ANALYSIS OF FATTY ACIDS COMPOSITION IN MARINE FISH (*genus Tenualosa*)

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ANALYSIS OF FATTY ACIDS COMPOSITION IN MARINE FISH (*genus Tenualosa*)

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A final year project report submitted in fulfillment of the requirement for the award of the degree of Bachelor in Resources Chemistry

(Resources Chemistry)

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"I hereby declare that this final year project report, submitted to Universiti Malaysia Sarawak as a partial fulfillment of the requirement for the degree of Bachelor of Resources Chemistry. I also certify that the work described here is entirely my own except for excerpts and summaries whose resources are appropriate cited in the references."

Signature : ……

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Date : 16 MAY 2013

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LIST OF ABBREVIATION

PUFA	Polyunsaturated fatty acids
MUFA	Monounsaturated fatty acid
n-6	Omega-6
n-3	Omega-3
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
ALA	A-linolenic acid
AA	Arachidonic acid
LA	Linoleic acid
FAME	Fatty acid methyl ester
GC-MS	Gas chromatography-mass spectrometer
GC	Gas chromatography

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ANALYSIS OF FATTY ACIDS COMPOSITION IN MARINE FISH (*genus Tenualosa*)

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ABSTRACT

The polyunsaturated fatty acids (PUFAs) were known for its function that can enhance human health. It is well known for its function that can reduce the cardiovascular diseases, improve the fetal development and much more. Therefore many researches had been done towards the PUFAs. Marine fish were known for its Omega 3 composition. Besides Omega 3, Omega 6 also one types of PUFAs that will give benefits towards the human health. DHA and EPA are the PUFAs that fall into the group of Omega 3. The extraction of the fish oil had been analysed using the Bligh and Dyers method. The composition of the extracted oil had been analysed using the Gas chromatography-Mass spectrometry. Therefore, the qualitative analysis of the composition of PUFA in the Tenualosa toli *(Terubok)* had been done. The Tenualosa toil (*Terubok)* obtained from the fisherman with enough information of the place of captured.

Keywords: PUFA, DHA, EPA, Bligh and Dyers, Omega 3, Omega 6, Gas chromatography and Tenulosa toil (terubok).

SINOPSIS KAJIAN

Rangkaian asid tidak tepu (PUFAs) terkenal dengan kelebihannya yang dapat meningkatkan kualiti kesihatan manusia. Asid ini begitu dikenali dengan kelebihannya yang dapat mangurangkan penyakit-penyakit jantung, meningkatkan perkembangan fetus dan lain-lain lagi. Oleh itu, pelbagai kajian telah dilakukan terhadap asid-asid ini. Ikan air masin sangat sinonim dengan minyak Omega 3. Selain Omega 3, Omega 6 juga merupakan salah satu jenis rangkaian asid tidak tepu yang dapat memberi manfaat terhadap kesihatan manusia. DHA dan EPA merupakan rangkaian asid tidak tepu yang digolongkan dalam kelompok Omega 3. Perahan pati minyak ikan air masin telah diperolehi menggunakan cara Bleigh and Dyers. Komposisi-komposisi pati minyak ikan ini telah dianalisis menggunakan kromatografi gas bagi mengenalpasti sama ada bahan-bahan yang terdapat di dalamnya merupakan DHA atau EPA. Oleh itu, kalitatif analisis terhadap komposisi rangkaian asid tidak tepu di dalam Tenualosa toli (Terubok) telah dijalankan. Tenualosa toli (Terubok) telah diperolehi daripada nelayan-nelayan yang telah sedia maklum mengenai lokasi penangkapan ikan.

Kata kunci: rangkaian asid tidak tepu, DHA, EPA, Bligh and Dyers, Omega 3, Omega 4, kromatografi gas dan Tenualosa toil (terubok).

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Fish are one of the healthy dietary alternative that can replace the meat since it's contain high protein content and low saturated fats (Domingo *et al*., 2007). Studied by Menzel and Olcott (1964) on fish liver oil found that 91% - 99% of the fatty acids in the α-position as unsaturated, while 36% - 86% of the fatty acids are in the β-position as saturated. Polyunsaturated fatty acids (PUFAs) that are present in the fish are essential for the human health with regular consumption especially the Omega-3 PUFA (Sidhu, 2003). Several studies have proven that PUFAs especially the n-3 (Omega-3) and the n-6 PUFA have a greater effect or beneficial in health such as cardiovascular diseases, neurodevelopment in infants, cancers and fat glycemic control (Kinsella *et al*., 1990). Fetal development can also be aided by the consumption of omega-3 fatty acids by pregnant women, as infant cognition was shown to increase proportionally with fish consumption by the mother (Oken *et al*., 2005).

