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DEVELOPING A NEW ESTERIFICATION METHOD FOR THE DETERMINATION OF FATTY ACID COMPOSITIONS OF MARINE OIL SUPPLEMENTS BY GAS CHROMATOGRAPHY WITH ORTHOGONAL CENTRAL COMPOSITE DESIGN AND RESPONSE SURFACE METHODOLOGY

İsmail Tarhan*
Selçuk University, Faculty of Science, Department of Biochemistry, Selçuklu, Konya, Turkey

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Tarhan, İ. (2020). Ortagonal merkezi kompozit tasarım ve yüzey cevap metodolojisinden faydalanarak balık yağı gıda takviyelerinin yağ asidi kompozisyonlarının gaz kromatografisi ile tayini için yeni bir esterleştirme metodunun geliştirilmesi. *GIDA* (2020) 45(3) 581-589 doi: 10.15237/gida.GD20043

ABSTRACT
In this study, an efficient, applicable, and special esterification method for the quantification of fatty acid compositions of MOs has been created with chemometric techniques. The esterification variables which are the temperature of esterification (A), the time of esterification (B), and the amount of hexane (C), were optimized by using the orthogonal central composite design (OCCD). The relationships of the variables with each other and with the response value (R) are interpreted with response surface methodology (RSM). The optimal conditions were as follows: the temperature of esterification, 60°C; the time of esterification, 40 min; and the amount of hexane, 4.3 mL. Even MOs that are difficult to esterify can be easily esterified and the fatty acid compositions of all MOs, especially important fatty acids such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6), have been successfully analyzed by gas chromatography with the new method developed.
Keywords: Chemometrics, esterification, fatty acid composition, gas chromatography, marine oil supplements.

ORTAGONAL MERKEZİ KOMPOZİT TASARIM VE YÜZEY CEVAP METODOLOJİSİNDEN FAYDALANARAK BALIK YAĞI GIDA TAKVİYELERİNİN YAĞ ASİDİ KOMPOZİSYONLARININ GAZ KROMATOGRAFİSİ İLE TAYİNİ İÇİN YENİ BİR ESTERLEŞTİRME METODUNUN GELİŞTİRİLMESİ

ÖZ
Bu çalışmada kemometrik tekniklerden faydalanılarak MO'ların yağ asidi kompozisyonlarının tayini için etkili, uygulanabilir ve özel bir esterleştirme yöntemi geliştirilmiştir. Esterifikasyon parametrelerinden esterleşme sıcaklığı (A), esterleşme süresi (B) ve hekzan miktarı (C) ortagonal merkezi kompozit tasarım (OCCD) kullanılarak optimize edilmişlerdir. İlgili parametrelerin birbirleriyle ve cevap değeri (R) ile ilişkileri cevap yüzey metodolojisinden (RSM) faydalanılarak yorumlanmıştır. İlgili parametrelerin tespit edilen optimum değerleri esterleşme sıcaklığı için 60°C, esterleşme süresi için 40 dakika ve hekzan miktarı için 4.3 mL olarak tespit edilmiştir. Gaz kromatografisi ve geliştirilen yeni metot ile esterleştirilmesi kolay olmayan MO numuneleri kolaylıkla esterleştirilebilmiş ve başta eikosapentanoik asit (20:5) ve dokosahekzaenoik asit (22:6) olmak üzere tüm önemli yağ asitleri analiz edilebilmiştir.
Anahtar kelimeler: Kemometri, esterleştirme, yağ asidi kompozisyonu, gaz kromatografisi, balık yağı gıda takviyesi.

