Regiospecific Analysis of Enzyme Interesterification of North

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Regiospecific Analysis of Enzyme Interesterification of North Sulawesi Skipjack (*Katsuwonus palamis*) Fish Oil with Lauric acid Using ^{1}H –NMR and ^{13}C - NMR

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Abstract : In the last decade quite a number of interest have been shown in a healthier food products and one of its efforts was to modify the fatty acids components into an unsaturated one,such product is known as Lipid Specific Structured (LSS). Synthesis of lipid structured using interesterification process will be beneficial to improve the functional properties and nutritive value of fat and oil as expected in some processed products. Enzymatic interesterification of North Sulawesi skipjack(*Katsuwonus palamis*) fish oil which extracted using wet rendering process and regiospecific analysis using ^{1}H-NMR and ^{13}C-NMR to identify fatty acid in Sn1, 2 and 3 had been studied. The analysis results showed that in position Sn-1 and Sn-3 were subtituted by lauric acid and in position Sn-2 was still occupied by the fatty acid of skipjack fish oil such as the one contain ω-3 – PUFA.
Keyword: Skipjack fish oil,Interesterification, Lauric acid, Regional specific, NMR.

Introduction

Internationally the concept of structured lipid have been developed aiming to nutritional and pharmacological applications, while the specific structured lipid by interesterification mainly aimed to its functional properties. Structured lipids are modified triacylglycerol by altering the fatty acids composition and/or their location in the glycerol backbone by chemical as well as enzymatic reactions[1,2,3]. Defined that structured lipids are triacylglycerol containing mixture of short and or medium chains of fatty acids and long chain fatty acids within same molecule of glycerol for its functional properties[2]. According some research about structured lipids can be produced by chemical or enzymatic reactions such as direct reaction between fatty acids and glycerol or by transfering acyl group between an acid and ester known as acidolysis as well as exchange of alkoxy group between an alcohol and an ester so called alcoholysis [3,4,5,6].

Some workers have successfully producing structured PUFA rich fish oil by incorporating caprylic acid via lipase acidylosis reaction [7,8,9]. This method had been successfully used for modification of plant oil fatty acid in producing structured lipid as reported [10,11,12].

Fish oil have been well known as polyunsaturated fatty acids (PUFA) sources especially in the form of docosahexaenoic acid (DHA;C22:6) and eicosapentaenoic acid (EPA;C20:5) and used as dietary supplement.Tuna fish oil also containing DHA 14.64% and EPA 3.64%, therefore this fish oil are reported as a good source of omega 3 fatty acids[13,14]. A regional specific to find out the distribution of fatty acid position in Sn1, 2 and 3 of fish oil had also been studied as reported some research[15-20]. Although an intensive studies had been made on enzymatic interesterification of fish oil, however there is limited information on the enzymatic interesterification of skipjack fish (*Katsuwonus palamis*) oil from North Sulawesi, hence the aim of this study

was to find out the distribution of fatty acid in Sn1,2 and 3 of North Sulawesi skipjack fish oil interesterified with lauric acid.

Materials and Methods.

Materials

Fish oil used as sample were obtained by extracting fish oil from skipjack tuna(*Katsuwonus palamis)* from Manado,North Sulawesi using wet rendering method as described [21], Specific 1,3 *Rhizomucor miehei* Lipase enzyme with optimum pH 8.0 and optimum temperature of 70°C (Novo Nordisk Denmark) and pure lauric acid ($CH_3(CH_2)_{10}COOH$) with molecular weight of 200.32(Sigma Aldrich)were bought from their local agents. All analytical grade of organic solvents (hexane,aceton, petroleum ether and formic acid) and chemical reagents (KOH, anhydrate Na_2SO_4) of Sigma Aldrich also bought from sigma local agent.