PUFAs that mainly found in the fish are n-3 (Omega-3) and n-6. Both the neutral lipids and the polar lipids of marine fish are rich in (n-3) polyunsaturated fatty acids (PUFA), with a ratio of (n-3)/(n-6) PUFA of l0-15 : 1 as studied by Ackman (1980). Nutritional studies with several species of marine have firmly established that long-chain (n-3) PUFA are essential dietary factors (Watanabe, 1982) but the essentiality of (n-6) PUFA is less clearly defined. Omega-3 (n-3) PUFA is the most common studied polyunsaturated fatty acids. Eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) are two types of Omega-3. Both of the Omega-3 will have their own benefit concerning the human health as mention above. EPA (20:5n3) can help in preventing arteriosclerosis and thrombosis including affecting the circulatory system. Meanwhile, DHA (22:6n3) is important for brain development since the normal adult will have more than 20g of DHA.

It is said that the composition of the fatty acids in fish are differ between species, place of origin and even the season of capturing. The content of marine n-3 PUFA varies between fish species being high in fatty fish like mackerel and salmon and low in lean fish such as flounder and cod. The content of n-3 PUFA in seafood varies considerably in relation to location and the season of capture **(**Erik *et al*., 2004). The composition of fatty

acids in marine and freshwater fish is different. The freshwater has lower level of Omega-3 compare to marine fish (Jankowska *et al*., 2003; Steffens, 1997; Vujkovic et al.,1999).

1.2 Statement of Problems

The PUFA can be classified into different types. This variation of the PUFA are differ depending on the types of fish that been studied. The habitat, diet and also the types of fish will determine the amount of PUFA present in the fish. This study will focus only on the marine fish that scientifically proven that contain huge amount of PUFA.

1.3 Objectives of the Project

The objectives of the study are:

a) to extract the PUFA from any selected marine fish using Bligh & Dyer method,
b) to derivative the fatty acids for identification in GC, and
c) to run the qualitative analysis towards the sample.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Fatty Acid and Polyunsaturated Fatty Acids in Marine Fish

Fatty acid is part of the compound that makes up a lipid together with other compound such as spingolipids, phospholipids, waxes and sterols. Fatty acid can be classified into several groups there are:

1. Saturated fatty acid

Straight chain fatty acid where the double bond is absent. Palmitic acid (16:0) is the saturated fatty acid that widely occurring and it present in fish oil with 10-30% of occurrences. Meanwhile up to 30% occurrences in milk, body fats of terrestrial animal and finally the most dominant in occurrences with 5-50% in vegetable fats. Besides that, stearic acid (18:0) is included in saturated fatty acid. It mostly found in tallows of ruminant animal with 5-40% of occurrences and also in tallows of some vegetables with 30-35% of occurrences like cocoa butter or shea butter (Gunstone , 1999).

2. Monounsaturated fatty acid

Fatty acids with one double bond located in its backbone. It is usually an olefinic compound with *cis (Z)* configuration but it also can be found in *trans (E)* configuration. The most commonly found monounsaturated fatty acid is oleic acid (18:1 *n* 9). The other example of monounsaturated fatty acid are Vaccenic acid (18:1 *n* 7) and also Myristoleic acid (14:1 *n* 5).

3. Polyunsaturated fatty acid (PUFAs)

Fatty acid with two or more double bond present in its double bond. It can occur in two types of configuration either in *cis (Z)* or *trans (E).* Omega-3 and Omega-6 are included in polyunsaturated fatty acid where the first double bond is located at carbon no 3 for Omega-3 and 6 for Omega-6 from the methyl end. Each of the double bond is conjugated with one CH_3 in between them. These fatty acid can be obtain from plants and also animal. Omega-3 is mainly found in animal meanwhile

Omega-6 is mainly found in plants. The examples of Omega-3 are Linolenic acid (18:3ω3), Eicosapentaenoic acid (20:5ω3) and also Docosahexaenoic acid (22:6ω3). The example of Omega-6 are Linoleic acid (18:2ω6), γ-Linolenic acid (18:3ω6) and Arachidonic acid (20:4ω6). PUFAs are essential in our daily life since the component or part of these fatty acids is needed in our daily life. Moreover, these fatty acids are needed to ensure a healthier life (Ruiz-Rodriguez *et al*., 2010).

2.2 Structure and Subdivision of Omega-3 and omega-6

Omega-3 PUFA is an essential fatty acid that has their first double bond on the carbon number three. The numbering method is started from the methyl end of the carbon chain that becomes the constituent of the backbone of the fatty acid as shown in **Figure 1**. Omega-3 can be categorized into two based on its origin whether it is derive from plant or seafood. Omega 3 that derived from plant is α-linolenic acid. Examples are canola oil, rapeseed oil, and linseed oil) and is composed of 18 carbon atoms with three double bonds (nomenclature; 18:3). Meanwhile, the omega 3 that derived from the seafood are eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) and these fatty acids is the major fatty acids in marine life. In humans, α-linolenic acid can elongate and desaturated to a limited extent and form EPA and DHA. Polyunsaturated fatty acids are obtains by the fish by consuming the algae in the water. Algae are the main source of Omega-3 since its pack with EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) (Schmidta *et al*., 2004**).**