* *Corresponding author* / Yazışmalardan sorumlu yazar
✉ ismtarhan@gmail.com, ✆ (+90) 332 223 3901 🖷 (+90) 332 241 2499
İsmail Tarhan; ORCID no: 0000-0003-3353-8635

INTRODUCTION

Fatty acids that include more than one double bond in their hydrocarbon chain are called as polyunsaturated fatty acids (PUFAs) and these compounds have many important bio compounds, such as essential fatty acids. PUFAs can be classified in three groups by their chemical structure. The first group is methylene-interrupted polyenes, the second group is conjugated fatty acids and the last group is other PUFA compounds. The omega-3 (n-3 or ω3) long-chain polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic (EPA, 20:5) and docosahexaenoic (DHA, 22:6) acids, have beneficial effects on human health and they have been recognized the essential ingredient to the human diet. PUFAs such as EPA and DHA, play a crucial role in health promotion and disease prevention. While EPA is one of the most important precursors of the signal molecules that regulate immune responses and ion transport (Chang et al., 2013; Li et al., 2015), DHA accounts for about 95% of the amount of PUFAs in the aminophospholipid structures of the cell membranes in the retina and brain (Mata et al., 2001; Niemoller and Bazan, 2010; SanGiovanni and Chew, 2005). EPA and DHA also can prevent the formation of many diseases in humans, especially cardiovascular ailments (Marchioli et al., 2002; Mozaffarian and Rimm, 2006). However, the irony is that PUFAs, which have such important benefits to human health, cannot be adequately synthesized by the human body (Barceló-Coblijn and Murphy, 2009).

The n-3 PUFAs needed are provided from the α-linolenic acid (18:3) taken into the body, mostly by consuming vegetable oils such as flaxseed oil, canola oil, and soybean oil (Olgunoglu, 2017), but the transformation of vegetable-derived n-3 PUFAs into EPA and DHA in human metabolism has a very low rate (Burdge and Calder, 2005). Therefore, it is understood that the essential fatty acids such as EPA and DHA should be obtained from different sources and at this point, marine oils (MOs) rich in these fatty acids, containing between 5.4-13.2% EPA and 7.6-11.5% DHA (Wang et al., 2010), come to the fore. While people living at the seaside can take EPA and DHA fatty acids that their bodies needed from kind of seafood, which constitutes an important part of their diets, other people have turned to omega-3 food supplements from MO to close this diet gap.

For this reason, MOs for providing rich PUFA have expanded in recent years and omega-3 food supplements from MO have become one of the popular dietary supplement products for consumers. Due to this high demand for these supplements, control analysis methods to ensure both label accuracy and product quality are of great importance in terms of protecting consumers who consume these products. Since the most important factor in determining the health-promoting properties and effectiveness of these MO products is the ratios of essential fatty acids such as EPA and DHA, the determination of fatty acid compositions with a suitable analytical technique is an important issue.

Currently, the composition of fatty acids in many samples such as biological samples and foods is commonly determined by gas chromatography (GC) with flame ionization detection (FID) due to its high sensitivity, selectivity, and wide analysis range. GC-FID is a unique technique for analysis of the fatty acid composition of edible oils and it is the highly recommended method of analysis in the edible oil industry and many research institutes (AOCS, 2017). In this method, the fatty acids in triglyceride form are first broken down into free fatty acids by a base-catalyzed reaction and then converted into volatile fatty acid methyl esters (FAME) derivatives with low molecular weight alcohol such as methanol. In this way, fatty acids converted into volatile ester derivatives can be separated by GC-FID employing a capillary GC column having high-polarity. Since all the fatty acids in the sample must be converted to the FAME derivatives, the most important step of this procedure is the correct esterification process. If the esterification of the oil sample is not done correctly, not all of the fatty acids can be converted to FAME derivatives and the analysis results having negative errors are obtained.

While the same GC-FID technique can be successfully applied for many edible oil samples, especially, the esterification step requires minor modifications because each edible oil has a different bioactive composition (Ayyildiz et al., 2015; Topkafa, 2016). Besides, food flavors that give to MOs a pleasant flavor, especially for children to willingly consume, and triglyceride preservatives are added to commercial MOs that are desired to have a long shelf life and, in this case, it is inevitable to develop a suitable and efficient esterification method for the edible oil to be analyzed.

Carvalho and Malcata (2005) studied the influence of various parameters in each step of derivatization reactions, using both cod liver oil and microalgal biomass. The accuracies of the methodologies were tested with AOCS standard method, whereas their reproducibility was assessed by analysis of variance. They showed that alkaline catalysts generated lower levels of long-chain unsaturated FAME than acidic ones. Among these, acetyl chloride and BF_3 were statistically equivalent to each other. The standard method provided equivalent results when compared with acidic methylation with BF_3 alone. However, they were not apply the method developed in their studies to any food supplements obtained marine oils and were not benefit from chemometric techniques.

