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Research Article

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GC Determination of Fatty Acids in Fish Supplements

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ABSTRACT

Omega-3 fatty acids (especially Docosahexaenoic acid and Eicosahexaenoic acid) are important for humans, due to their health benefits that include: supporting neurodevelopment, preventing from aging process and cognitive decline, reducing the abnormal heart rhythm and stroke in people with heart disease, possessing hypotriglyceridemic effect on type 2 diabetes, and decreasing inflammation. The aim of the current study was the application of optimized GC method for complete separation of fatty acids methyl esters in different trade supplements with fish oil, and their determination by percentage method at the following chromatographic conditions: flow rate of hydrogen: 45 ml/min, inlet pressure of carrier gas: 15 Psi and temperature program: holding on at 140 ℃ for 5 min, increasing the temperature to 240 ℃ at a rate of 4 ℃/min., maintaining at 240 ℃ for 20 min, increasing the temperature to 280 ℃ at a rate of 6 ℃/min and holding on at 280 ℃ for 10 min. After hydrolysis of esters of fatty acids, the free forms in supplements with fish oil were preesterificated with methanol solution of 14 % boron trifluoride. The content of methyl esters of fatty acids in fish food supplements was obtained by the application of percentage method. The experimental results showed that in comparison with other supplements, in Omega-3 Fish Oil Softgel, the highest concentrations of the following acids: Linolenic acid (50.71 %), Stearic acid (10.71 %) and Linoleic acid (15.49 %), were found. The suitability of the system was confirmed by the lack of a statistically significant difference between the values of the chromatographic parameter retention time in the analysis of 3 samples of t_R. The advantages of the current study were: the determination of fatty acids without derivatization and application of not expensive hydrogen as a carrier gas. The advantage of the percentage method which was used was providing very fast complete quantitative analysis of all the fatty acids. The GC percentage method which was described can be applied for routine analysis of fatty acids in fish oil food additives, after derivatization to the respective methyl esters.

Key words: *GC, Fatty Acids, Percentage Method, Determinatio*

INTRODUCTION

Fatty acids represent a substantial part of lipids in human body, and are important sources of energy. Most fish species contain the following fatty acids: Palmitic acid (C 16: 0; 15.97 % - 31.04 %), Palmitoleic acid (C 16: 1; 1.48 % - 19.61 %), Stearic acid (C 18: 0; 2.79 % - 11.20 %), Oleic acid (C 18: 1 n-9; 2.44 % - 28.97 %), Linoleic acid (C 18: 2 n-6; 0.06 % - 3.48 %), Arachidonic acid (C 20: 4 n-6, 0.12 % - 10.72 %), cis-5,8,11,14,17-Eicosapentaenoic acid (C 20: 5 n-3, 1.94 % - 10 %) and cis-4,7,10,13,16,19-Docosahexaenoic acid (C 22: 6 n-3, 3.31 % - 31.03 %). The maximum content found in fishes have been: Docosahexaenoic acid (up to 31.03 %), Palmitic acid (up to 31.04 %), Oleic acid (up to 28.97 %) and Palmitoleic acid (up to 19.61 %), and the minimun concentration has been observed for Linoleic acid (0.06 % - 3.48 %) [1].

Palmitoleic acid is an omega-7 monounsaturated fatty acid, and is a constituent of the glycerides of human adipose tissue and liver. It is biosynthesized from Palmitic acid by the action of the enzyme Stearoyl-CoA desaturase [2]. In clinical trial in humans, it has been proved that Palmitoleic acid reduces the high sensitivity of C-reactive protein and serum lipids and decreases 15 % of plasma triglycerides [3]. Palmitoleic acid increases insulin sensitivity, lowers plasma glucose and insulin levels [4], and prevents glucose induced beta-cell apoptosis [5]. Other positive effects have

been reported including: lowering the low-density lipoprotein (LDL) cholesterol, and increasing the high-density lipoprotein cholesterol (HDL) [6].

Oleic acid is a common monounsaturated fatty acid in human diet. Monounsaturated fat consumption has been associated with decreased LDL cholesterol, and possibly increased HDL cholesterol. Oleic acid is responsible for the hypotensive effect of olive oil [7]. The consumption of oleate in olive oil has been associated with a decreased risk of breast cancer [8].

Stearic acid (Octadecanoic acid) lowers LDL cholesterol [9] and reduces cholesterol absorption in animal study [10].

The essential polyunsaturated fatty acids: Linoleic (omega-6) and α-Linolenic (omega-3) acid cannot be synthesized in the body and must be obtained from food. These fatty acids are used for formation of omega-3 and omega-6 fatty acids, which are important for many functions of all tissues of the body. Deficiencies of Linoleic acid and α-Linolenic acid lead to disorders in immune function, and are connected with the development of the different diseases of liver and kidneys and depression. In opposite, the intake of Linoleic acid and α-Linolenic acid with food supplements, results in health benefits: protection from atherosclerosis, ulcerative colitis and joint pain, and prevention of heart diseases and stroke. The best sources of fish oil are cold-water fatty fish. Some of the best fish oil are from wild-caught salmon, herring, white fish, sardines and mackerel. The fish oil benefits include decreasing the risk of heart diseases and stroke, helping in symptoms of depression, hypertension, attention deficit hyperactivity disorder, joint pain, arthritis and eczema. Fish oil intake has been associated with increased energy [11].