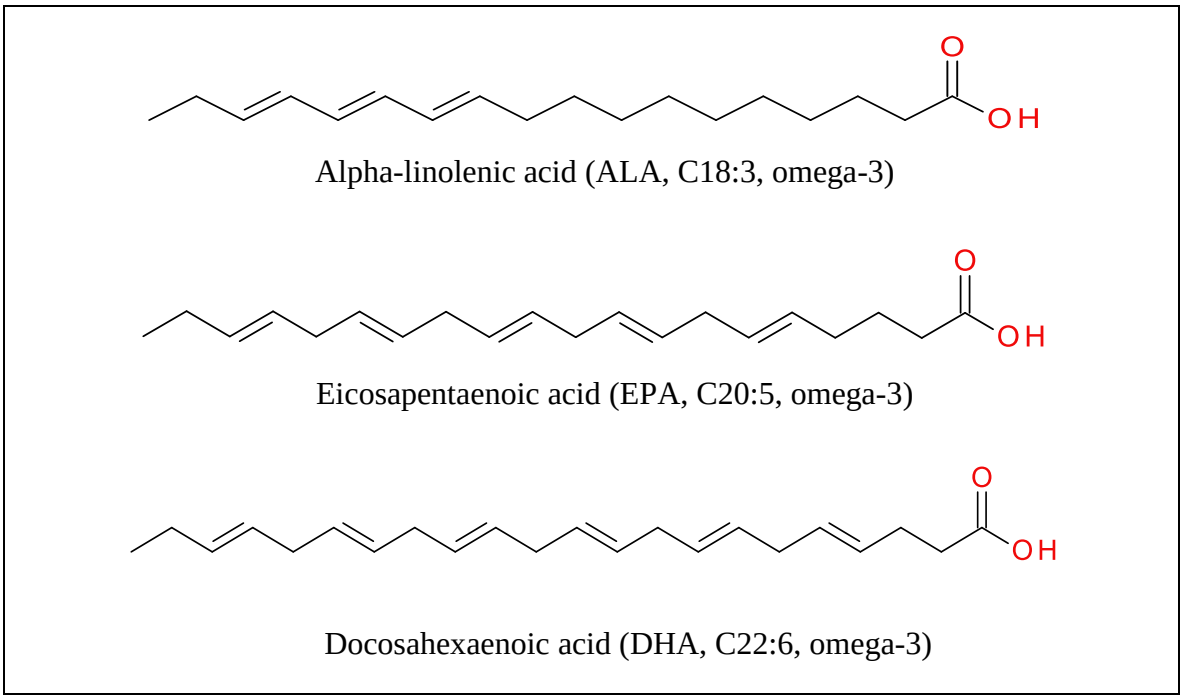

Figure 1 : Omega 3 Polyunsaturated Fatty Acid

In contrast, Omega 6 can be subdivided into two types that are linoleic acid (LA) and arachidonic acid (AA). Omega 6 is fatty acids where the first double bond is on the carbon number 6 from the methyl end as shown in **Figure 2**. These fatty acids will not be further explained due to the fact that these fatty acids are normally found in the plant.

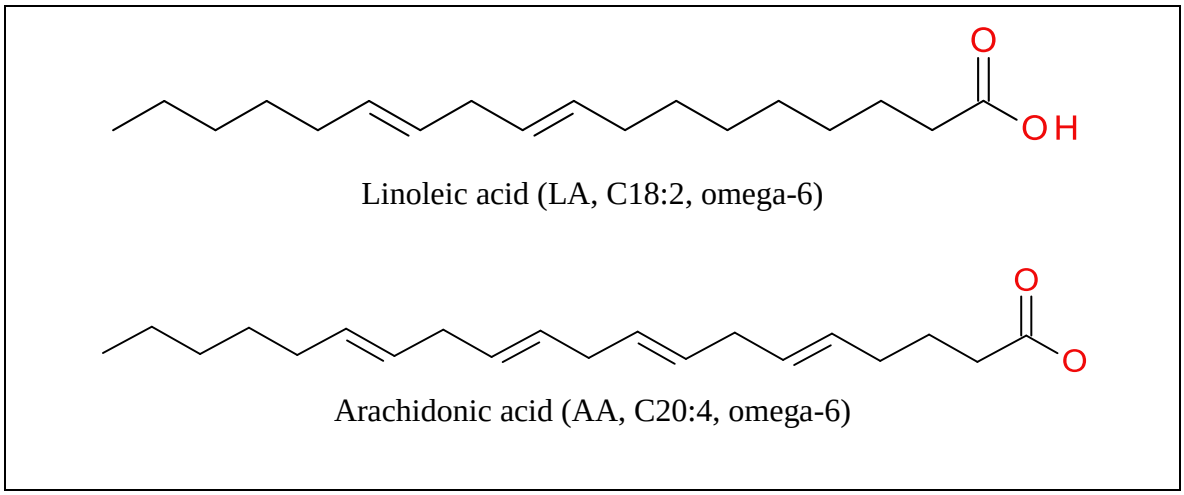

Figure 2 : Omega 6 Polyunsaturated Fatty Acid

Basically, the analysis of polyunsaturated fatty acids in marine fish, the main focus for the extraction is the Omega 3 PUFA. This is due to the fact that this type of PUFA will usually found in the marine fish extraction.

