

Article

Quantitative determination of EPA and DHA in fish oil capsules for cardiovascular disease therapy in Indonesia by GC-MS

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Abstract

Background: The consumption of EPA (Eicosapentaenoic acid), and DHA (docosahexaenoic acid), from fish oil, in the long run, has been observed to have a positive impact on patients with coronary heart disease. Fish oil products, with so much EPA and DHA content are available, and have very variable prices. Therefore, as a therapy to be used for long-term treatment, the cost factor is to be considered.

Design and methods: This study analyzed the content of EPA and DHA, using GC-MS. The sample to be analyzed was the fish oil that has the lowest price (Product A1), and that of the highest (Product A2). Furthermore, the macroscopic analysis was performed, by observing the physical form including organoleptic and qualitative tests, by reading the fragments identified by EPA and DHA.

Results: Clinical trials were conducted on patients (about 46 people), with risk factors and dyslipidemia. Product A1 showed EPA at $t_R=15.574$ min (relative%= 88.49%, similarity= 95%), and DHA at $t_R=21.714$ min (relative%= 88.92%, similarity= 93%). Product A2 showed EPA at $t_R=28.719$ min (relative%= 22.58%, similarity= 89%), and DHA at $t_R=32.327$ min (relative%= 22.87%, similarity= 90%), which meant that both had EPA and DHA contents, in accordance with their labels. Both products were confirmed to reduce total cholesterol in 4weeks ($p=0.000$, $p=0.000$), with no significant difference in their effectiveness ($p=0.652$).

Conclusion: The results showed that both the A1 and A2 products, had the EPA and DHA contents in accordance with their respective labels. However, with the A2 product having a percentage relatively higher than that of the A1 brand, both are equally very effective.

Introduction

World Health Organization described cardiovascular disease as the top leading cause of death in the world. In 2005, an estimated number of 17 million people died of cardiovascular disease, representing 30% of the total global mortality. About 80% of these

deaths occurred in low- and middle-income countries.¹ In Indonesia, this condition is included in the top 10 non-communicable diseases, experienced by many people. Furthermore, cardiovascular disease often encountered by adult, is coronary heart illness and failure.² Deaths caused by this condition, especially coronary heart disease (CHD) and stroke, keep on increasing, and predicted to reach 23.3 million mortality by 2030.³

The biggest risk factors for CHD are, obesity and diabetes, with overweight adolescents, which increase the number of obese, by an additional 5%-15% in 2035, resulting to 100,000 new cases.⁴ The heart obtains lipids from circulating non-esterified fatty acids (FFAs), and esterified FFAs, bound to lipoprotein. The abnormal regulation of lipid uptake, or its intracellular metabolism, play an important role in heart diseases.⁵ This supports the theory that, there is a relationship between cholesterol level (LDL), and the risk of cardiovascular disease (CVD).⁶

Also, blood cholesterol is related to the amount of fat, in the composition of daily food (diet). Cholesterol-lowering diets include delicacies that are low in fat, replacing food composition with those containing unsaturated fats, or rich in omega-3.^{7,8} Omega-3 polyunsaturated fatty acids (n-3 PUFAs), consists of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) contents. It is widely contained in fish, possessing protective effects, against cardiovascular disease and hyperlipidemia.^{9,10} DHA and EPA are important food nutrients for human health, which are defined as essential fatty acids, because they are not synthesized by the body, and are often obtained from the diet.¹¹ These nutrients play an important role in the human body, by reducing bad cholesterol, in a bid to prevent CHD,^{12,13} through pleiotropic effects on cell function, which does affect blood lipids, inflammation, with endothelial cell and cardiomyocyte roles.¹⁴

EPA and DHA consumption in the long run, has been observed to have a positive effect on patients with CHD, to reduce the risk of sudden death.¹⁵ Intake of fish oil containing EPA and DHA 1g/day, has been widely recommended for CHD patients. However, intake in excess of 3 g/day is also not recommended, because it can reduce immune system function, as it lowers body inflammatory responses, and increases bleeding time due to high dosage.¹⁶

Significance for public health

Omega-3 consists of EPA and DHA, that provides significant benefits in lowering total cholesterol, which also, reduces the risk of cardiovascular disease. The omega-3 products in the market have relative percentage variability that affects the purification formulas of EPA and DHA, which often cause very large price variations. Furthermore, it is necessary to identify, whether the price difference also affects its effectiveness.

