characterize the atherogenic and thrombogenic potential of the fatty acids relative to other indices. Lower values of both indices

indicate better nutritional value of fatty acids, so diets with lower AI and TI values may reduce the potential risk of coronary heart disease (Karimian-Khosroshahi et al., 2016). Very low values are recommended for AI (<1) and TI (<1), indicating positive health benefits from the product (Krešić et al., 2019). In this study, significantly higher ($p<0.05$) AI indices were observed in both AS and WR extraction methods exceeding the expected range for fish oils. In this context, coconut oil was reported to be a highly atherogenic food with an AI value of 13.63. While raw mackerel, olive and sunflower oil with AI values of 0.28, 0.14 and 0.07, respectively, have been reported to be low atherogenic foods (de Alba et al., 2019). Meanwhile, microwave assisted, wet rendered and solvent extracted fish oils had similar TI index except for AS method. Oils extracted by SE method had the lowest TI index due to oil absorption (Koubaa et al., 2012). However, the TI values detected in the current study are in the expected range. Previously, raw mackerel was reported to be highly antithrombogenic with a TI value of 0.16, followed by sunflower oil (0.28) and olive oil (0.32), respectively (de Alba et al., 2019).

The effect of specific fatty acids on cholesterol metabolism can be represented by hypo-to-hyper cholesterolamic ratio (HH) (Santos-Silva et al., 2002). A higher HH ratio is desirable as it represents higher nutritional value. In this study, the highest HH value was reported in SE (3.703±0.182) method followed by WR (2.048±0.188), MAE (1.865±0.302) and AS (0.845±0.076) extraction method. In other studies, the HH ratio in fish and fishery products was found between 0.25 − 4.83 (Hosseini et al., 2014; Testi et al., 2006). MAE and WR extraction methods showed non-significant effects ($p>0.05$) on HH ratio. In contrast, SE method significantly increased the HH ratio, while AS showed the least HH ratio. However, the HH ratio of all extracted oils was beyond the optimum value (HH>1) as described by Krešić et al., (2019).

The health-promoting index (HPI) was proposed by Chen et al., (2004) to evaluate the nutritional value of dietary fat focusing on the effect of fatty acid composition on cardio-vascular diseases (CVD). It is believed that fats or oils with high a HPI values are more beneficial to human health. HPI is the inverse of the IA. This study showed significant differences ($p<0.05$) in HPI indices dependent of the extraction methods. However, as reported by Chen et al., (2004) and Bobe et al., (2007), the obtained values of the HPI indices are much larger than those of butter (0.37 to 0.66) and cheese (0.29 to 0.46), respectively.

FLQ was primarily used to determine fish lipid quality (Łuczyńska et al., 2017). FLQ calculates the sum of EPA and DHA as a percentage of total fatty acids. FLQ is more suitable for seafood due to its high EPA and DHA content. In this study, all the extraction methods showed significant differences ($p<0.05$) in terms of calculated FLQ. However, previously reported FLQ value ranged from 13.01 to 36.37 for various fish species that are far beyond the obtained results (Chen and Liu, 2020).

The ratio of linoleic acid (LA, C18:2 n-6)/α-linolenic acid (ALA, C18:3 n-3) is often used to reflect the quality of milk. It was also developed to guide infant formula (Chen and Liu, 2020). However, in the present study all the extraction methods except SE showed non-significant differences ($p>0.05$) in terms of the calculated LA/ALA ratio. However, the obtained values of LA/ALA indices are much higher than the values in milk fat (2.464 ± 0.147) as reported by Sharma et al., (2018).