2.3 Correlation between Marine Fish and Polyunsaturated Fatty Acids.

The compositions of fatty acids are varies depending on the species of the fish, the habitat and the season. Diet, location and season are the major factors effecting the fatty acids composition (Gruger, 1967). The ratio of total ω-3 to ω-6 fatty acids is much higher for marine fish rather than freshwater fish, varying from 5 to 10 or more. Even though the marine fish contain much more fatty acids composition than fresh water, but the freshwater fish can synthesize the C_{18} EFA, 18:3n-3 and 18:2n-6 into a more longer, unsaturated and physiology important eicosapentaenoic acid (20:5n-3 ; EPA), docosahexaenoic acid (22:6n-3;DHA) and arachidonic acid (20:4n-6;AA) by the fatty acid desaturase and elongase enzymes and so only require the C_{18} PUFA (Henderson and Tocher, 1987; Sangent *et al*., 1989,1995). On the other hand, marine fish cannot do the conversion process. A hypothesis stated that marine fish that consumed a carnivorous diet that naturally rich in HUFA, result in an evolutionary down-regulation of the desaturase and or elongase enzymes activity required for the conversion of the C_{18} PUFA to HUFA (Sergent *et al*., 1995). Fatty acid such as EPA and DHA that been found in marine fish are originally obtain from the phytoplankton and also seaweed that include in their food chain (Cavington,2004).Thus, the marine fish does not require the conversion process the same as the freshwater fish done, instead they just obtain the fatty acids from their diet.

2.4 The *genus Tenualosa.*

Tenualosa comes from the Clupeidae family that includes herrings, shads, and sardines. It inhabits the fast-flowing, turbid estuary and adjacent shallow coastal waters of Sarawak. It is an anothropes species where they spawning in fresh water and growing in salt water. **Figure 3** will act as a guide to show the pathway for their migration that will be stated below. *T. toli* is protandrous hermaphrodite where this species can change their sex at certain point of their life span. The male in this *genus* will spawn near the end of their first year and change sex and spawn as female at the second year. Spawning process occurs in the middle rich of estuaries and the female will spawn their egg at once. **Figure 4** shows *T. toli* life cycle (Babler, 1997).

Figure 3: Map to show migration pathway

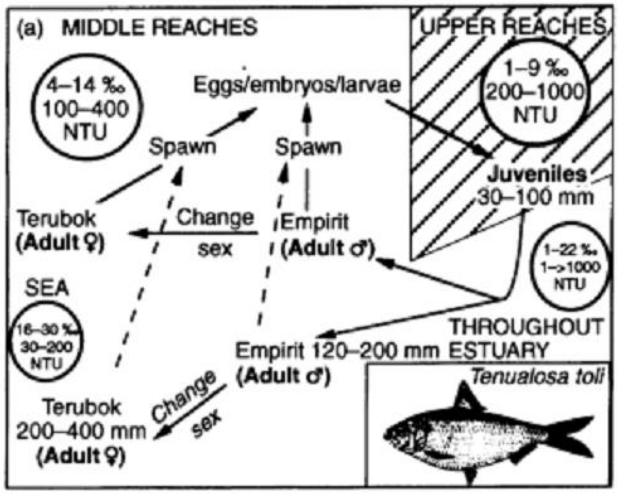

Figure 4: Life cycle of *Tenualosa toli*

Variation in physical appearance will distinguish between the *Tenualosa genuses* and have visible variation between them. *T. thiabaudeaui* is an endemic species to the Mekong, in Vietnam. This species migrates upstream to Thailand and Loas around Chinese New Year time (Robert, 1993). **Figure 4** shows physical appearance of *T. thiabaudeaui.* The upper jaw symphysis with notch for lower jaw occlusion [1] and mouth terminal or pointing obliquely upwards [2]. Besides that, *T. thiabaudeaui* has dark spots on top of its body along the flank. It can grow up to 30 cm.