Fish oil products that have so much EPA and DHA contents, are available, and have very variable prices. Furthermore, as a therapy to be used for long-term treatment, the cost factor is to be considered, because it is related to compliance,¹⁷ economic burden,¹⁸ and quality of life.¹⁹ Therefore, in this study, quality control needs to be conducted, in the form of a qualitative test, to see the suitability of the EPA and DHA contents on the label.

The profiles of the chemical compounds are to be identified by using GC-MS chromatography method. The GC-MS method, used in fish oil analysis for the determination of EPA and DHA, has been confirmed to be effective and accurate.^{20,21} Also, EPA and DHA are to be analyzed, by using High Performance Liquid Chromatography (HPLC), and Liquid Chromatography Mass Spectrometry (LC-MS), with the HPLC not as sensitive enough like the GC-MS, while requiring very high costs when using LC-MS.²⁰ Also, clinical trials are to be conducted, in comparing the two products, to observe the effects of EPA and DHA, in the reduction of blood cholesterol levels (LDL, total cholesterol, and triglycerides). Therefore, the aim of this study is to determine the composition of EPA and DHA in fish oil products, for cardiovascular disease treatment, using GC-MS (Gas Chromatography-Mass Spectrometry).

Design and Methods

Study design

This study analyzed the contents of EPA and DHA from two fish oil products, for cardiovascular disease therapy in Indonesia, through qualitative analysis, using GC-MS, in April to December 2017. The sample to be investigated, was the fish oil that had the lowest price (Product A1, Batch Number, 604683 and Exp.Date, January 2019), and the highest rate (Product A2, Batch Number, OCT16AO1 and Exp.Date, February 2019). The study was conducted in a quantitative analysis chemistry laboratory, at the Faculty of Pharmacy, University of Surabaya, Indonesia.

Chemicals and equipment

The chemicals used in this study included n-hexane p.a, saturated sodium chloride p.a, potassium hydroxide p.a, methanol p.a, boron-trifluoride (BF₃), and distilled water. The equipment used in this study included beaker glass, measuring cup, closed test tube, 5 ml/cc injection syringe, vials, membrane filter, millipore holder, Shimadzu QP-2010 SE brand GC-MS, Japan-using capillary column RTX-5SM (60 m x 0.25 mm, layer thickness 0.25 μm), with minimum column temperature of 330°C-350°C, used for sample separation. For temperature programming, the oven was maintained at 80°C for one min, increased at intervals of 10°C/min to 250°C, then reduced to 8°C/min to 280°C, and balanced for 5 min. Split injection was performed at a ratio of 1:200, as an helium carrier gas, with 1 μl injection volume of 0.73 ml/min was used. The mass spectrum, was operated in electron impact mode (EI). Furthermore, other parameters included, pressure 100 Kpa, injection temperature 250°C, ion source EI 200°C, interface temperature 220°C, electron energy 70 eV, delay solvent 5 min. For the qualitative analysis the full scan mode used for variously was 40-400m/z (Figure 1).

Qualitative test

Qualitative tests carried out included, organoleptic observation (visual conditions based on shape, smell, taste, and color), and

sample making. A 3 ml fish oil in soft capsules, was obtained, and placed into a centrifuge tube, with 3 ml of potassium hydroxide methanol (0.5 M) solution added to it. The solution was mixed together vigorously, until it became homogeneous, then heated in a 60°C water bath, hydrolyzed with nitrogen gas flow, while being stirred three times, for 20 min. Furthermore, 3 ml of BF₃ was added, after which the solution was cooled. The solution was heated at 60°C of water bath, and hydrolyzed again with nitrogen gas, for 5 min. Saturated sodium chloride solution of 2 ml, and 2 ml n-hexane were added, mixed vigorously and centrifuged at 4000 rpm, for 10 min. Supernatant was also obtained for use, as a sample solution. An aliquot of 1 μl supernatant, was then injected for GC-MS analysis (Figure 2).