CHAPTER-6: CONCLUSION AND RECOMMENDATION

With the aim of achieving the premium quality of fish oil using different extraction methods, the microwave assisted extraction (MAE) demonstrated to be a very efficient method on the recovery of oils from Pangus fish. This method showed the highest extraction yield and resulted into oil with best physical properties such as melting point and viscosity, oxidative stability (AV, PV, FFV), contained important fatty acids (higher content of MUFAs, EPA, DPA and lower content of SFA with optimal nutritional quality indices (NQI), since it is easy, fast, efficient, and safe for consumption. Furthermore, SE could be another method to increase the nutritional quality of fish oil by enhancing the amount of PUFAs content, especially EPA and DHA; though it has some issues regarding human health. However, obtained results from this study suggest that lipids of Pangus fish could be used directly in human diet or as supplementary edible oils as it contains good quality fatty acids, especially PUFAs. In future study it is essential to identify the optimal extraction conditions such as microwave power, extraction time and temperature. However, we recommend that future researchers can apply these nutritional values of FAs and NQI as for clinical evidences to explore their potential usage in disease prevention and treatment. Furthermore, future use of MAE method could help in real time, the routine extraction of edible fish oil both in research laboratories and in the food and pharmaceutical industries. In addition, fish oil refining methods have significant potential for new technologies, but are still based on traditional steps. Similarly, various PUFA concentration methods have been proposed, such as enzymatic and chromatographic methods, winterization, supercritical fluid and membrane filtration. However, currently the combination of various greener methods appears to provide a good alternative for improving the purity and performance of these components.

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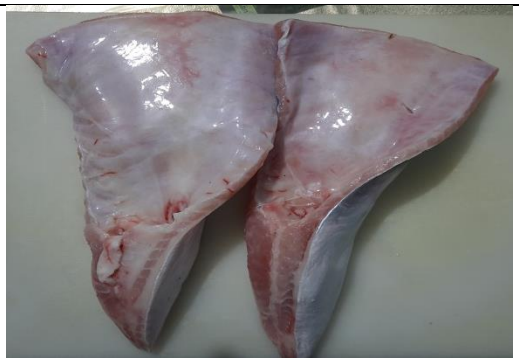
Appendix A: Pangus fish collection point from Sandwip Channel in Chattogram, Bangladesh

Appendix B: Pangus fish oil extraction using different extraction methods

	
Length measurement of raw fish	**Width measurement of raw fish**
	
Muscle from belly flap	**Cubed shaped muscle**
	
Soxhlet extraction	**Wet rendering**

Acid silage

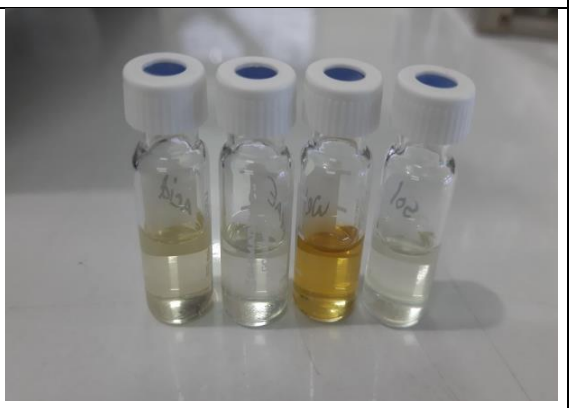
Microwave assisted extraction

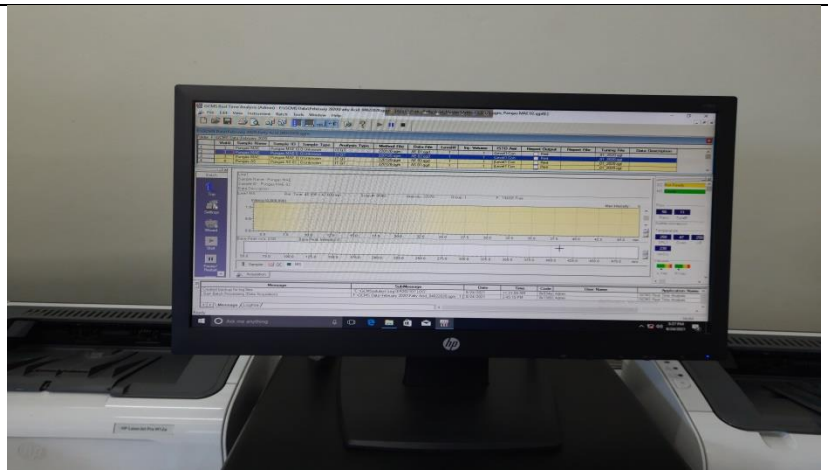
Separation of oil

Yield measurement

Extracted oils using four different extraction methods

Appendix C: Characterization of extracted fish oils quality

	
Analysis of chemical properties	**Measurement of refractive index**
	
Viscosity measurement	**Density measurement**
	
Ultrasonic heating of oils	**FAME solution for FA profiling**



GC-MS analysis

Analysis of Fatty acid profile

Appendix D: Experimental data

a) Yield percentage of oil

Extraction methods	Yield (%)
Solvent Extraction (SE)	13.07
	13.56
	13.88
Wet rendering (WR)	19.89
	19.28
	18.57
Acid silage (AS)	9.86
	10.56
	10.28
Microwave assisted extraction (MAE)	22.05
	21.76
	21.59