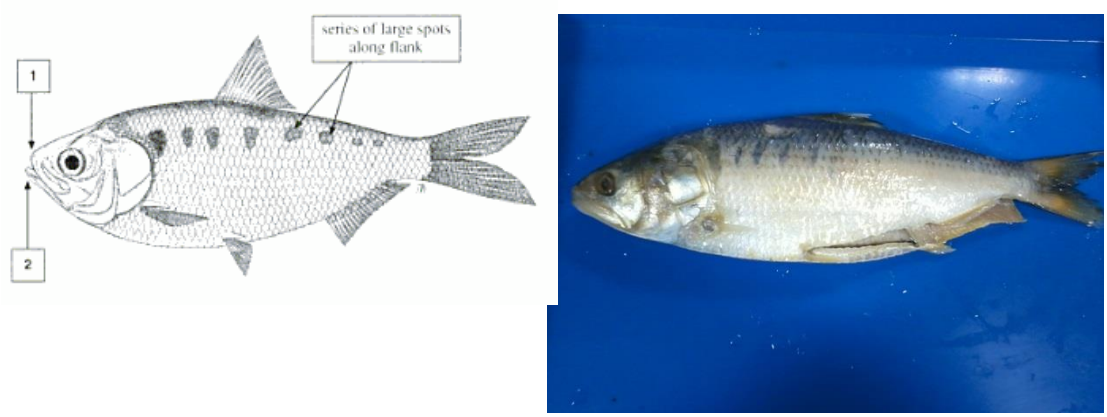

Figure 5: *Tenualosa thiabaudeaui*

T. toli can be obtained from Indonesia to India. It also can be found in Cambodian Mekong near Vietnam border. It can grow up to 60 cm. Compared to the *T. Thiabaudeaui, T. toli* do not possess the dark spot along its flanks instead it has the dark spot behind the operculum. **Figure 5** shows the physical appearance of *T. toli.*

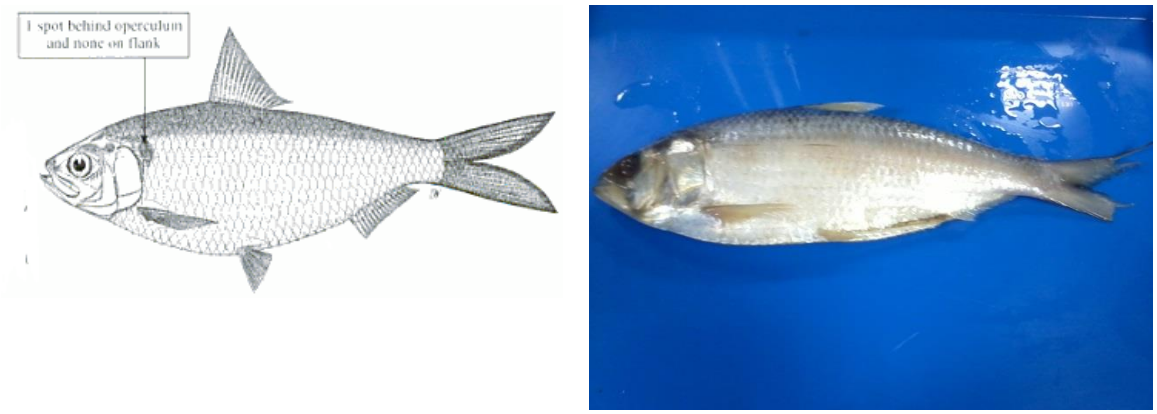

Figure 6: *Tenualosa toli*

CHAPTER 3
MATERIALS AND METHODS

3.1 Sample Collection

The sample that been used this research is the *genus Tenualosa.* All of this fish from this *genus* are obtain from the market near Samarahan. The physical appearance of the fish including the information from the fisherman helps to distinguish the species types. There are three types of fish from this *genus* are used. The species are *Tenualosa toli, Tenualosa thiabaudeaui* and finally terubok that cannot be identified the species but according to the fisherman the fish had been obtain from Burma. Figures below show the picture of the sample. The average size of the fish that been used is 32 cm.

Tenualosa toli (Sarawak)

T.spp (Burma)

Tenualosa thiabaudeaui (India)

3.2 Extraction of the PUFA

The PUFA had been extracted using the method developed by Bligh & Dyer (Bligh and Dyer,1959). Approximately, 30 g of the sample had been weighted and homogenized in 90 ml mixture of methanol: chloroform (3:1; v/v) for 2 minutes. 1 volume of chloroform (30ml) was added together with 30 ml of distilled water. Then homogenate had been stirred using the glass rod followed with filtering using the Advantec 5B 110mm filter paper on a Buchner funnel with slight suction. After that the filtrate was transferred into the separatory funnel. The lower clear phase was drained into 250 ml round-bottom flask and concentrated with a rotary evaporator at 40^{o}C. The concentrated oil from the round bottom flask has been diluted with dichloromethane using the sonicator. Then, the diluted oil sample had been transferred into a vial and nitrogen will be blown slightly before storage to avoid oxidation towards the samples. Later, the oil sample will be derivatize for GC-MS analysis.

3.3 Derivatization of the PUFAs

Prior to gas chromatographic analysis, fatty acids had been derivatized into fatty acid methyl esters in order to improve volatility of the fatty acid compounds. Derivatization was carried out by adding 1 mL of hexane to about 200 µl of lipid extract followed by additional of 0.2 mL of 2M NaOH-methanol reagent. Then, the mixture solution was subjected to 10 seconds shaking by vortex before placing in water bath at 50°C for 20 seconds. After cooling for about 1 to 2 minutes, the mixture was subjected to 10 seconds shaking by vortex again. Finally, 0.2 mL of 2M HCl-methanol reagent was added. The mixture undergo vortex for 10 seconds and left to separate into layers. If the layers did not separate well, the mixture solution was subjected to centrifugation and the supernatant was collected for gas chromatography (GC) analysis. The supernatant known as FAME was diluted before sent for analysis. The diluted FAME not be stored instead they was sent directly for GC-MS analysis in order to avoid unwanted reaction to the FAME.