Clinical trials

The clinical trials in humans used the randomized controlled trial (RCT) method. The research subjects were adults aged >18-year-old, and dyslipidemic patients having ≥1 of CVD risk factors, with CHD uncertainty. The assessment of CVD risk factors in the next 10 years are conducted, using the Framingham risk score.²² This includes body mass index (BMI), blood pressure, smokers, physical activity, and increased blood sugar.²³⁻²⁵ The criteria for the study subjects were, no history of kidney and heart disease. The research subjects were divided into two groups namely, those that use A1 and A2 products routinely, everyday. Each group watched for 4 weeks, and consisted of 23 people, with a purposive sampling method. Further test was carried out for total cholesterol (mg/dl), using Accutrend® GCT (Figure 3).

The sample size used in this study was 23 people, taking into account the value of Z=1.96 and p=0.015, which is the proportion for certain properties, estimated from Indonesian Heart Association (2017).²⁶

Results and Discussions

Identification of omega 3 in fish oil

The organoleptic observations of product A1 and A2 included, shape, color, odor, and taste. The results showed that, both products have the same form (oil), color (clear yellow), smell (fishy), and taste (like fish). The results of the study by GC-MS, obtained

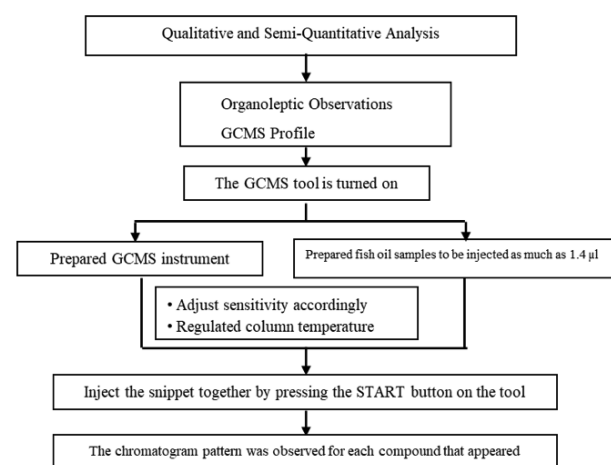


Figure 1. EPA and DHA test scheme on fish oil with GCMS.

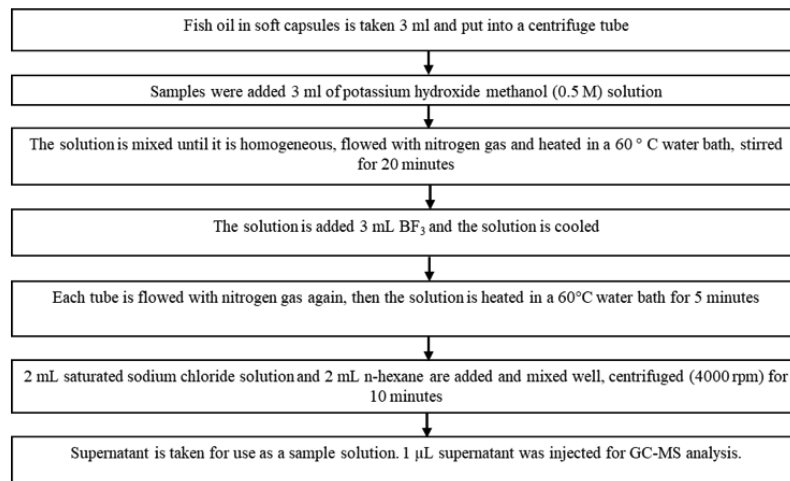


Figure 2. Work scheme preparation of fish oil samples.

two chromatograms and mass spectra, which were derived as products from the analysis. The identification of fish oil using Gas Chromatography-Mass Spectroscopy (GC-MS), showed 49 peaks on the chromatogram profile shown in Figure 4, with the highest observed at 28 and 32. The mass spectrum of the compound at peak 28, with $t_R=28.719$ min (22.58%), showed Ethyl 5,8,11,14,17 Eicosapentaenoate (Figure 5a). Based on data from the NIST08.LIB library, Ethyl 5,8,11,14,17 Eicosapentaenoate has the molecular formula $C_{21}H_{32}O_2$, with a molecular weight of 316, and a basic peak at m/z 79. The structure shown is clearly the same as that of the EPA in Figure 5a, with 89% similarity.