To the best of our knowledge, there is no study involving an esterification method specifically developed for the determination of fatty acid compositions of MO supplements. In this study, an efficient, applicable, and special esterification method for the quantification of fatty acid compositions of MOs has been created. The esterification parameters, which are the temperature of esterification (A), the time of esterification (B), and the amount of hexane (C), were optimized within the framework of chemometric approaches by response surface methodology (RSM).

MATERIALS AND METHODS

Samples and Reagents

A total of 18 MO dietary supplements samples of different brands were purchased from the local markets in Turkey. All chemicals and solvents of the analytical chromatographic grade used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA). The standard of FAME containing 37 compounds was purchased from Supelco (Bellefonte, PA, USA).

Fatty Acid Composition by GC-FID

For the determination of fatty acid content of MOs, AOCS standard method (AOCS 2017) with some modifications was used. Briefly, the triglyceride structures of MOs were esterified by adding 1 mL of 2 N methanolic-KOH solution to the 1g of MO sample in a glass test tube. The mixture was heated in a magnetic stirrer and FAMEs were extracted using hexane. The temperature of esterification, the time of esterification, and the amount of hexane of the esterification procedure were changed according to the chemometric design. Then the mixture was dried using a small amount of anhydrous sodium sulfate. 1.5 mL of the dried mixture was filtered by 0.45 μm nylon syringe filter and injected into GC-FID system in triplicate.

GC-FID system used in this study was employed consisting of an FID detector, thermostated column oven, and Agilent (CA, USA) HP-88 capillary column (100m x 0.25mm x 0.25μm, 88%-cyanopropyl)aryl-polysiloxane). The operating conditions were as follows: The temperature program of the column oven was held at 50°C for 2 min, and then increased at 4 °C/min from 50°C to 240°C, held at 240°C for 10 min. The temperature of the injection block was 250°C; the carrier gas was hydrogen; the flow rate was 1.0 mL/min; and the sample injection volume was 1.0 μL (splitless). FAME peaks of the samples were identified based on comparing their retention times with FAME standard. The detector response was recorded on the software of the Chemstation data processor (Ver. B.03.02).

Chemometric Experimental Design

RSM is one of the most relevant multivariate statistical technique in analytical method optimization and it is a hybrid of statistical and mathematical branches based on the fit of a polynomial model to the experimental data (Arslan et al., 2013; Memon et al., 2015; Tarhan et

al., 2017; Tarhan and Kara, 2016; Tarhan and Kara, 2019). Thanks to RSM, the behaviors of a data group are successfully defined by making statistical predictions. In order to develop a new esterification method for the quantification of fatty acid compositions of MOs, the esterification parameters that are the temperature of esterification (A), the time of esterification (B), and the amount of hexane (C), were optimized with RSM. The calibration design, three variables including A, B, and C was used based on the orthogonal central composite design (OCCD) by employing a three-variables, five-levels. The parameters and their coded level values are given in Table 1. The experimental designs generated and RSM graphics were achieved using the Design-Expert software (MN, USA, version 11). The level of 1.68179 represents the axis point of OCCD for three independent variables.

Firstly, 18 MO samples were esterified according to the standard method and the sample with the lowest esterification efficiency, MO1, was chosen for the experimental design. The sample of MO1 was esterified according to each experimental design given in Table 1 and injected into GC-FID system. The average peak areas of EPA and DHA fatty acids were calculated by using the chromatograms obtained from each analysis in Table 1 and the response value (R) was generated for each experimental design. The design with the highest R is considered the best esterification condition.