a) Physical properties of extracted oils

Physical properties	Extraction methods			
	SE	WR	AS	MAE
Density	0.91	0.912	0.913	0.908
	0.911	0.912	0.912	0.91
	0.908	0.911	0.914	0.909
Refractive Index	1.458	1.461	1.458	1.461
	1.456	1.461	1.458	1.453
	1.453	1.458	1.461	1.456
Melting point	32.5	34.2	33.4	33
	32	34	33	34
	33	34.5	34	33.5
Viscosity	46	48	52	43
	45.2	47.5	52.4	42.5
	47.8	48.5	51.6	43.5

b) Chemical properties of extracted oils

Chemical properties	Extraction methods			
	SE	WR	AS	MAE
Acid Value	1.472	1.522	1.444	1.403
	1.463	1.532	1.433	1.393
	1.473	1.516	1.449	1.409
Free fatty acid	0.739	0.765	0.726	0.705
	0.735	0.77	0.72	0.7
	0.74	0.762	0.728	0.708
Saponification value	162.967	186.701	177.859	195.698
	162.96	186.705	177.83	194.532
	162.965	186.685	176.976	195.629
Saponification equivalent	344.241	300.48	315.418	286.666
	344.256	300.474	315.469	288.384
	344.246	300.506	316.992	286.767
Iodine Value	61.443	49.929	51.449	54.101
	60.568	48.96	51.55	55.121
	61.550	49.146	52.125	54.095
Peroxide Value	4.197	3.295	4.615	1.962
	4.096	3.325	4.857	2.158
	4.145	3.305	4.463	2.122
Ester Value	161.495	185.179	176.415	194.295
	161.497	185.173	176.397	193.139
	161.492	185.169	175.527	194.22

c) Fatty acid profile of extracted oils

Fatty Acids (FAs)	Extraction methods			
	SE	**WR**	**AS**	**MAE**
Saturated fatty acids (SFAs)				
Caprylic acid (C8:0)	0.1116	0.0525	0.4699	0.0557
	0.1075	0.0436	0.4833	0.0804
	0.1061	0.0442	0.4524	0.0782
Capric acid (C10:0)	0.1944	0.0166	0.1040	0.0442
	0.1958	0.0445	0.1097	0.0676
	0.1978	0.0235	0.1106	0.0559
Lauric acid (C12:0)	0.6493	0.7781	0.5927	0.355
	0.4232	1.0173	0.9511	0.6725
	0.7912	0.8761	0.8415	0.6908
Tridecyclic acid (C13:0)	0.0934	0.1396	0.0361	0.0621
	0.1377	0.0921	0.0528	0.0682
	0.1111	0.0999	0.0696	0.0591
Myristic acid (C14:0)	4.0404	4.0853	1.9495	2.1623
	4.5238	3.1824	2.6798	1.5655
	3.5557	4.9881	1.5083	0.8447
Palmitic acid (C16:0)	0.9257	2.1796	8.252	7.7707
	0.5525	1.6371	7.2507	6.4015
	0.7794	1.0945	6.9326	5.0323
Margaric acid (C17:0)	0.0154	0.12	0.0229	0.0102
	0.0273	0.1231	0.0301	0.013
	0.0392	0.1137	0.0296	0.0158
Stearic acid (C18:0)	0.0887	0.1306	0.5945	0.0254
	0.1085	0.1923	0.6564	0.0289
	0.069	0.2218	0.5968	0.0325
Arachidic acid (C20:0)	0.0957	0.0716	0.0059	0.0265
	0.0918	0.0625	0.0126	0.0121
	0.1043	0.0808	0.0143	0.0092
	2.844	2.5663	0.8755	2.834