α-Linolenic acid is essential for normal growth, and is especially important for the development of the brain and the retina [11], possesses antiarrhythmic effect [12], prevents against cardiovascular diseases [13], and protects cardiac arrest in patients with ischemic heart disease [11]. The intake of α-Linolenic acid in the diet protects against fatal ischemic heart disease, and this protection results from its antiarrhythmic effect [12], which is mediated through the syntheses of Eicosapentaenoic acid and Docosahexaenoic acid. A direct antiarrhythmic effect of Eicosapentaenoic acid has been proven. Cardiac arrhythmias can be protected not only by using food supplements, containing α-Linolenic acid, but also through the consumption of fish and fish oil, which contain large quantities of both Eicosapentaenoic and Docosahexaenoic acids (300 mg in 1g fish oil) [11]. Docosahexaenoic and Eicosapentaenoic have beneficial effects on health, and on the control of chronic diseases [14], preventing aging process and cognitive decline, supporting neurodevelopment, decreasing inflammation, possessing hypotriglyceridemic effect in type 2 diabetes. Deficiency of α-Linolenic acid causes impaired cognition and behaviour, reduces vision, and leads to abnormal electroretinogram results [11].

Linoleic acid is a polyunsaturated omega-6 fatty acid, and is used in the biosynthesis of Arachidonic acid, leukotriene A, leukotriene B, leukotriene C and thromboxane. Linoleic acid is a part of cell components, and is used to manufacture signaling molecules in the body. Deficiency of the Linoleic acid leads to poor growth, fatty liver, skin lesions, and reproductive failure [11]. Western diets contain too much Linoleic acid relative to Linolenic acid. Current diets have an omega-6: omega-3 ratio of about 20: 1, while optimal ratios should be closer to 1.5: 1. Increasing omega-3 fat intake more than omega-6 intake has been recommended [14].

Linoleic acid is a precursor of prostaglandin E2, especially in the absence of other polyunsaturated fatty acids which inhibit this conversion. Prostaglandin E2 increases the production of intrleukin-4, and decreases the formation of interleukin-1, interleukin-2 and tumor – necrosis factor-α. Through this mechanism, Linoleic acid causes the suppression of the type 1 immune response, which is the reason for autoimmune disorders: alopecia areata, Crohn's disease, insulin-dependent diabetes mellitus, multiple sclerosis, psoriasis and rheumatoid arthritis [15].

Different GC methods have been reported for the analysis of fatty acid methyl esters: gas chromatography/mass spectrometry (GC/MS) in fish oils [16], GC with flame ionization detector: in cod liver oil [17]. For the assay of fatty acids in fish oil, GC/MS method coupled with chemometric resolution techniques has been developed [18].

For identification and quantitative measurement of fish oils and fatty acids methyl esters in salmon, mackerel, catfish, phytophagous fish, cod liver, the bream GC/MS method on a capillary column: (100 m length × 0.25 mm inner diameter × 0.2 μm film thickness) has been used. By this method, polyunsaturated fatty acids which are important for human health including: Eicosapentaenoic and Docosahexaenoic acids were identified and quantified [16].

Fatty acids contained in cod liver oil were separated by gas chromatographic polar microbore column of (10 m × 0.1 mm) [17].

The characterization and determination of a complex mixture of Palmitoleic, Oleic and Stearic acid methyl esters have been performed for commercial fish oil using GC/MS method [18].

In different food samples: edible oil and dairy products, after extraction of triglycerides and their conversion to corresponding methyl esters using methanolic solution of potassium hydroxide (trans-esterification), fatty acids methyl esters were separated by GC method including: cyanopropyl capillary column DB (30 m × 0.25 mm); injector temperature: 250 °C; temperature program: 60 °C rising to 220 °C at the rate of 7 °C/min; carrier gas: helium; flow rate: 1 ml/min., flame ionization detector with temperature: 280 °C [19].
The aim of the current study was the application of optimized GC method for complete separation of fatty acids methyl esters in different trade supplements with fish oil, and their determination by percentage method.

MATERIALS

I. Food supplements, containing fatty acids.

Table 1 shows the food supplements containing fatty acids.

Table 1. Food supplements containing fatty acids

Food supplement	Produser	Content
Omega-3 Forte 1000 mg caps.	Adipharm	1000 mg Omega-3 (Salmon oil) 120 mg DHA (12 %), 180 mg EPA (18 %) 10 mg Alfa-tocopherol acetate
Norwegian Fish Oil 12/18 1000 mg caps.	ABO Pharma	Fish Oil 1000 mg 120 mg DHA (12 %), 180 mg EPA (18 %)
Omega-3 Fish Oil	Now Foods	Fish Oil 1 g polyunsaturated fatty acids 0.5 g saturated fatty acids 0.5 g monounsaturated fatty acids 120 mg DHA (12 %), 180 mg EPA (18 %)
Doppelherz aktiv Omega-3 + Vit. E caps.	Doppelherz	1000 mg Omega-3 (Salmon oil) 120 mg DHA (12 %), 180 mg EPA (18 %) 12 mg Alfa-tocopherol acetate
Omega-3 Select Softgel	Jamieson	Fish Oil 200 mg DHA, 300 mg EPA

II. Reference substances:

The reference substances included: Methyl Linoleate (Methyl *cis*,*cis*-9,12-octadecadienoate) (Serva, N:L 1876), Methyl Linolenate (Methyl *cis*,*cis*,*cis*-9,12,15-octadecatrienoate (Serva, N:62200), Methyl Nonadecanoate (Serva, N:74208), Methyl Palmitoleate (Methyl *cis*-9-hexadecenoate) (Serva, N:76176), Methyl Palmitate (Methylhexadecanoate) (Serva, N:P 5177), Methyl Stearate (Methyl octadecanoate) (Serva, N:S 5376), Methyl Oleate (Methyl *cis*-9 octadecenoate) (Serva, N:75160), Nonadecanoic acid (Serva, N:72332) (Figure 1.)