Methods

Interesterification of skipjack fish oil with lauric acid using microbial lipase enzyme were carried out using the method with slightly modification [22]. In the first experiment 1.74 g of skipjack fish oil mixed with 3.32 g lauric acid(fish oil molarity and lauric acid ratio was 1: 5) in erlenmeyer flask added with 0.50 g lipozyme (10% of substrate) and 8.1 ml hexane. This mixture was then incubated in shaking waterbath (120 rpm) for 24 hours at 50°C and then the mixture was filtered using Whatman filter paper No.2 to separate immobile lipase to stop the reaction. Furthermore 20 ml of mixture of ethanol and alcohol (1 : 1 v/v) were added to prevent emulsion formation during free fatty acids neutralization as well as inactivate the enzyme in case there were still leftover enzyme. The free fatty acids were neutralized by titration with 0.6M KOH and Phenolphtalein as indicator until pink colour of solution was observed.Asilglycerol were extracted from the mixture using 35 ml hexana,and after hexana added then the mixture were carefully transfered into separate flask. Two layers were formed namely water fraction and hexane fraction where water fraction were discarded and into hexane fraction were added with anhydrate natrium sulphate to remove the leftover water before evaporated using vacuum evaporator at 335 mmHg and 40°C to obtained the asilglycerol and asilglycerol fraction which were free from organic solvent stored in small bottle before regiospecific analysis was carried out by ^{1}H-NMR and ^{13}C-NMR.

Regiospecific Analysis

The regiospecific analysis of lauric acid in triglyceride of skipjack oil was carried out using NMR spectroscopy (JEOL, ECA 500, magnetic field 500MHz, spectrophotometer: DELTA2 NMR)) using chloroform D as solvent and type of analysis single pulse, Dim_title: 1H, relaxation delay: 5 seconds, replication time: 6.63577856 seconds at 24.6 °C.

Results and Discussion.

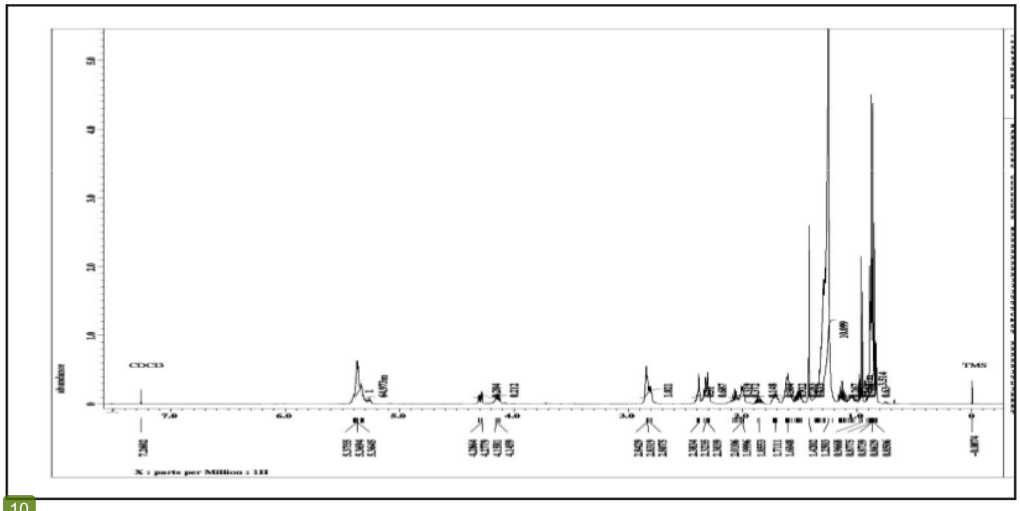


Figure 1a. ^{1}H -NMR spectra of skipjack fish oil.

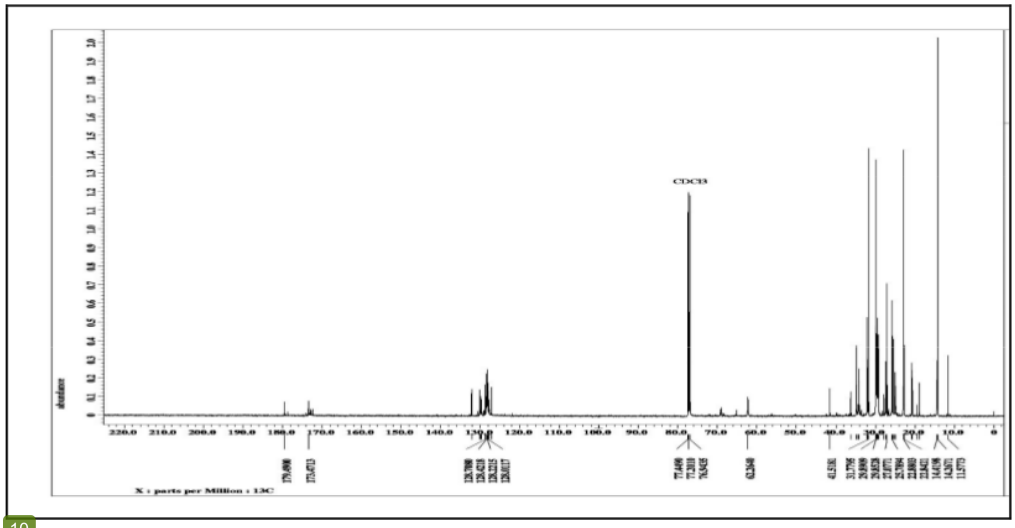


Figure 1b. ^{13}C - NMR spectra of skipjack fish oil.