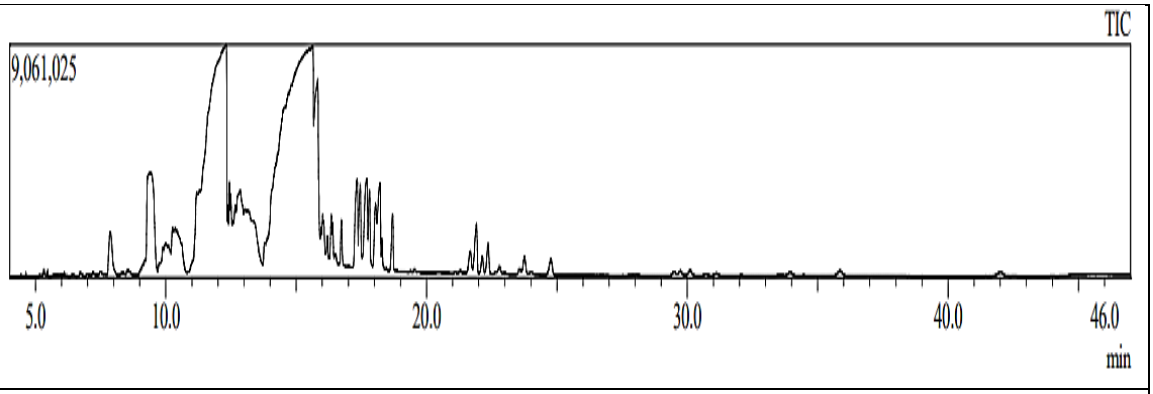
Heneicosylic acid (C21:0)	2.1349	2.2292	0.7202	2.4503
	1.4258	2.2592	0.7577	2.0665
Behenic acid (C22:0)	0.2004	0.7242	2.4962	1.6847
	0.2731	0.7359	2.9846	2.05
	0.1277	0.7477	2.0078	1.3194
Tricosanoic acid (C23:0)	0.0565	0.1135	1.1665	0.2752
	0.0714	0.1908	0.9706	0.1614
	0.0417	0.1727	0.9244	0.2437
Lignoceric acid (C24:0)	0.0931	0.1461	1.5429	0.5048
	0.1015	0.2641	1.3865	0.4643
	0.0847	0.2415	1.3461	0.4238
ΣSFA	9.4086	11.1234	18.1086	15.8108
	8.749	9.8149	18.2884	14.0357
	7.4337	10.9637	15.5917	10.8719
Monounsaturated fatty acids (MUFAs)				
Palmitoleic acid (C16:1)	0.213	0.3195	0.1957	0.0278
	0.5033	0.4741	0.1886	0.0217
	0.3581	0.3968	0.188	0.0333
Oleic acid(C18:1n-9)	0.9968	0.431	0.0716	3.3413
	1.066	0.5008	0.0796	3.2278
	0.6814	0.3612	0.0877	3.4549
Eicosenoic acid (C20:1n-9)	0.3942	0.2258	0.8674	2.7817
	0.4142	0.3278	1.3709	3.2705
	0.3741	0.364	0.9094	2.2928
Erucic acid (C22:1n-9)	0.8776	0.4135	0.6097	0.8793
	0.742	0.3974	1.0211	0.7316
	0.5336	0.3792	0.9781	1.027
Nervonic acid (C24:1n-9)	0.3001	0.0234	2.4336	1.1476
	0.4021	0.0161	2.9614	0.7669
	0.398	0.0307	2.6163	0.9861
ΣMUFA	2.7817	1.4132	4.178	8.1777
	3.1276	1.7162	5.6216	8.0185

	2.3452	1.5319	4.7795	7.7941
Polyunsaturated fatty acids (PUFAs)				
Linoleic acid (C18:2n-6)	19.6244	9.9367	4.4436	9.183
	17.2466	10.3606	5.585	8.2903
	15.6222	11.5128	5.0143	7.3977
α-Linolenic acid (C18:3n-3)	0.0699	1.0689	1.4874	0.6556
	0.068	1.1137	1.4975	0.6195
	0.0719	1.0912	1.4772	0.6918
Arachidonic acid (C20:4n-6)	0.0204	0.**2**641	0.0706	0.0070
	0.0315	0.2745	0.0713	0.0010
	0.032	0.295	0.07	0.0080
Eicosapentaenoic acid (C20:5n-3)	0.0311	0.1998	0.1063	0.2308
	0.038	0.2327	0.0953	0.2328
	0.0374	0.2099	0.0924	0.2225
Eicosatrienoic acid (C20:3n-3)	0.712	0.3064	0.1402	0.1743
	1.0468	0.3042	0.1539	0.1766
	1.0226	0.3739	0.1548	0.2061
Docosahexaenoic acid (C22:6n-3)	0.0**5**64	0.0952	0.4207	0.0664
	0.0635	0.1108	0.4464	0.0714
	0.0707	0.103	0.395	0.0539
Docosapentaenoic acid (C22:5n-3)	0.4143	0.699	1.4919	2.3741
	0.5362	0.6329	1.3986	3.586
	0.6581	0.5222	1.189	2.1622
ΣPUFA	20.9285	12.5701	8.1607	12.6912
	19.0306	13.0294	9.248	12.9776
	17.5149	14.108	8.3927	10.7422