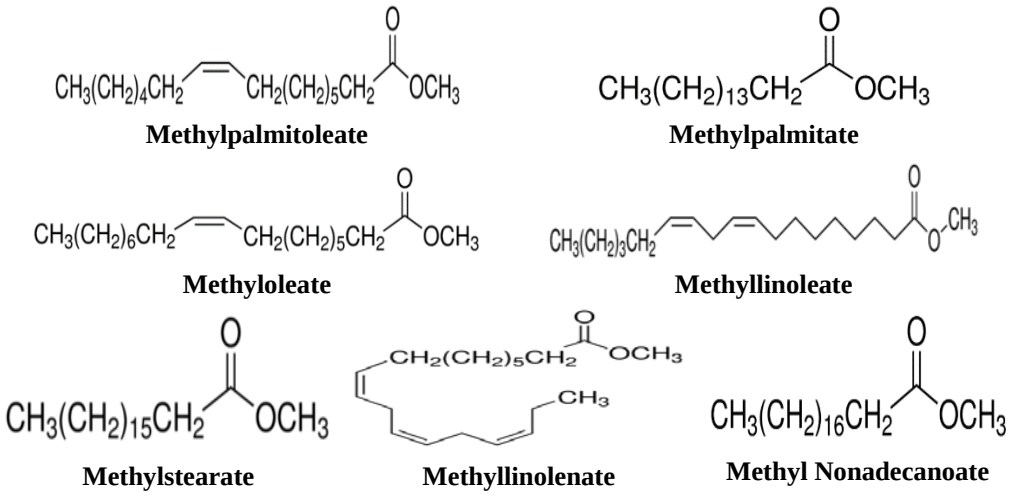

Figure 1: Fatty acid methyl esters.

III. Reagents with analytical grade quality:

The reagents with analytical grade quality included:
Derivatizing reagent: boron trifluoride (99.5 %) (Sigma Aldrich, N:339963)
n-hexane (Valerus, N:UN 1208)
isooctane (99.7 %) (Sigma Aldrich, N:59030)
methanol (99.9 %) (Sigma Aldrich, N:SZBD 063AV UN 1230)
nitrogen (Messer grisheim, N:00474)
potassium hydroxide (Fluka, N:757 551)
toluene (99.8 %) (Sigma Aldrich, N:244511).

METHODS: Gas chromatography

I. Equipment:

A gas chromatograph "Autosystem" (Perkin Elmer, USA), equipped with: a split-splitter injector, a flame ionization detector, a capillary column of ZB-1701 (cyanopropylmethylsilylsiloxane) (30 m × 0.25 mm × 0.25 μm) (Phenomenex Inc.),a hydrogen generator (HGH-300E, Beijing uiland, China), a compressor for compressed air with a system of filters; an analytical balance; and an air thermostat was used in the present study.

II. Gas chromatographic conditions.

Chromatograms of mixtures of fatty acids methyl esters were obtained at a flow rate of hydrogen: 45 ml/min, and air flow of : 450 ml/min, inlet pressure of carrier gas was 15 Psi and temperature program was at 140 °C holding for 5 min., increasing the temperature to 240 °C at a rate of 4 °C/min., holding at 240 °C for 20 min, increasing the temperature to 280 °C at a rate of 6 °C/min. and holding at 280 °C for 10 min.

III. Preparation of sample for determination of total fatty acid content.

Hydrolysis of esters of fatty acids and methylation of the free forms of fatty acids in the sample of fish oil were accomplished by preesterification with methanol solution of 14 % boron trifluoride by the following analytical procedure: in a glass reaction vessel of 6 ml, 200 μl of toluene solution of Nonadecanoic acid (C19/0) was introduced, and it was used as an internal standard.

The solvent was removed by gently flushing with nitrogen at the room temperature, and then into the vessel 2 ml of precooled reagent: 14 % boron trifluoride in methanol was added. The reaction vessels were purged with nitrogen. Purging should be short in order to avoid reducing the concentration of boron trifluoride in methanol. Into the vessel, 5 μl of sample of fish oil was added. The samples were incubated for 12 min. At 65 °C and after cooling, 1 ml distilled water and 1 ml n-hexane were added. The samples were shaken for 1-2 min. For an extraction of the methylated acids, they were transferred rapidly to 10 ml tubes, and after the separation of the phases, 200 μl of n-hexane extract was introduced in an autosampler container. Aliquots of the methylated acid extract (1 μl) were introduced into the gas chromatograph for the analysis.

RESULTS AND DISCUSSION

The development of methods for fatty acids analysis has been very important due to the requirements of health agencies to control the lipid composition in foods and food supplements in connection with the prevention of cardiovascular diseases [20], ulcerative colitis [21], anti-inflammatory potential [22] and mood disorders [23]. In the natural products, fatty acids have been present in free form and in connected forms, such as triglycerides, phospholipids, and others. The determination of the content fatty acids required hydrolysis of the connected forms and methylation of the free fatty acids. The very often method applied for hydrolysis has been with 0.5 N potassium hydroxide in methanol. For the further methylation, the methanol solution of boron thifluoride (AOAC) has been often used [24].

Marine fatty fish species are dietary sources of fatty acids. The gas chromatographic analysis has been often applied for the identification and determination of fatty acids content in marine organisms and in different food supplements, containing fish oil. The most applied technique has been the derivatization of fatty acid to respective methyl esters by boron trifluorode. For the detection of fatty acids, a flame ionization detector or mass detector has been used often [25].

For the determination of the fatty acid content in food, gas chromatography/electron ionization-mass spectrometry (GC/EI-MS) method in the selected ion monitoring mode was developed, after derivatization of fatty acids to methyl esters [26].