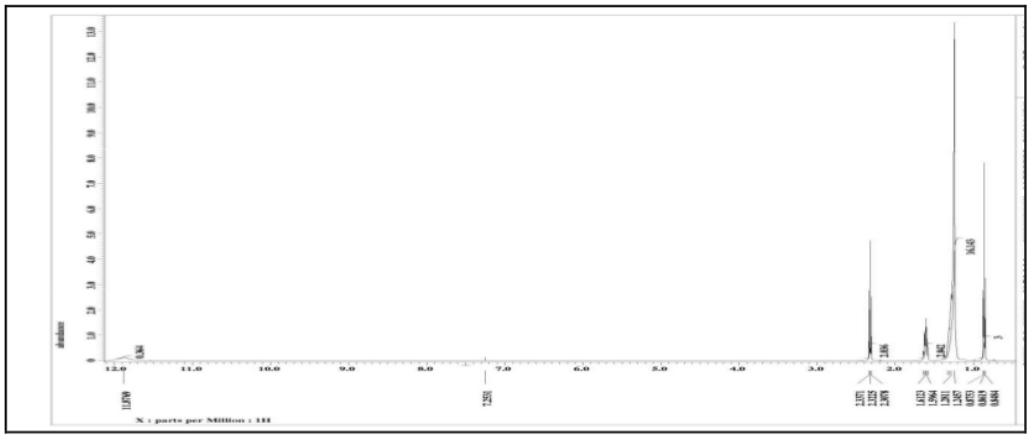


Figure 2a. ^{1}H – NMR spectra of lauric acid

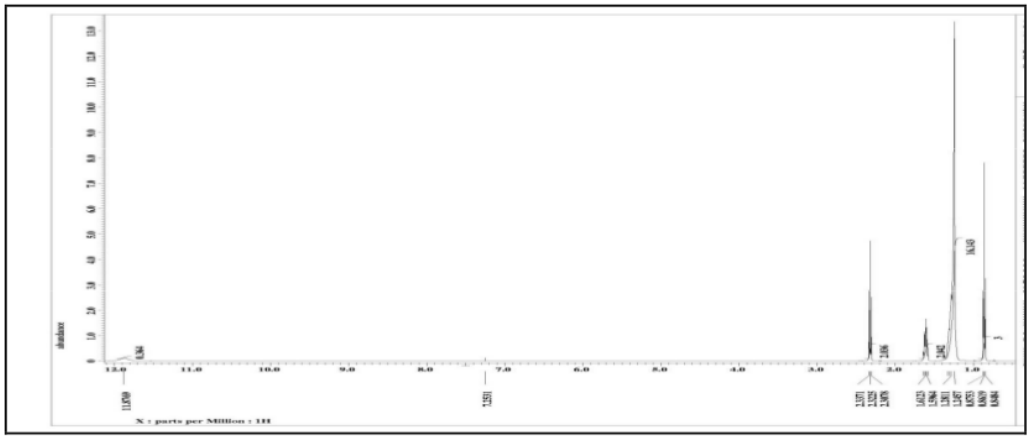


Figure 2b. ^{13}C - NMR spectra of lauric acid

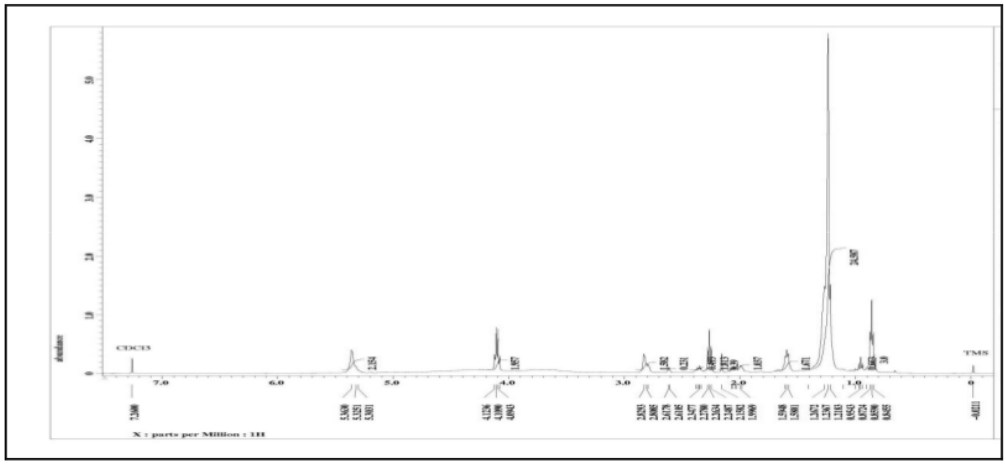


Figure 3a. ^{1}H - NMR spectra of reaction result of skipjack fish oil and lauric acid.

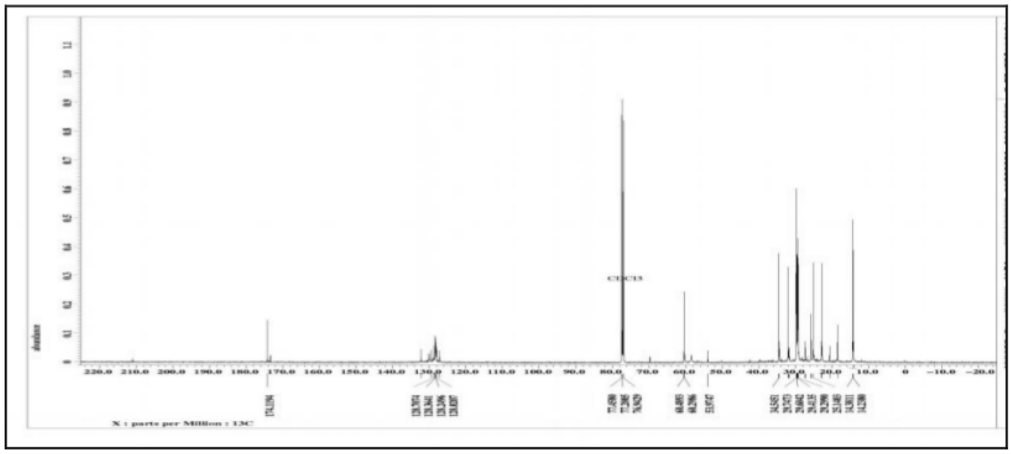


Figure 3b. ^{13}C - NMR spectra of reaction result between skipjack fish oil and lauric acid.

The oil sample used in regiospecific analysis by NMR spectrophotometry were restructured lipid obtained from the highest incorporation of skipjack (*Katsuwonus palamis*) fish oil with lauric acid at 50^{o}C for 24 hours and substrate ratio skipjack fish oil and lauric acid = 1:5 catalysed by lipase enzyme from *Rhizomucor miehei* (10% of substrate). The ^{1}H- NMR and ^{13}C - NMR spectra of skipjack fish oil, lauric acid and restructured lipid are presented in Figure 1a , 1b, 2a, 2b, 3a and 3b.

The NMR spectra in Figure 1 - 3 showed proton integration between skipjack fish oil, lauric acid and restructured lipid and Figure 1a indicated that skipjack fish oil consist of mixture of acids and ester compound. There were glycerol part which formed triglyseride which was observed at chemical shift between δ 4.0 – 5.5 ppm, and up field chemical shift was part of acid group. Double bond of PUFA group was observed also at around δ 5.5 ppm as in this area have more proton than at δ 4.2 ppm. Whilst in Figure 1b showed that skipjack fish oil were observed in the form of acid at chemical shift ± δ 173.47 ppm and this mixture consist of some acids, either saturated acids such as palmitic acid and polyunsaturated acid (PUFA, DHA). The other unsaturated or polyunsaturated fatty acids were observed from peak of unsaturated bond at ± δ 128 ppm, while glycerol group from triglyceride were observed at δ 60 -70 ppm.

Figure 2 a and 2 b showed that lauric acid used in this study were pure acid indicated by chemical shift δ 180 ppm from acid functional group and not found at δ 174 as ester group. The functional glyceride group at chemical shift of δ 4.0 – 5.5 ppm were also not detected, so with at δ 60 – 70 ppm, hence it can be concluded that lauric acid used is a pure lauric acid and in Figure 2b chemical shift at δ 14.24 ppm of ^{13}C – NMR is specific for lauric acid.