RESULTS AND DISCUSSION

Optimization of The Proposed Esterification Method Parameters

The experimental design given in Table 1 was done to determine of optimum values of the developed esterification method parameters. Rs obtained from the chromatographic separation of EPA and DHA were determined in Table 1. From the variable values given in Table 1, equation 1 was generated and RSM plots (Figure 1) were generated using that equation.

$$R = 284.6490 - 113.6160A - 8.1134B + 18.9436C - 44.4725AB + 32.3450AC - 30.9500BC - 90.4758A^2 - 31.5862B^2 + 130.6970C^2 \quad (Equation\ 1)$$

Figure 1a displays the effect of A and B on the relative response value. With a given A value up to the level of 0 (60°C), the relative response value increased and slowly decreased above the level of 0 (60°C) of A. This result indicates that the R value is the best for the esterification of the fatty acids at 60°C as the temperature of esterification. For B parameter, there is an increase of the relative response value, with the increase of B up to the level of 0 (40 min). The maximum response obtained from these levels was observed as 60°C and 40 min for A and B, respectively.

The effect of A and C on the relative response value shown in Figure 2b. With a given A value up to the level of 1.68179 (94°C), the relative response value decreased. But for C parameter, the relative response value was decreased up to the level of 0 (6 mL) and increased above the level of 0. The maximum response obtained from these levels was observed as 40°C and 4.3 mL for A and C, respectively.

The effect of B and C on the relative response value shown in Figure 3b is similar to the effect of A and C. The maximum response obtained from these levels was observed as 40 min and 7.7 mL for B and C, respectively.

The optimal conditions obtained using RSM and OCCD for the esterification of MO1 sample were as follows: the temperature of esterification, 60°C; the time of esterification, 40 min; and the amount of hexane, 4.3 mL. MO1 sample was esterified according to the optimal conditions and injected into GC-FID system. It was seen that all fatty acids of MO1 were successfully esterified and separated in Figure 1d. With the standard esterification method, only 16% of 18 MO samples gave usable results. The method we developed within the scope of the study has successfully esterified 88% of 18 MO samples.

Fatty Acid Compositions of MO Samples with the Developed Esterification Method

18 MO samples were esterified according to the optimal conditions and injected into GC-FID system in order to validate. The fatty acid composition results obtained are given in

Supplementary files. 88% of all MO samples except MO6, MO7, MO11, and MO12 samples were successfully analyzed. It has been interpreted that this situation in the samples that failed to esterification is due to the different preservative, flavoring or coloring contents contained in the relevant samples. The rate of degradation and utilization levels in human metabolism of this type of marine oils, whose triglyceride structure cannot be broken down even under harsh esterification conditions, is a matter of separate discussion.