The mass spectrum of the compound at peak 32 with $t_R=32.327$ min (22.88%), showed 4,7,10,13,16,19 Docosahexaenoic acid methyl ester, as shown in Figure 5b. Based on data from the NIST08.LIB library, 4,7,10,13,16,19 Docosahexaenoic acid methyl ester, has a molecular formula $C_{23}H_{34}O_2$, with a molecular weight of 342, and a base peak at m/z 79. The structure that shown is clearly observed in the appendix, equal to that of the DHA, as shown in Figure 5b, with 90% similarity (Figure 5b).

Identification of fish oil using Gas Chromatography-Mass Spectroscopy (GC-MS), showed 50 peaks on the chromatogram profile shown in Figure 6, with the highest observed at 4 and 22, respectively (Figure 6). The mass spectrum of the compound, at peak 4 with $t_R=15.574$ min (88.49%), showed Ethyl 5,8,11,14,17 Eicosapentaenoate (Figure 7a). Based on data from the NIST08.LIB library, Ethyl 5,8,11,14,17 Eicosapentaenoate has the molecular formula $C_{21}H_{32}O_2$, with a molecular weight of 316, and a basic peak at m/z 79. The structure shown is more clearly the same as that of the EPA in Figure 7a, with 95% similarity.

The mass spectrum of the compound at peak 32 with $t_R=32.327$ mins (22.88%), showed 4,7,10,13,16,19 Docosahexaenoic acid methyl ester, as shown in Figure 5b. Based on data from the NIST08.LIB library, 4,7,10,13,16,19 Docosahexaenoic acid methyl ester, has a molecular formula $C_{23}H_{34}O_2$, with a molecular weight of 342, and a base peak at m/z 79. The structure shown is more clearly observed in appendix, equal to that of the DHA in Figure 5b, with 93% similarity (Figure 7b).

Data analysis

The calculations to determine the level of compounds contained in a sample, is conducted, using the equation, %relative=(area÷area total) × 100% (Table 1). The product A1, with the lowest price after being identified using GC-MS, showed EPA at $t_R=$



Figure 3. Cholesterol measurement tool.

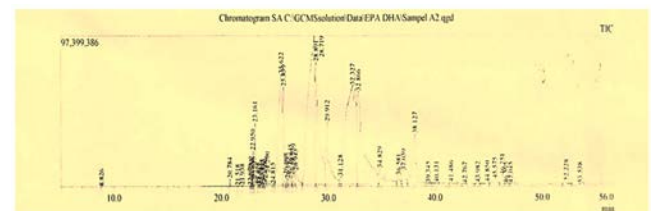


Figure 4. Chromatography profile of Product A1.

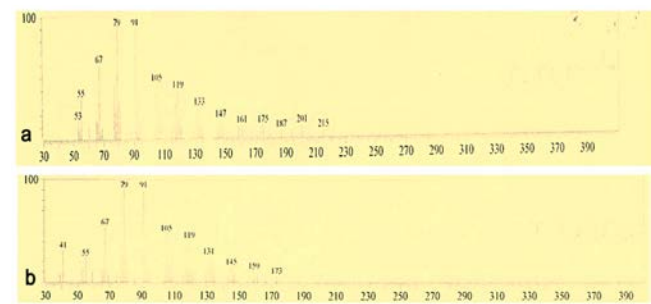


Figure 5. a) EPA peak mass spectrum highest area. b) DHA mass spectrum peaks the highest area of sample A1.

28.719mins, with relative%= 22.58%, having a similarity of 89%, and DHA at $t_{R}= 32,327$ mins, with relative%= 22.87%, possessing a 90% similarity, which means that both contents are compatible with the label (Figure 8a). Furthermore, product A2, with the highest price, showed EPA at $t_{R}=15.574$ min, with relative%= 88.49%, having a 95% similarity, and DHA at $t_{R}= 21.714$ min, with relative% = 88.92%, possessing a 93% similarity, which means that both contents are also compatible with the label (Figure 8b).