It is interesting to note that at δ 180 ppm (acid group) was not found in ^{13}C – NMR spectra of restructured lipid prepared using skipjack fish oil and lauric acid (Figure 3b) and only ester group observed at δ 174.11 ppm; this condition indicated that all reaction results between skipjack fish oil and lauric acid were in ester form. In this interesterification reaction there was a substitution of acids group of triglyceride with lauric acid at position Sn-1 and Sn- 3. The stronger peak chemical shifts of lauric acid which almost double higher compared to non lauric acid substitution in position Sn-2. Symetrical form of molecules occured in subtituted position Sn-1 and Sn-3 and resonance peaks of lauric acid at position Sn-1 and Sn-3 gave a similar chemical shift. If substution occured in position Sn-1 and Sn-2, there are no symetrical molecules formed and lauric acid chemical shift will be different with chemical shift at position Sn-2. The chemical shifts at ± δ 14,24; 24.84; 29.25; 29.45; 29.54; 29.64; 32.11 and 34.32 ppm have intensity two times bigger than the others indicated interesterification process occured in position Sn-3. Therefore it can be concluded that lauric acid substitution was not occured in position Sn- 2.

However acid group observed in position Sn-2 was not yet identified and confirmed, it is possible a part of PUFA in considering that unsaturated group appeared at chemical shift of ^{13}C – NMR spectra at δ 128.08 – 128.76 ppm, whilst at δ 53.97 – 60.48 ppm was glycerol group from triglyceride. Although there is also a possibility that acid group at position Sn-2 were other saturated acids such as palmitic acid, but to lead to this conclusion there must be esters of unsaturated acid group gave chemical shift peaks at δ 128.02 – 128.76 as impurities.

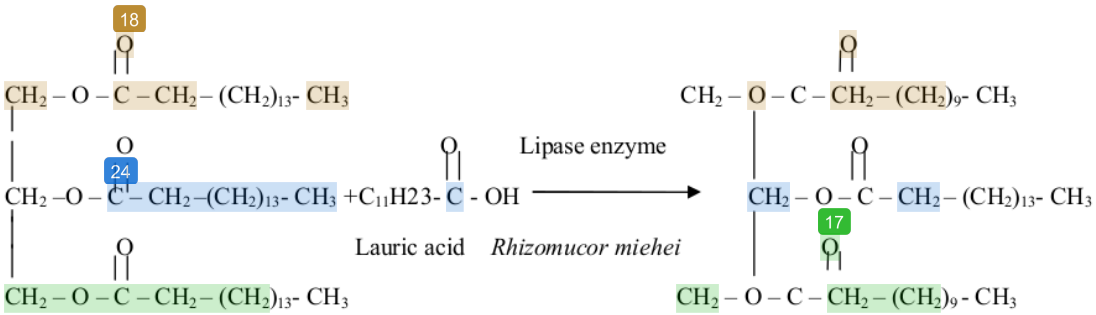
While chemical shift (δ) of proton of fatty acids mixture and restructured lipid by interesterification between skipjack fish oil and lauric acid are presented in Table 1.

Table 1. Chemical shift (δ) of proton from fatty acids mixture.

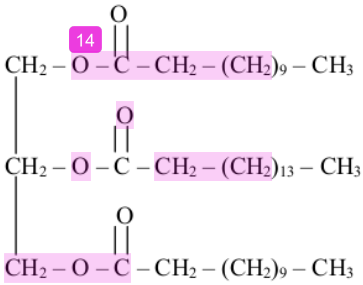
No.	(δ) value (ppm)		
1	5.32	6H	3xCH_2 from trigliseride group
1	4.109	5H	2xCH_2 plus 1xCH from trigliseride group
2	2.83	4H	2xCH_2 from palmitic acid
3	2.61	*Impurities*	*Impurities*
4	2.34	2H	CH_2 from lauric acid
2	2.26	4H	CH_2 from palmitic acid
3	2.16	*Impurities*	*Impurities*
4	1.99	*Impurities*	*Impurities*
2	1.59	4H	2xCH_2 position of C1 dan C3 (from lauric acid)
3	1.26	72H	36 CH_2 2groups of lauric acid C1, C3 (from lauric acid) 10 CH_2 Palmitic/miristic acid
4	0.86	9H	3 CH_3