In the previous study done by the researchers, for separation of the methyl esters of the fatty acids (after derivatization with boron thifluoride) GC method with the following conditions was used: capillary column (30 m × 0.25 mm × 0.25 μm) with a chemically bonded stationary phase ZB-1701 (polycyanopropylmethylsiloxane), the inlet pressure of carrier gas hydrogen: 10 Psi, and the temperature gradient: from 60 °C to 135 °C. By application of this method in the mixture of Methyl Laurate, Methyl Palmitate, Methyl Heptadecanoate, Methyl Stearate, Methyl Nonadecanoate, Methyl Behenate, Methyl Lignocerate, Methyl Hexacosanoate, Methyl Palmitoleate, Methyl Oleate; Methyl Linolenate, was obtained with a good separation for all methyl esters, excluding the critical couple of methyl esters of Stearic acid and Linoleic acid. For the optimization of separation of Stearic acid and Linoleic acid, more suitable conditions as following were found: the inlet pressure of carrier gas was changed from 10 Psi to 15 Ps, and the temperature gradient was modified from 60 °C to 160 °C. By this method, methyl esters of Stearic acid and Linolenic acid were separated completely, but the separation of the esters of Oleic acid and Linoleic acid was not satisfactory. For completely separation of all the fatty acids, methyl esters, including methyl esters of Oleic acid and Linoleic acid in the previous study the following GC conditions were applied: the inlet pressure of the carrier gas was 15 Psi, and the temperature program was from 140 °C to 240 °C [27].

In current study, the analysis of the food products processed by the procedure was described, and the samples were analyzed by gas chromatography. The obtained chromatograms of samples have been presented in Fig. 2- Fig.6.