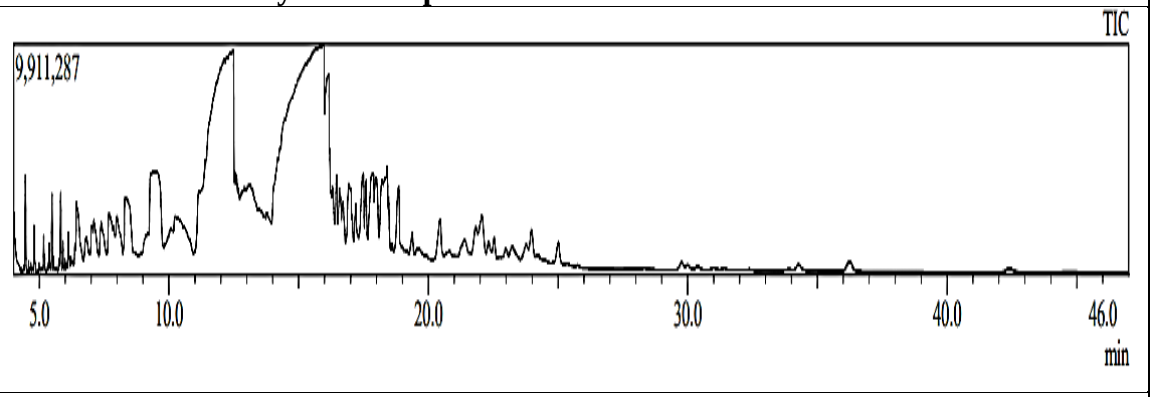
d) Nutritional quality indices (NQI) in extracted oils

NQI	Extraction methods			
	SE	**WR**	**AS**	**MAE**
PUFA/SFA	2.2244	1.1301	0.4507	0.8027
	2.1752	1.3275	0.5057	0.9246
	2.3561	1.2868	0.5383	0.9881
EPA+DHA	0.0875	0.295	0.527	0.2972
	0.1015	0.3435	0.5417	0.3042
	0.1081	0.3129	0.4874	0.2764
AI	0.7481	1.3801	1.3488	0.8038
	0.8775	1.0433	1.2725	0.6352
	0.7955	1.4017	1.0482	0.5649
TI	0.4323	0.6375	0.9964	0.2540
	0.4836	0.4691	0.8738	0.7244
	0.4611	0.5731	0.8061	0.5558
HH	3.9045	1.8459	0.7627	1.5584
	3.6543	2.2181	0.8572	1.8757
	3.5496	2.0793	0.9136	2.1616
HPI	1.3368	0.7246	0.7414	1.2440
	1.1619	0.9585	0.7859	1.5744
	1.2575	0.7134	0.9540	2.0612
n-3/n-6	0.0080	0.1337	0.4462	0.4462
	0.0098	0.1370	0.3605	0.3605
	0.0115	0.1189	0.3864	0.3864
LA/ALA	280.7496	9.2962	2.9875	14.0070
	253.6265	9.3029	3.7295	13.3822
	217.2768	10.5506	3.3945	10.6934
FLQ	0.2642	1.1749	1.7309	1.0524
	0.3284	1.3986	1.6337	0.8684
	0.3961	1.1762	1.6945	0.9399