Data in Table 1 proofed that lauric acid really had substituted fatty acids of skipjack fish oil possibly in position Sn-1 and Sn-3 based on integrated atom H calculation. The triplet peak at 0.8 – 0.9 ppm indicated methyl (CH_3) group of fatty acid, and highest peak slightly broading at 1.2 – 1.3 ppm showed methylen (CH_2) group of fatty acid because broading was the accumulation of more than one CH_2 peaks which chemical shifts were not much difference. If comparing some fatty acids such as palmitic acid, lauric acid, stearic acid and observing based on peaks and/or chemical shifts values of those compounds all of them gave splitting and chemical shift values also number of same peak until could not differentiated. However because of number of methylen (CH_2) group present by each compound are different therefore it could be differentiated by specific integration value in area 1.2 – 1.3 pm.

In this study some assumption were taken i.e. 1) Lipase enzyme of *Rhizomucor meihei* specifically only work at atom C_1 (Sn-1) and C_3 (Sn - 3); 2) based on GC-MS analysis results which indicated the most dominant fatty acid was palmitic acid, therefore reaction was observed on triglyceride component of palmitic acid at skipjack fish oil triglyceride chain. In accordance to the mentioned assumption hence the reaction occured between skipjack fish oil and lauiric acid were as follow:



Lauric acid ($C_{12}H_{24}O_2$) were substituted at position C1 and C3 therefore skipjack fish oil after reaction with lauric acid forming a new compound namely 2 palmito, 1.3 laurat as follow :



2 palmito, 1.3 laurat

This formula proofed that palmitic acid in skipjack fish oil were substituted with lauric acid catalysed by lipase enzyme from *Rhizomucor miehei*, but there is possibility that such reaction could occur also in PUFA.

General PA and DHA were more concentrated in position Sn-2 in fish oil triglyceride[23] ; while reported that in tuna fish oil which contained 35.83% palmitic acid and after interesterification lauric acid substituted palmitic acid in position Sn1 following the reaction as mentioned above[24].

Substitution of lauric acid in skipjack fish oil triglyceride at position Sn-1 and Sn-2 can be calculated by the amount of atom H i.e. in Sn-1: 9 x 2 = 18H and Sn-3: 9 x 2 = 18H while in Sn-2: 13 x 2 = 26H as palmitic acid. Therefore it can be concluded that based on this calculation and accordance to data in ^{1}H – NMR and ^{13}C – NMR spectra it proofed that lauric acid really substituted fatty acids of skipjack fish oil at Sn-1 and Sn-3 via interesterification which are the work of lipase enzyme of *Rhizomucor miehei* through specific acidolisis process. While in palmitic acid(saturated fatty acid) and PUFA in this case EPA (unsaturated fatty acid) were in Sn-2 position.

That a combination of ^{1}H – NMR and ^{13}C – NMR could be used for determining Sn-1 monoacylglycerols, Sn -1,2 and 1,3 diacylglycerol adducts and could also determined trans-fatty acids, free glycerol, cholesterol and added vitamins A and E as minor components[25]. While studied the DHA content and fatty acids of ω-3 in fish oil and determined the DHA lipid oxidation[26,27]. Furthermore [28,29] had used ^{1}H - NMR for fatty acids profile identification of mixture of triglyceride and plant cooking oil.

According NMR spectroscopy [30] have a high resolution to determine the changes of acyl groups in fish oil, molar proportion of ω-3 PUFA and DHA could be measured and determinition using NMR need a shorter time of analysis and also no purification and derivatisation of samples before analysis and no fatty acids standard were needed. Monounsaturated or saturated fatty acids of regiospecific of Alantic salmon (*Salmo salar* L), mackerel (*Scomber scombus*) and herring (*Clupea harengus*) regiospecific distribution were determined using the carbonyl region of ^{13}C – NMR spectra [19]. Whilst studied quantitative determination of fatty acids from fish oils using GC – MS method [31] and ^{1}H - NMR spectroscopy and found that fish oil samples

were recorded on triglycerides and two classes of fatty acids (unsaturated as total ω-3 and saturated as DHA) were determined.

Conclusion

The regiospecific analysis of restructured lipid from skipjack fish oil and lauric acid catalysed by lipase enzyme from *Rhizomucor miehei* through interesterification showed lauric acid substituted in position Sn-1 and Sn-3, while PUFA- ω-3(EPA and DHA) found in position Sn-2.

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