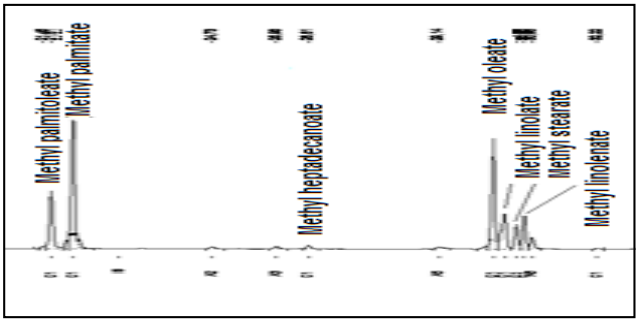

Figure 2. Chromatograms of Omega-3 Forte 1000 mg caps

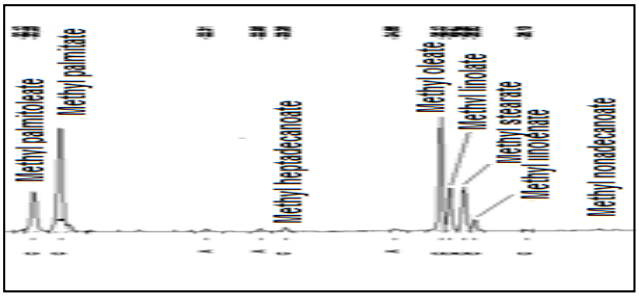

Figure 3. Chromatograms of Norwegian Fish Oil 12/18 1000 mg caps

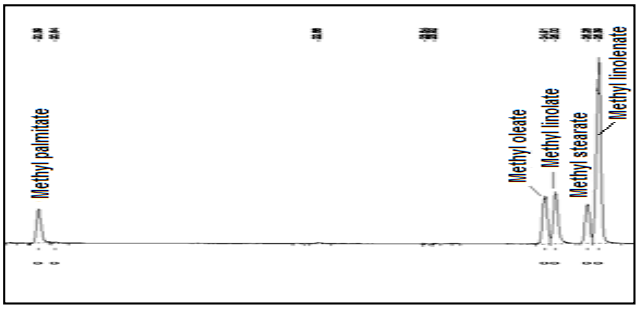

Figure 4. Chromatogram of Omega-3 Fish Oil Softgel

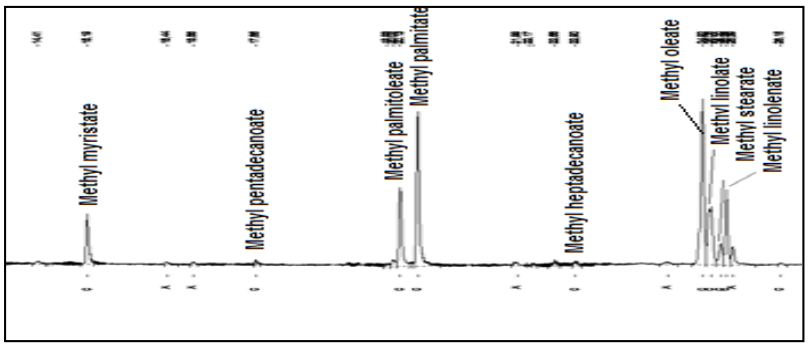

Figure 5. Chromatogram of Doppelherz Omega-3 aktiv + Vit. E caps

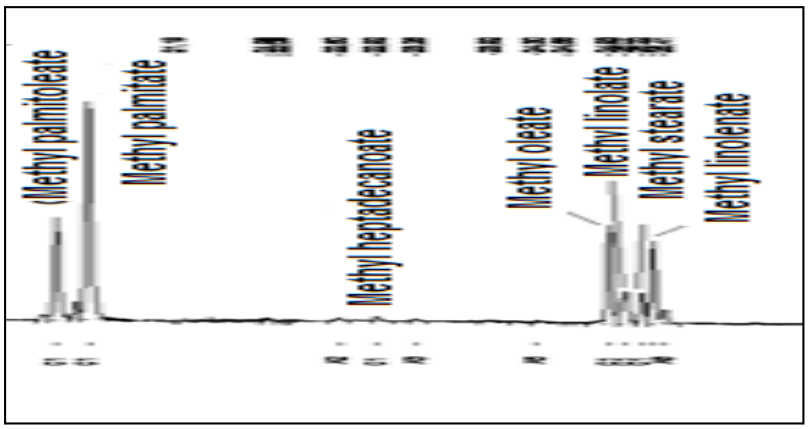

Figure 6. Chromatogram of Omega-3 Select Softgel

I. Test for system suitability

Table 2. (Methyloleate, Methyllinoleate, Methylstearate, Methyllinolenate) and Table 3. (Methyldocosahexanoate, Methyleicosapentaenoate) are summarized results for Chauvenet's criterion for t_R**.** For all of the samples the calculated related standard deviation was lower than 2, which proved the system suitability [28].

Table 2. System suitability for retention times for Methylpalmitoleate, Methylpalmitate and Methyloleate

	Methylpalmitoleate t_R [min.]	Methylpalmitate t_R [min.]	Methyloleate t_R [min.]
Norwegian Fish Oil 12/18 1000 mg caps.	20.235	20.554	25.104
Omega-3 Fish Oil Softgel	20.540	20.393	24.906
Doppelherz Омега-3 Aktiv + Vit. E caps.	20.134	20.416	24.921
Omega-3 Select Softgel	20.150	20.427	24.929
$\overline{X}$	20.265	20 448	24.965
SD	0.19	0.07	0.09
RSD [%]	0.94	0.34	0.36

Table 3. System suitability for retention times for Methyllinoleate, Methylstearate and Methyllinolenate.

	Methyllinoleate t_R [min.]	Methylstearate t_R [min.]	Methyllinolenate t_R [min.]

Norwegian Fish Oil 12/18 1000 mg caps.	25.210	25.382	25.505
Omega-3 Fish Oil Softgel	24.997	24.284	25.386
Doppelherz Омега-Aktiv + Vit. E caps.	25.055	25.204	25.295
Omega-3 Select Softgel	25.072	25.216	25.304
$\overline{X}$	25.084	25.022	25.373
SD	0.09	0.5	0.1
RSD [%]	0.36	2.0	0.39

The suitability of the system was confirmed by the lack of a statistically significant difference between the values of the chromatographic parameter retention time in the analysis of 3 samples of t_R (Table 2 and Table 3).
The experimental results for retention time, area and concentration of methyl ester in samples have been presented in Table 4 for Omega 3 Forte 1000 mg caps and Norwegian Fish Oil 12/18 1000 mg caps. Table 5 represents these items for Doppelherz Omega-3 aktiv + Vit. E caps and Omega-3 Select Softgel, and Table 6. for Omega-3 Fish Oil Softgel.

Table 4. Retention time, area and concentration of methyl ester in Omega 3 Forte 1000 mg caps. and Norwegian Fish Oil 12/18 1000 mg caps

Methyl ester	t_R [min.]	A	A [%]	C [%]
Omega 3 Forte 1000 mg caps.				
Methylpalmitoleate	21.610	5877.85	7.67	7.80
Methylpalmitate	22.037	13477.27	17.58	17.89
Methyloleate	30.201	10377.76	13.54	13.77
Methyllinoleate	30.410	4867.06	6.35	6.46
Methylstearate	30.648	2396.39	3.13	3.18
Methyllinolenate	30.796	3101.66	4.05	4.12
Norwegian Fish Oil 12/18 1000 mg caps.				
Methylpalmitoleate	20.235	3923.56	7.42	7.54
Methylpalmitate	20.554	9348.43	17.68	17.97
Methyloleate	25.104	6870.19	13.00	13.21
Methyllinoleate	25.210	3122.04	5.91	6.00
Methylstearate	25.382	3492.16	6.61	6.71
Methyllinolenate	25.505	756.97	1.43	1.46

Table 5. Retention time, area and concentration of methyl ester in Doppelherz Omega-3 aktiv + Vit. E caps., and Omega-3 Select Softgel

Methyl ester	t_R [min.]	A	A [%]	C [%]
Doppelherz Omega-3 aktiv + Vit. E caps.				
Methylpalmitoleate	20.134	13241.99	7.52	7.57
Methylpalmitate	20.416	24524.27	13.92	14.02
Methyloleate	24.921	28745.76	16.31	16.43
Methyllinoleate	25.055	15665.47	8.89	8.95
Methylstearate	25.204	4164.23	2.36	2.38
Methyllinolenate	25.295	10632.79	6.03	6.08
Methyl ester	tR [min.]	A	A [%]	C [%]
Omega-3 Select Softgel				
Methylpalmitoleate	20.150	21395.91	8.69	8.83
Methylpalmitate	20.427	49028.20	19.92	20.22
Methyloleate	24.929	22218.21	9.03	9.16
Methyllinoleate	25.072	10572.70	4.30	4.36
Methylstearate	25.216	6373.50	2.59	2.63
Methyllinolenate	25.304	16259.73	6.61	6.71

Table 6. Retention time, area and concentration of methyl ester in Omega-3Fish Oil Softgel

Methyl ester	t_R [min.]	A	A [%]	C [%]

Methylpalmitoleate	20.540	1418.22	0.49	0.49
Methylpalmitate	20.393	26534.51	9.21	9.21
Methyloleate	24.906	39010.69	13.54	13.54
Methyllinoleate	24.997	44622.86	15.49	15.49
Methylstearate	24.284	30848.63	10.71	10.71
Methyllinolenate	25.386	14573.40	50.57	50.57