3.4 Gas Chromatography Analysis

Analysis of fatty acid methyl esters will be performed on Perkin Elmer-GCMS gas chromatographic technique equipped with column BPX-5 (0.25µm thickness × 0.25mm diameter × 29.4m length) in a splitless mode. The injector temperature was 260°C while the column temperature was programmed at an initial temperature 90° C to a final temperature of 300°C. Interface temperature has been set at 320°C. The initial time is 5.00 minutes while final time is 10.00 minutes. Helium has been used as carrier gas.

3.5 Semi-quantitative analysis

All the data that been obtained in this experiment will be calculated based on semi-quantitative calculation. The percentage of composition of the fatty acids that been identified will be calculated using its area. The calculation is as follow:

$$Composition\ \% = \frac{area\ of\ individual\ fatty\ acids}{\sum all\ area\ of\ fatty\ acids}$$

As for the percentage yield of the isolated oil or lipids, the weight of extracted oil and the weight of isolated oil are used.

$$Isolated\ oil\ \% = \frac{weight\ of\ isolated\ oil(g)}{weight\ of\ sample(g)}$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 Oil yield.

The extraction of the sample *T.toli, T.spp* and *T. thiabaudeaui* shows different amount of extracted oil and also the concentrated oil obtain. The extraction had been replicated for 3 times for each sample. The weight of concentrated oil and also the extracted oil is tabulated in the Table 4.1 below. The % yield of the lipid had been calculated and tabulated in Table 4.1. As shown in table 4.1, the greatest amount among the entire sample is *T.toli* and *T.thiabaudeaui* with 6% followed *T.spp* with 3%. Based on this data, *T.toli* and *T.thiabaudeaui* has the greatest amount of isolated oil. the isolated oil was derivatized and analysis in GC-MS to identify the compound that are present.

Table 4.1: Amount of concentrated oil and extracted oil for each sample

sample	*T.toli*			*T.spp*			*T.thiabaudeaui*		
isolated oil	1.15	1.65	1.29	1.25	1.71	1.18	1.58	1.95	1.63
% yeild	6	12	1	3	4	3	6	4	4
average % yield	0.06 ± 0.04			0.03 ± 0.02			0.06 ± 0.05		

4.2 Fatty Acid Composition in Oils.

Figure shows the GC-MS result that showing the peak of each compound that been detected in the sample.