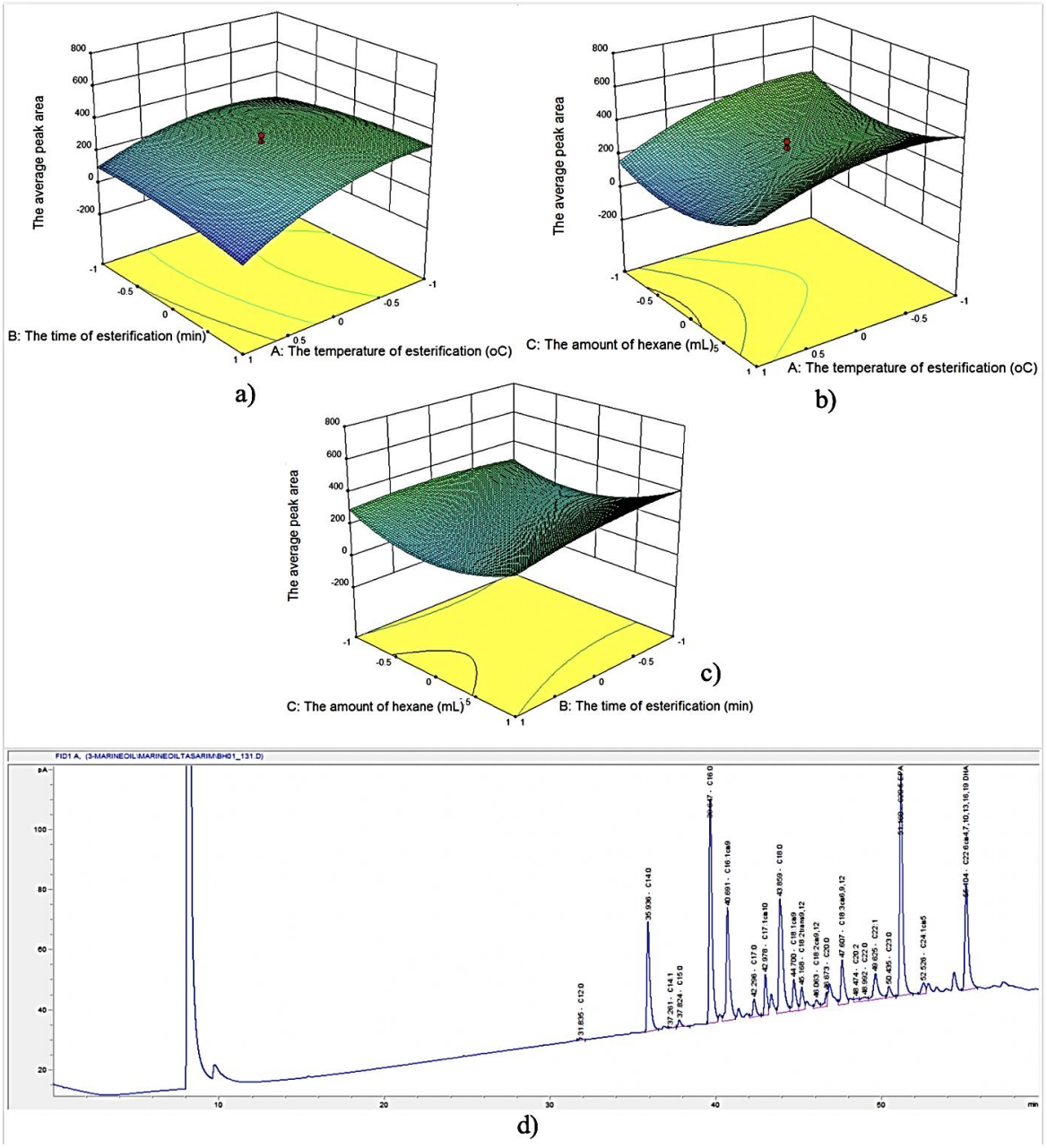

Figure 1. RSM profiles affected by the developed method parameters for the esterification of the fatty acids and their chromatogram obtained (a) A (the temperature of esterification) and B (the time of esterification), (b) B and C (the amount of hexane), (c) A and C, and (d) the chromatogram of the fatty acid composition of MO1 sample.

Table 1. OCCD and the values of the parameters used in RSM for (A: The temperature of esterification, °C; B: The time of esterification, min; C: The amount of hexane, mL; and R: Response value)

	Coded levels and the values of the parameters						
Experiment number	Level for A	A (°C)	Level for B	B (min)	Level for B	B (mL)	R
1	-1	40	-1	20	-1	5	391.13
2	1	80	-1	20	-1	5	15.60
3	-1	40	1	60	-1	5	434.23
4	1	80	1	60	-1	5	1.25
5	-1	40	-1	20	1	7	452.93
6	1	80	-1	20	1	7	327.22
7	-1	40	1	60	1	7	492.67
8	1	80	1	60	1	7	68.63
9	-1.68179	26	0	40	0	6	114.98
10	1.68179	94	0	40	0	6	0.00
11	0	60	-1.68179	6	0	6	200.48
12	0	60	1.68179	74	0	6	247.63
13	0	60	0	40	-1.68179	4.3	754.57
14	0	60	0	40	1.68179	7.7	611.55
15	0	60	0	40	0	6	298.28
16	0	60	0	40	0	6	338.52
17	0	60	0	40	0	6	252.98
18	0	60	0	40	0	6	263.53
19	0	60	0	40	0	6	279.32
20	0	60	0	40	0	6	265.40

Fatty acid compositions of MO samples were evaluated using 11 different fatty acids and 4 different total saturation levels. Among them, stearic acid (C18:0) and EPA (20:5) were the major compounds as expected and it was followed by DHA (22:6) and palmitic acid (C16:0). The compound of stearic, EPA, DHA, and palmitic acids varied from 10.34% (MO3) to 37.68% (MO2), 0.00% (M11 and MO12) to 20.27% (MO16), 0.00% (MO6, MO7, MO11, MO12, and MO17) to 17.60% (MO18), and 5.48% (MO12) to 23.18% (MO7), respectively. The total ratio of saturated fatty acids (SFAs), the total ratio of monounsaturated fatty acids (MUFAs), the total ratio of polyunsaturated fatty acids (PUFAs), and the total ratio of omega-3 fatty acids were found to be highest in MO2 (57.59%), MO5 (18.48%), MO18 (36.59%), and MO18 (36.59%), respectively (Supplementary files).