Product effectiveness data

Based on the characteristics shown by the respondents, majority were women, with an average age, early adult, and are students. The average pre-post total cholesterol in both product group, is above the normal average value (<200 mg/dl) (Table 2). Based on Table 3, both product groups showed changes in pre (p=0.000), and post (p=0.000), meaning they are equally effective in reducing total cholesterol in the blood. However, the two products showed no difference in their effectiveness, when reducing total cholesterol (p=0.652).

The study showed that 3 ml of fish oil in soft capsule, was inserted into a centrifuge tube. The sample mixed with 3 ml of KOH, was useful for hydrolyzing into esters, or called derivatization. Both EPA and DHA which were then heated in 60°C water, completely hydrolyzed and flowed with nitrogen gas, to avoid being oxidized, and stirred three times for 20 min. The fish oil was known to have a high vapor point, because the constituent substance was triacylglycerol. Therefore, before being analyzed by GC-MS, the methyl ester form was changed, using methanol and Boron trifluoride (BF₃) as a catalyst. This form of fatty acid methyl ester, was then injected into the GC-MS device. BF₃ was added to the sample, and the solution was cooled.

BF₃ bind well to atoms with high electronegativity, to form complexes. The solution was heated over a 60°C water bath for 5mins. Saturated sodium chloride solution of 2 ml and 2 ml n-hexane were added together, mixed vigorously, and centrifuged (4000 rpm) for 10 min. N-hexane is a solvent that was used, because of its low boiling point, lightness in removing the oil contained, and volatility in making extraction easy. Saturated NaCl was added to reach salting in the centrifuge, with n-hexane dissolved phase injected into GC-MS, as much as 1 µl.

The results of the test with GC-MS, obtained two chro-

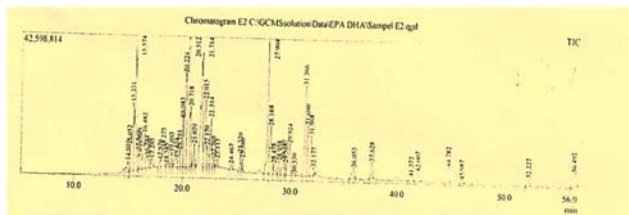


Figure 6. Chromatography profile of Product A2.

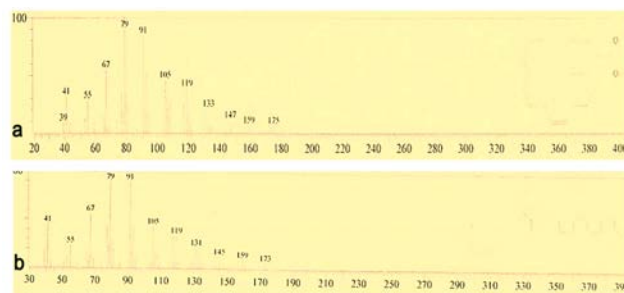


Figure 7. a) EPA peak mass spectrum highest area. b) DHA mass spectrum peaks the highest area of sample A2.



Figure 8. a) Label of Product A1 (1200 mg: 360 EPA and 240 DHA). b) Label of Product A2 (1000 mg: 180 EPA and 120 DHA).

Table 1. Comparison of EPA and DHA in Product A1 and A2.

Components	Product A1		Components	Product A2	
	Area	% Relative		Area	% Relative
Ethyl 5,8,11,14,17 Eicosapentaenoate	4073005037	22.58%	Ethyl 5,8,11,14,17 Icosapentaenoate	449971252	88.49%
4,7,10,13,16,19 Docosahexaenoic acid, methyl ester	4124358876	22.87%	4,7,10,13,16,19 Docosahexaenoic acid, methyl ester	451560536	88.92%

Table 2. Characteristics of respondents.