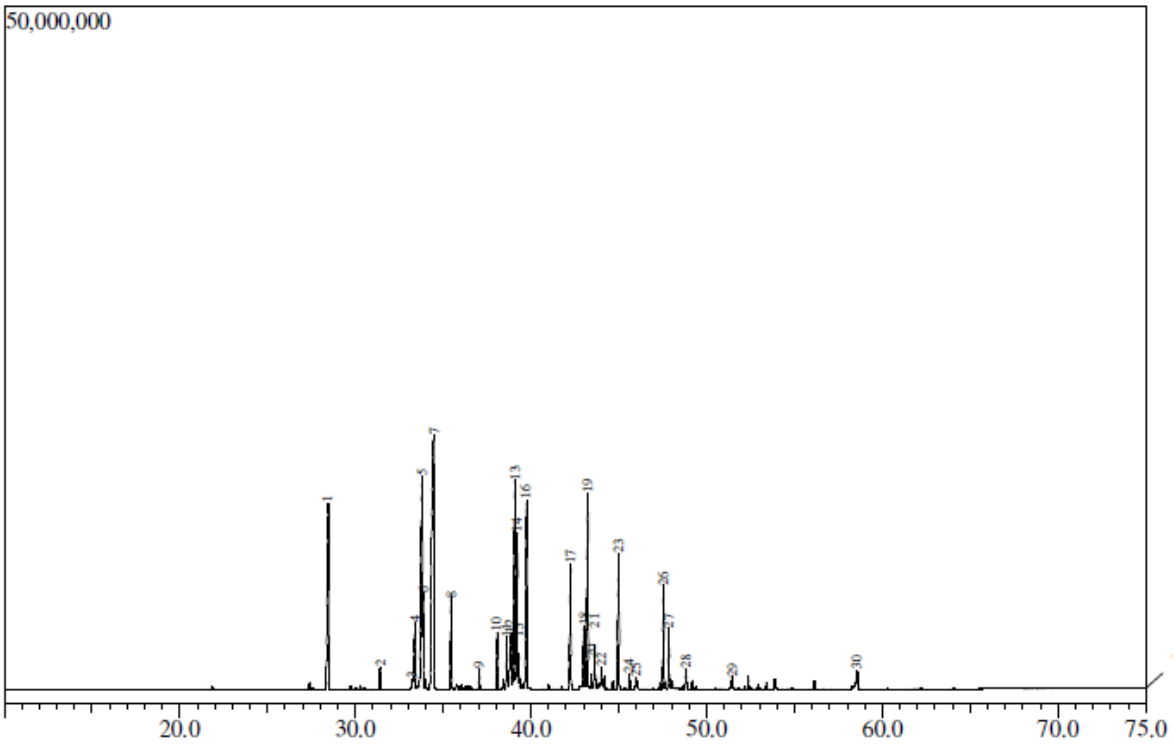

Figure 7: GC-MS result of *T.toli.*

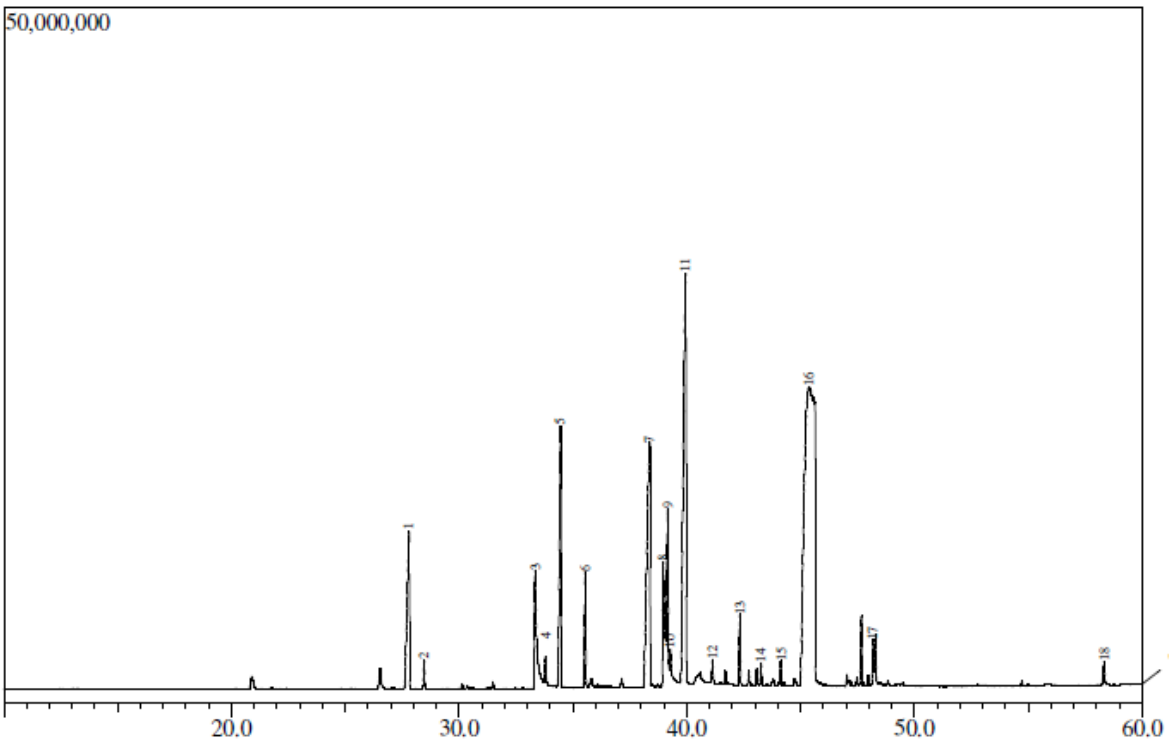

Figure 8: GC-MS result of *T.spp.*

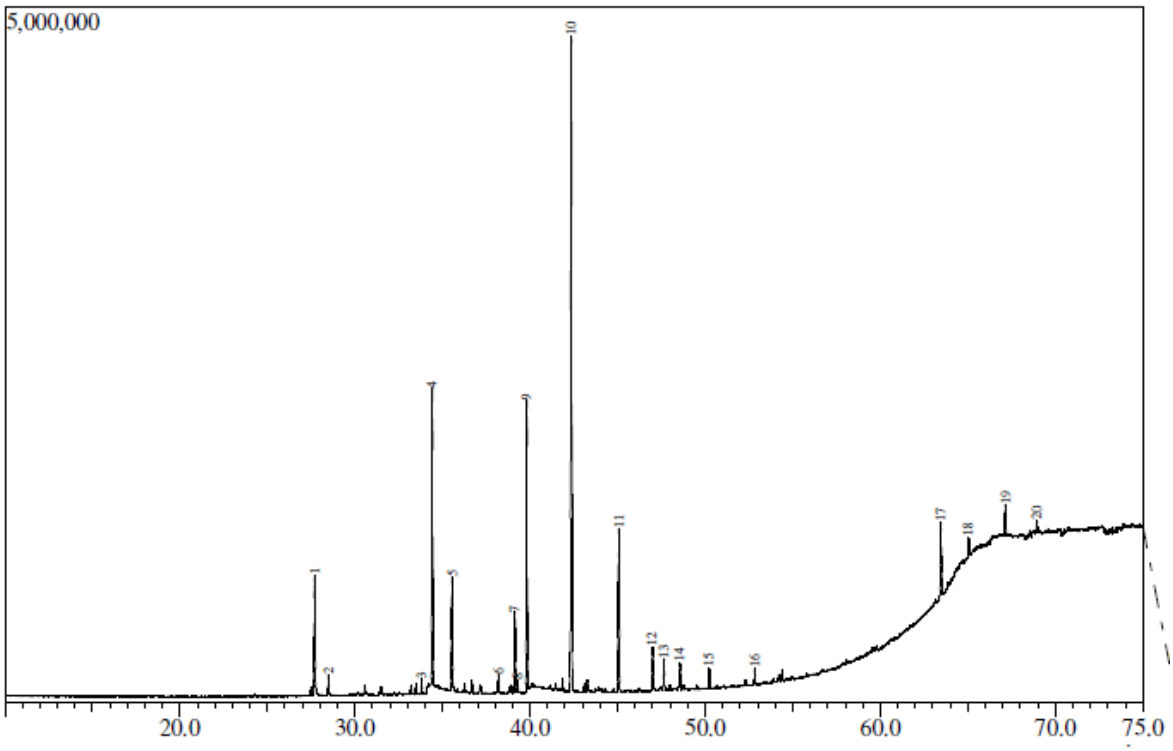

Figure 9: GC-MS result of *T.thiabaudeaui*

Based on Figure 7, Figure 8 and Figure 9, the chromatogram shows that there are 30, 18 and 20 peaks respectively that are detected in each sample but only several peaks had been chosen and included in the sample due to the fact that the other peak is not fatty acids. The entire peaks that are present are identified and determine for compound that its represents based on its retention time.

Based on the peak obtain from the chromatogram, the fatty acids composition in each of the sample had been identified. The fatty acids identified had been classified according to their properties either saturated fatty acids, monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs). Table 4.2 shows the list of fatty acids that been identified in each sample based on the GC-MS result. The extracted oil had been undergoing derivatization as a requirement for GC-MS analysis. Due to derivatization, the fatty acids that been found in each of the sample will be in the form of fatty acids methyl ester (FAME).

Table 4.2: Fatty acids composition in all sample

	R.time	Compound Name	Composition %				
			T.toli	*T.spp*	*T.thia baude aui*	(Airina , 2011)	(Asyikin , 2006)
Saturated Fatty Acids							
C14:0	28.49	tetradecanoic acid methyl ester	9.28	2.04	5.71	nd	9.22