CONCLUSION

In this study, a new esterification method for the esterification of fatty acids of MOs has been successfully developed by employing the chemometric techniques. The method is effective, applicable, and simple as well as avoiding the use of excessive chemicals. The developed method has great potential for the routine analysis of MO samples and certification of the commercial MO products. As a result, the proposed esterification method could be easily used for effective determination of the fatty acid composition of MOs with GC-FID system.

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Supplementary file 1. Fatty acid (%) composition[a] of MO samples

Sample	C14:0 (Rt:35.9)	C16:0 (Rt:39.6)	C16:1 (Rt:40.7)	C18:0 (Rt:43.9)	C18:1 (Rt:44.7)	C18:2 (Rt:46.0)	C18:3 (Rt:47.6)	C20:0 (Rt:46.7)	C22:1 (Rt:49.6)	C20:5 EPA (Rt:51.2)	C22:6 DHA (Rt:55.1)
MO1	7.69	15.25	8.75	11.09	2.48	0.76	0.93	3.04	3.68	15.88	9.07
SD±	0.27	0.34	0.20	0.17	0.02	0.04	0.05	0.27	0.10	0.33	0.26
MO2	5.49	12.18	5.54	37.86	1.90	0.00	2.05	1.22	1.69	8.12	5.01
SD±	0.44	0.46	0.44	5.21	0.15	0.00	1.04	0.46	0.25	1.47	0.93
MO3	4.59	13.59	6.05	10.34	1.01	0.41	2.35	3.75	1.96	9.56	13.69
SD±	0.43	1.40	0.54	0.76	0.14	0.13	0.05	0.89	0.07	0.81	1.98
MO4	6.46	14.22	8.68	11.21	2.17	0.60	1.49	1.95	4.38	14.39	7.45
SD±	1.33	2.38	1.41	1.39	0.20	0.19	0.67	0.43	0.73	1.42	0.97
MO5	3.80	8.44	9.60	19.94	0.53	0.38	15.14	8.34	2.95	7.52	8.26
SD±	0.21	0.19	0.22	0.22	0.01	0.02	0.09	0.16	0.02	0.09	0.13
MO6	0.00	12.92	0.00	17.88	0.00	0.00	0.00	3.66	0.00	8.76	0.00
SD±	0.00	3.81	0.00	5.94	0.00	0.00	0.00	6.34	0.00	5.54	0.00
MO7	0.00	23.18	0.00	20.50	0.00	0.00	0.00	0.00	0.00	3.96	0.00
SD±	0.00	10.84	0.00	17.80	0.00	0.00	0.00	0.00	0.00	3.45	0.00
MO8	6.62	21.42	8.49	11.57	1.84	0.00	2.47	2.07	2.75	14.52	7.28
SD±	0.36	0.02	0.20	0.12	0.30	0.00	0.26	0.16	0.08	0.84	0.64
MO9	7.49	16.37	9.00	11.58	2.14	0.57	2.87	3.35	3.04	16.19	10.32
SD±	0.58	0.33	0.23	0.15	0.07	0.02	0.11	0.37	0.05	0.36	0.23
MO10	7.03	14.78	9.59	11.95	2.84	0.55	0.63	2.43	2.82	16.19	6.02
SD±	0.39	0.37	0.24	0.11	0.04	0.06	0.05	0.32	0.08	0.39	0.30
MO11	0.00	6.22	0.00	11.65	1.15	0.00	0.00	1.05	0.00	0.00	0.00
SD±	0.00	0.32	0.00	1.58	0.07	0.00	0.00	1.81	0.00	0.00	0.00
MO12	0.00	5.48	0.00	12.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD±	0.00	0.15	0.00	0.42	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MO13	2.29	14.14	6.04	16.84	0.89	0.75	4.50	6.81	2.06	13.13	12.80
SD±	0.19	0.30	0.19	0.46	0.32	0.13	0.13	1.11	0.01	0.34	0.25
MO14	5.08	13.50	8.71	12.34	2.12	1.08	2.92	4.62	3.23	17.32	5.11
SD±	0.72	1.11	1.01	0.67	0.16	0.37	0.30	1.29	0.47	1.74	0.85
MO15	3.74	13.15	8.30	12.68	1.94	0.63	3.23	4.15	3.24	19.30	6.96
SD±	0.23	1.10	0.76	0.41	0.13	0.06	0.20	0.36	0.02	0.78	0.67
MO16	0.94	10.20	4.00	15.50	0.00	0.00	0.00	8.48	0.78	20.27	14.90
SD±	1.63	1.05	0.56	1.80	0.00	0.00	0.00	0.58	1.35	1.48	3.27
MO17	0.00	20.06	0.00	35.73	0.00	0.00	0.00	0.00	0.00	3.00	0.00
SD±	0.00	0.07	0.00	1.08	0.00	0.00	0.00	0.00	0.00	5.19	0.00
MO18	0.00	12.93	4.43	21.25	0.00	0.00	0.00	7.44	0.00	18.99	17.60
SD±	0.00	0.56	0.21	0.65	0.00	0.00	0.00	0.12	0.00	0.21	0.53