In comparison with other supplements in Omega-3 Fish, Oil Softgel was found the highest concentration in the following acids: Linolenic acid (50.71 %), Linoleic acid (15.49 %) and Stearic acid (10.71 %). The experimental results showed that the quantity of Palmitoleic acid in analysed food supplements were included in interval (7.54 % ÷ 8.83 %) and only Omega-3 Fish Oil Softgel contains 0.49 % Palmitoleic acid. In Omega 3 Forte 1000 mg caps, the maximum content was for Palmitic acid (17.89 %) followed by Oleic acid (13.77 %). The same data were obtained for Norwegian Fish Oil 12/18 1000 mg caps.: Palmitic acid (17.97 %) followed by Oleic acid (13.21 %). In Doppelherz Omega-3 aktiv + Vit. E caps, the results were opposite : maximum content for Oleic acid (16.43 %) followed by Palmitic acid (14.02 %).

In comparison with other examined food supplements, Omega-3 Select Softgel was found as the maximum content of Palmitic acid (20.22 %) and the minimum content of Oleic acid (9.16 %). The experimental results showed that for Linoleic acid, the maximum concentration (15.49 %) was observed in Omega-3 Fish Oil Softgel, and the minimum content (4.36 %) was in Omega-3 Select Softgel. In comparison with other analyzed food supplements, Omega-3 Fish Oil Softgel was found as the maximum concentration of Stearic acid (10.71 %), while in Doppelherz Omega-3, aktiv + Vit. E caps were the maximum. Stearic acid was included in minimum content (2.36 %). The maximum content of Linolenic acid (50.71 %) was found in Omega-3 Fish Oil Softgel, and the minimum quantity (1.46 %) was included in Norwegian Fish Oil 12/18 1000 mg caps.

From the experimental data, it was obvious that the obtained quantities for the examined fatty acids in the investigated food supplements corresponded to the often presented content in the most fish species [1] (Table 7.).

Table 7. Fatty acid experimental result and content in the most fish species [1]

Fatty acid	Experimental result	Content in the most fish species
Palmitoleic acid	0.49 % ÷ 8.83 %	1.48 % - 19.61 %
Palmitic acid	9.21 % ÷ 20.22 %	15.97 % ÷ 31.04 %
Oleic acid	9.16 % ÷ 16.43 %	2.44 % - 28.97 %
Stearic acid	2.38 % ÷ 10.71 %	2.79 % - 11.20 %
Linoleic acid	4.36 % ÷ 15.49 %	0.06 % - 3.48 %

In the literature of the separation of Palmitoleic acid, Palmitic acid, Stearic acid, Oleic acid, Linoleic acid and Linolenic acid have been described, by using a DB-FFAP (nitroterephthalic acid modified polyethylene glycol) capillary GC column (30 m × 0.32 mm), which differed from this study's column: ZB-1701 (polycyanopropylmethylsiloxane). In investigation of [29], three inlet temperatures (210 °C, 230 °C and 250 °C) were evaluated. The authors proved that at the low inlet temperature of 210 °C, some fatty acids with high boiling points could not be eluted due to the lack of energy to vaporize. The experimental results showed that at the high inlet temperature of 250 °C, the recoveries of saturated fatty acids were better than 210 °C, but due to the thermal degradation, the peak areas of some unsaturated fatty acids decreased. At the chosen optimal inlet temperature of 230 °C, no thermal degradation was observed.

A GC/MS method for the analysis of Palmitic acid, Stearic acid, Oleic acid, Linoleic acid, after derivatization with quaternary ammonium reagent m-tri-fluoromethylphenyltrimethylammonium hydroxide has been proposed [30]. In comparison with GC/MS methods with derivatization, the difference and the advantage of the current study was the determination of fatty acids without derivatization, which is inexpensive.

In compound hepar extract preparations, Palmitic acid and Stearic acid were analyzed after extraction by ultrasonic wave by GC method at the following chromatographic conditions: HP-5 MS capillary column (30 m × 0.25 mm × 0.25 μm), column temperature: raised by programming, injector temperature: 250 °C, carrier gas: helium (1.0 ml/min). Palmitic acid and Stearic acid were confirmed by GC-MS and quantitatively analyzed with standard curve method [31]. In comparison with this method, in this current analysis, hydrogen was applied as a carrier gas instead of helium [31], and its advantage is being inexpensive.

GC method was applied for fatty acid profile in Sardina pilchardus [32], Cyprinus carpio [33], Callista umbonella [34], Portunus pelagicus [35], Rachycentron canadum [36] and Rutilus frisii kutum [37].

The fatty acid composition of Sardina pilchardus showed a high content of polyunsaturated fatty acids dominated by omega-3 fatty acids Eicosapentaenoic (15.75 %) and Docosahexaenoic acid (33.42 %). The saturated fatty acid content in different samples of sardines were from 35.50 % to 41.32 %. In this work, fatty acid methyl esters of sardine samples were prepared using a solution of 0.5 N potassium hydroxide in methanol, and were separated and quantified by gas chromatography with a split/splitless injector (split ratio of 1 : 50), fused silica capillary column (60 m × 0.25 mm × 0.25 μm), oven temperatures programmed as follows: 120 °C, held for 5 min., raised to 180 °C at 10 °C/min., then to 220 °C at 20 °C/min. and finally passed isothermal process at 220 °C for 30 min., the injector port temperature was: 220 °C, flame ionisation detector was with temperature of : 225 °C, carrier gas: helium was with a linear flow rate of 1 ml/min. [32].