C15:0	31.42	pentadecanoic acid methyl ester	0.61	nd	1.05	nd	nd
C16:0	34.42	hexadecanoic acid methyl ester	31.15	36.97	32.55	21.22	31.46
C17:0	37.12	heptadecanoic acid methyl ester	0.56	nd	1.02	nd	nd
C18:0	39.82	octadecanoic acid methyl ester	9.06	36.89	18.62	5.51	6.54
Subtotal			50.67	75.9	58.95	26.73	47.22
Monounsaturated Fatty Acids (MUFAs)							
C16:1	33.80	9-hexadecenoic acid methyl ester	14.76	nd	nd	12.57	10.28
C16:1c	33.81	cis-9-hexadecenoic acid methyl ester	15.44	1.56	5.56	nd	nd
C18:1c	39.13	cis-9-octadecenoic acid methyl ester	10.94	10.79	12.34	nd	nd
C18:1t	39.28	trans-10-octadecanoic acid methyl ester	4.43	1.82	2.25	nd	nd
C18:1t	39.22	trans-11-octadecenoic acid methyl ester	12.08	17.7	9.3	nd	nd
C20:1c	44.01	cis-11-eicosenoic acid methyl ester	0.83	nd	nd	0.48	nd
Subtotal			58.48	31.86	29.45	13.05	10.28
Polyunsaturated Fatty Acids (PUFAs)							
C16:2t	33.80	trans-9,12-hexadecadienoic acid methyl ester	1.8	nd	nd	nd	nd
C18:2ω6	39.32	trans-9,12-octadecadienoic acid methyl ester	3.16	nd	nd	2.14	1.56
C20:3t	43.44	trans-7,10,13-eicosatrienoic acid methyl ester	0.52	nd	nd	nd	nd
C20:3ω6	58.56	cis-8,11,14-eicosatrienoic acid methyl ester	1.05	nd	nd	1.5	nd
C20:4ω3	45.60	cis-5,11,14,17-eicosatetraenoic acid methyl ester	2.66	nd	nd	0.51	nd
C20:4ω6	38.62	cis-5,8,11,14-eicosatetraenoic acid methyl ester	1.96	nd	2.12	0.16	1.30
C20:5ω3	45.68	cis-5,8,11,14,17-eicosapentaenoic acid methyl ester	9.67	1.44	7.08	9.93	8.63
C22:6ω3	47.65	cis-4,7,10,13,16,19-docosahexaenoic acid methyl ester	3.13	9.98	8.98	2.50	9.20
Subtotal			26.09	11.42	19.72	16.74	20.69