[a]Values are means of three measurements and ± standard deviation

MO: Marine oil EPA: Eicosapentaenoic acid DHA: Docosahexaenoic acid SD±: Standard deviation

Rt: Retention time (min)

Supplementary file2. Fatty acid (%) composition[a] of MO samples (contunied)

Sample	Other	Total SFA	Total MUFA	Total PUFA	Total ω3
MO1	21.37	34.97	14.26	29.40	28.64
SD±	0.76	0.65	0.16	0.56	0.57
MO2	18.95	57.59	8.66	14.81	14.81
SD±	9.83	7.01	0.20	2.65	2.65
MO3	32.70	30.86	10.81	25.62	25.21
SD±	5.69	2.53	0.88	2.62	2.75
MO4	27.01	33.38	12.79	26.82	26.23
SD±	7.35	4.47	1.17	1.97	2.04
MO5	15.11	47.32	18.48	19.10	18.72
SD±	0.40	0.56	0.06	0.22	0.19
MO6	56.79	30.80	3.66	8.76	8.76
SD±	6.92	9.55	6.34	5.54	5.54
MO7	52.35	43.68	0.00	3.96	3.96
SD±	11.04	7.71	0.00	3.45	3.45
MO8	20.97	42.08	12.40	24.55	24.55
SD±	0.86	0.43	0.35	1.52	1.52
MO9	17.08	38.31	14.49	30.12	29.54
SD±	0.30	0.76	0.23	0.65	0.65
MO10	25.17	34.39	14.86	25.58	25.03
SD±	0.69	0.79	0.23	0.81	0.75
MO11	79.93	17.87	2.20	0.00	0.00
SD±	0.61	1.87	1.78	0.00	0.00
MO12	81.68	18.32	0.00	0.00	0.00
SD±	0.56	0.56	0.00	0.00	0.00
MO13	19.74	37.78	13.74	28.74	27.99
SD±	0.61	0.17	0.75	0.59	0.57
MO14	23.97	33.85	15.45	26.74	25.66
SD±	1.66	1.87	1.43	2.19	2.26
MO15	22.70	32.79	14.39	30.12	29.49
SD±	0.71	1.52	0.57	1.44	1.39
MO16	24.93	26.64	12.48	35.95	35.95
SD±	1.22	4.28	0.66	5.76	5.76
MO17	41.21	55.79	0.00	3.00	3.00
SD±	4.14	1.05	0.00	5.19	5.19
MO18	17.37	34.18	11.86	36.59	36.59
SD±	1.16	1.21	0.30	0.37	0.37

[a]Values are means of three measurements and ± standard deviation
MO: Marine oil
SFA: Saturated fatty acid
MUFA: Mono unsaturated fatty acid
PUFA: Poly unsaturated fatty acid
ω3: Omega-3