In samples of Cyprinus carpio L, fatty acid methyl esters were prepared by transesterification using trimethylsulfonium hydroxide, and determined by GC method: silica cyanopropyl HP-88 column (100 m × 0.25 mm × 0.2 μm) [33]. In this work, the port temperature used was: 250 °C as the difference from the method of Benguendouz (220 °C) [32]. Other differences were in: flame ionization detector temperature: 280 °C [33] and 225 °C [32], and carrier gas: nitrogen at a flow rate: 1.33 ml/min. [33], carrier ga: helium with a linear flow rate of 1 ml/min [32]. In the current investigation for methylation, boron trifluoride in methanol was applied as the difference from trimethylsulfonium hydroxide [33].

In the samples of Callista umbonella, the most important fatty acids were Oleic, Palmitic, Myristic and Nonadecanoic, and were identified by GC-MS: capillary column HP-5 (5 % diphenyl 95 % dimethyl siloxane copolymer (30 m × 320 μm × 1 μm); helium as the carrier gas:; with injector temperature of 200 °C; and detector temperature of 280 °C. In this method, oven temperature programme (from 75 °C/min. to 270 °C at 30 °C/min was used, and the final temperature was held at 270 °C for 7 min) was different from the method proposed by [32], which was: from 120 °C to finally isothermal at 220 °C for 30 min. Fatty acid identification was done by GC/MS method using standards. From this experiment, it was proven that in Callista umbonella, the most important fatty acids were Oleic acid and Palmitic acid. The content of α-Linolenic acid was the minimum percentage (0.135 % in summer and 0.163 % in winter) [34].

Stearic acid, Oleic acid, Docosahexaenoic acid and Eicosapentaenoic acid in Portunus pelagicus were investigated by GC on DB-Wax capillary column (30 m × 0.25 mm × 0.25 mm). The initial column temperature was maintained at 100 °C for 1 min, and then at 25 °C/min, it was raised to 190 °C, and held for 10 min. and then raised to 220 °C and held for 5 min.; nitrogen at flow rates of 1 ml/min was used as the carrier gas; the injector temperature was 250 °C; the temperature for flame ionization detector was 260 °C [35]. Again in this method, the difference was observed for flame ionization detector temperature which was 260 °C [35] in comparison with 280 °C [33] and 225 °C [32]. Another difference was observed in the oven temperature programme from 100 °C to 220 °C [34] in comparison with the conditions of other methods: in which, it was from 75 °C/min to 270 °C [33] and from 120 °C to finally isothermal at 220 °C [32].

In comparison with other methods, in the current method, the temperature programme was from 140 °C to 280 °C.

Lipid extracts of Rachycentron canadum were saponified with 0.5 N methanolic sodium hydroxide and further methylated with boron trifluoride in methanol [24, 36], which was the same methylation in the current study. For the separation of the resulting fatty acid methyl esters, GC method was proposed with the following chromatographic conditions: SGE column of (30 m × 0.25 mm); helium as the carrier gas; the initial temperature of (175 °C), heating rate of (1 °C/min.), the final temperature of (220 °C); the injector temperature of (250 °C), and the flame ionization detector temperature of (270 °C). The experimental results of [36] showed that in Rachycentron canadum, the maximum content was for Palmitic acid, Oleic acid, Stearic acid, Linoleic acid and Docosahexaenoic acid.

In the samples of Rutilus frisii kutum, the fatty acid methyl esters were obtained on GC equipped with a BPX 70 SGE capillary column (60 m × 0.25 mm × 0.25 μm), the oven temperature of initially 150 ºC, rising to 190 ºC at a 2 ºC min. rate; rising to 245 ºC at a 20 ºC min, by nitrogen as the gas carrier; with the injection temperature of 230 ºC, and the flame ionization detection temperature of 260 ºC. Oleic acid and Palmitoleic acid were the predominant monounsaturated fatty acid, and Docosahexaenoic acid and Eicosapentaenoic acid were the major polyunsaturated fatty acids in the samples of Rutilus frisii kutum [37]. The flame ionization detector temperature applied in this study was used in the method of [35], 10 °C lower then used in [36], and 20 °C lower in comparison with [33] in which it was 280 °C.

GC method was developed for fatty acid composition of species of freshwater fish, marine fish and brackish water fish. From the experimental results, it has been shown that the saturated fatty acids including Palmitic and Stearic acids were present in fish species in high content; Palmitic acid was 50 % - 55 %. Palmitoleic acid and Oleic acid were the most distributed monounsaturated fatty acids, and from polyunsaturated fatty acids, the most important ones were Linoleic acid, Arachidonic acid Eicosapentaenoic acid and Docosahexaenoic acid. The highest amount of Palmitoleic acid (16.52 %) was found in stinging cat fish. The authors proved that the ratio of Oleic acid/Linoleic acid was higher in marine fish than in the freshwater fish [38].

In freshwater and marine fish species, the fatty acids methyl esters were determined by GC/MS at the following conditions: DB capillary column of (30 × 0.25 mm × 0.25 μm); column temperature of 150 °C; and helium as the carrier gas. The major saturated fatty acid was Palmitic acid, the prominent monounsaturated fatty acid was Oleic acid, and among the polyunsaturated fatty acids, omega-6 acids were dominant. The significance of this study was that it showed marine fishes were better sources of omega-3 acids while freshwater fish were good sources of omega-6 based on [39].

GC analysis of methyl esters in marine fish was performed by a capillary gas chromatograph equipped with a split-splitless injector, and the separation was done by using apolar HP88 column (100 m × 0.25 mm × 0.2 μm); 200 °C column temperature; helium as the carrier gas: at a linear velocity of 30.0 ml/min.; with injection port temperature of 250 °C, and the flame ionization detector temperature of 250 °C [40]. In comparison with all of these methods in which helium was applied as the carrier gas [34, 36, 39, 40], and nitrogen was used in [37], in the current analysis, hydrogen was used, which advantage is that it is not expensive.