		Groups	
		Product A1 (N=23)	Product A2 (N=23)
Gender	Male	3 (13.04%)	4 (17.39%)
	Female	20 (86.96%)	19 (82.61%)
Age (years)	Early adulthood (26-35)	20 (86.96%)	21 (91.30%)
	Late adulthood (36-45)	3 (13.04%)	2 (8.70%)
Occupation	College student	18 (78.26%)	18 (78.26%)
	Employee	5 (21.74%)	5 (21.74%)
Average of total Cholesterol (normal: <200mg/dL)	Baseline (pre)	245.1	241.7
	Post	211.3	205.4

Table 3. Measurement of total cholesterol profile

Groups	Changes in the value of total cholesterol			p-value (pre-post)	p-value (post A1 vs Post A2)
	Increase	Fixed	Decrease		
Product A1 (n:23)	1	4	18	0.000	0.652
Product A2 (n:23)	0	4	19	0.000	

p<0.05 means that there are significant differences.

matograms, for gas chromatography (GC), and mass spectra, for that of mass spectroscopy (MS) analysis, functioning as x-axis (intensity) and y-axis (mass per charge (m/z)), respectively. The results showed that, both the A1 and A2 products had the EPA and DHA contents, in accordance with their respective labels. Furthermore, the A2 product has a relatively higher percentage than the A1 brand, which then shows that the compound content, contained in the A2 sample is very high. Therefore, it can be concluded that, there is a relationship between price and both product contents, as observed from the relative percentage. Product A1 (with the lowest price), has relatively lower percentage, compared to brand A2 (with the highest price), which has relatively higher grade, although the dosage stated on the label of A1, is higher than that of A2. The result in Table 3, showed that the two therapy groups were equally effective at lowering total cholesterol, and there was no significant difference in effectiveness. Also, gender did not affect the risk of dyslipidemia.²⁷ Furthermore, race factors affected the treatment of dyslipidemia more. Also, the result of the study explained that hyperlipidemia is more aggressively treated and controlled in white men, than in white women. However, black women are at the highest risk of CHD, than others.²⁸ The age and educational level also affects dyslipidemia treatments, with the most at risk in the 50-59 years-old group. Dyslipidemia, is mostly served by patients with low levels of education.²⁹

The systematic review by Nejad and Bikdeli,³⁰ in an early study, showed that supplementation with 1g of omega-3 per day, significantly reduced mortality. A double-blind randomized in France, showed that, in 633 patients taking 600mg of omega-3 daily, for an average of 4.7 years, did not find significant decrease in major cardiovascular events. The same is the case with other studies in Netherlands, with randomized double-blind placebo-controlled trials, showing that, in 1,192 CVD patients using supplements with an average of 226 mg EPA, and 150 mg DHA per day, did not show significant decrease in major cardiovascular events. While another study in Germany, found no additional benefit from 1g of omega-3 per day, for one year, against sudden cardiac death, and other cardiovascular events. This study had limitations, which is, not being able to know how significant the relative percentage difference is. Therefore, it is recommended for further research to determine the levels of product contents in a fish oil product, with a comparison of pure EPA and DHA. Furthermore, research is also needed to observe cost-effective events between the cost of drugs, with effectiveness in reducing cholesterol levels. Also, factors of race and occupation/education, related to health affects the risk of dyslipidemia, and adherence to medication. Further research is also needed, to compare natural resources with supplements. Omega-3 sources are more recommended in the form of food intake from fish, rather than in the form of supplements.³¹ Also, to lower cholesterol in the blood, omega-3s reduces blood pressure,³² with further research needed, to observe changes in the system. Also, future clinical trials need to pay attention to genetic related factors including, apolipoprotein AI (apoA-I), ATP-binding cassette transporter A1 (ABCA1), ABCG1, apoE, scavenger receptor class B type I (SR-BI), cholesteryl ester transfer protein (CETP), low-density lipoprotein receptor (LDLr), cholesterol 7 alpha-hydroxylase (CYP7A1), and ABCG5/G8.³³

Conclusions

The results showed that both the A1 and A2 products had the EPA and DHA contents in accordance with their respective labels. Also, with the A2 product having a relatively higher percentage than the A1 brand, it is shown that the compound content in the A2 sample, is high. Therefore, it can be concluded that, there is a relationship between price with, EPA and DHA content, as observed from relative percentage. Product A1 (with the lowest price), has relatively lower percentage, compared to brand A2 (with the highest price), with relatively higher grade. Therefore, both products are confirmed to reduce total cholesterol in 4 weeks, with no significant difference in their effectiveness.

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