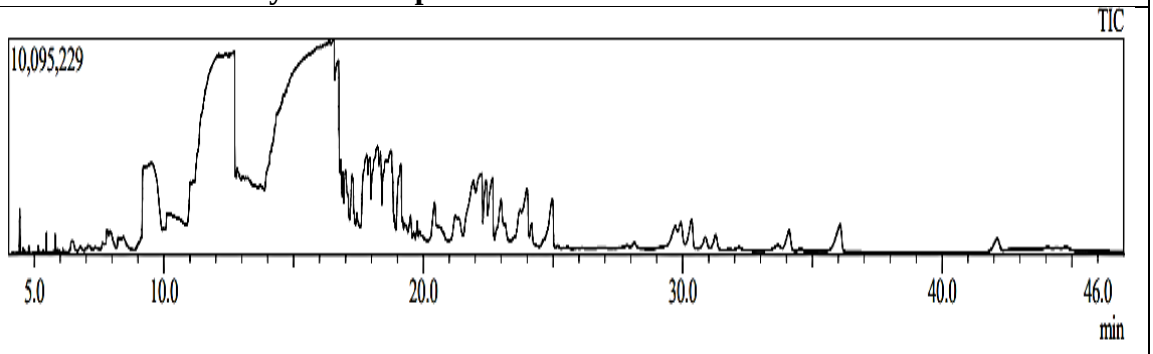
Appendix E: GC-MS Chromatograms



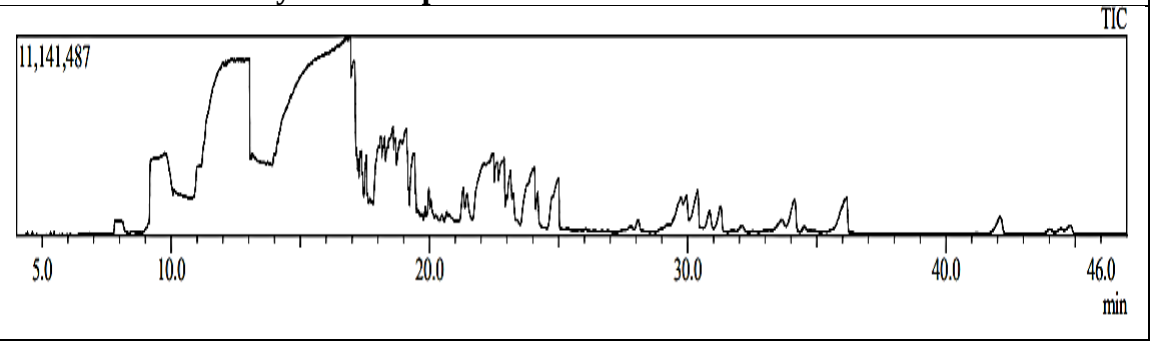
Fatty acid composition of fish oils from SE method



Fatty acid composition of fish oils from WR method



Fatty acid composition of fish oils from AS method



Fatty acid composition of fish oils from MAE method

Brief biography

Nahidur Rahman has completed his B.Sc. (Hon's) in Food Science and Technology from Faculty of Food Science and Technology, Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, Bangladesh obtaining CGPA 3.93 in the scale of 4.00. He has been nominated for the prestigious Prime Minister Gold Medal-2019 and also received the prestigious Dean's award based on academic excellences (holding 1st position) in undergraduate studies. For the time being, he admitted himself as an aspirant for the degree of MS in Food Processing and Engineering in Department of Food Processing and Engineering, Chattogram Veterinary and Animal Sciences University (CVASU), Bangladesh. He formerly did two academic internships at Universiti Malaysia Terengganu (UMT), Malaysia and Institute of Nutrigenetics (ION), India. He also received Elementary Food Safety Trainings from Food Tech Club, West Bengal. Besides, his ties and trainings from different food industries and Training Institute for Chemical Industries (TICI) have made him confident enough to tackle challenges in the field of food processing, quality control and engineering. He is passionate about teaching and research. His research interests are mainly focused on food safety and public health, probiotics and functional food, nutritional genomics and gene-diet interaction. He was a Research Fellow at the Department of Food Processing and Engineering, Chattogram Veterinary and Animal Sciences University (CVASU), and the Brac James P Grant School of Public Health at Brac University. He has five peer-reviewed publications so far. His areas of expertise are phytochemical screening and extraction, instrumental and statistical analysis (UV-Vis, AAS, GC-MS), epigenetics of diseases, interpreting genetic report and dietary recommendations. He possesses deep interest and pleasure in imparting knowledge, academic research and would welcome any further opportunities to contribute.