In the current study in the analyzed food supplements, the peaks of the methylated fatty acids were identified by the retention times by comparison with the available reference standards including: methyl esters of Palmitic acid, Palmitoleic acid, Stearic acid, Oleic acid, Linoleic acid, and Linolenic acid. The quantitative analysis of the compounds was accomplished by the comparison of the chromatographic peak areas of the components of the test sample with the comparative reference standard, having a known quantity, by applying the methods of the external standard or the internal standard, and the application of the method of the assessment of the relative values of the chromatographic peak areas of analytes to the sum of the areas in all of the components of the analyzed sample, when they all were exhibited in the chromatogram, and their analytical signals were comparable in intensity.

In the case of analysis of the fatty acids in the objects, that were in the whole composition of lipids (which were investigated in the present work preparations), and using the flame ionization detector, which had practically the same factors of the signals (response factors), for the determination of the content of the methyl esters of the hydrolyzed, and methylated samples in the investigated preparations were suitable and appropriate for administrating the percentage method.

The percentage method for quantitative analysis was based on measuring the area of all the peaks in the chromatogram of the hydrolyzed and methylated samples, and calculating their sum (excluding the peak area of the solvent), whereby the area of each peak was calculated as the percentage of the total area of the chromatographic peaks. For analytical samples with entirely volatile components, and in case of using a flame ionization detector, relative peak areas corresponded to their percentage content in the analyzed object. The advantage of percentage method used in the current study was that it provided very fast complete quantitative analysis of all the fatty acids.

In comparison with Callista umbonella, α-Linolenic acid content was (0.135 % in summer and 0.163 % in winter) [37], in the investigated food supplements, α-Linolenic acid was found in higher concentration: 50.71 % in Omega-3 Fish Oil Softgel, and even in Norwegian Fish Oil 12/18 1000 mg caps. This acid presented in higher quantity (1.46 %) than in Callista umbonella [34]. The quantity of Palmitoleic acid was (7.54 % ÷ 8.83 %) in the analyzed food supplements of this study, which was about 2 times lower than the content of this fatty acid (16.52 %) which was found in stinging cat fish [38].

In the work of [41], the fatty acid composition of different fish oils as softshell turtle oil, freshwater eel oil, shark liver oil, tuna oil, and lemuru oil were analyzed and quantified using gas chromatography by applying the same capillary column as in the current investigation: cyanopropyl methyl silylsiloxane column. The authors proved that the major free fatty acids in softshell turtle oil were Oleic acid (32.22 %), Palmitic acid (19.95 %) and Linoleic acid (7.77 %). Palmitic acid (10.41 %), Oleic acid (28.25 %) and Linoleic acid (4.27 %) were also included in freshwater eel oil. Palmitic acid (9.76 %) was included in the highest concentration of Lemuru oil [41].

As a difference from the work done by [41], in the current investigation, other fatty acids were found in the highest concentrations including: Linolenic acid (50.71 %) and Stearic acid (10.71 %) (Omega-3 Fish Oil Softgel). Oleic acid

was the predominant fatty acid in sofshell turtle oil (32.22 %) and freshwater eel oil (28.25 %) [41], while in the current study, in the food supplements contained fish oil, the concentration of this fatty acid was about 2 times lower (9.16 % ÷ 16.43 %). In comparison with α-Linolenic acid content in softshell turtle oil, freshwater eel oil, shark liver oil, tuna oil, and lemuru oil in the current investigation of food supplements, α-Linolenic acid was found in higher concentration of: 50.71 % in Omega-3 Fish Oil Softgel.

CONCLUSION

The sensitivity of the developed GC method of the analysis was high enough – that could determine the low concentrations of fatty acids. Retention times of peaks of esters were closer, but the peaks were well-separated.
The suitability of the system was confirmed by the lack of a statistically significant difference between the values of the chromatographic parameter retention time in the analysis of 3 samples of $t_{R.}$
In comparison with GC/MS methods with derivatization, the difference and the advantage of the current study was the determination of fatty acids without derivatization, which was easier and inexpensive.
In comparison with other methods in which helium and nitrogen were used, in the current analysis, hydrogen was applied as the carrier gas, and its advantage was being inexpensive.
The advantage of percentage method used in current study was that it provided very fast complete quantitative analysis of all the fatty acids.
The method used in the current study can be applied in routine analysis for the identification and determination of fatty acids in food additives after derivatization to methyl esters.

Conflict of interests

All authors had none to declare.

REFERENCES

1. Ozogul, Y., Ozogul, F., Ciçek, E., Polat, A., Kuley, E. Fat content and fatty acid compositions of 34 marine water fish species from the Mediterranean Sea. *Int. J. Food Sci. Nutr.*, 2009, 60(6), 464-475.
2. Hodson, L., Fielding, B.A. Stearoyl-CoA desaturase: rogue or innocent bystander? *Prog. Lipid Res.*, 2013, 52(1), 15-42.
3. Bernstein, A.M., Roizen, M.F., Martinez, L. Purified Palmitoleic acid for the reduction of highsensitivity C-reactive protein and serum lipids: a double-blinded, randomized, placebo controlled study. *J. Clin. Lipidol.*, 2014, 8(6), 612-617.
4. Yang, Z.H., Miyahara, H., Hatanaka, A. Chronic administration of Palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay mice with genetic type 2 diabetes. *Lipids Health Dis.*, 2011, 10(1), 1-8.
5. Morgan, N.G., Dhayal, S. Unsaturated fatty acids as cytoprotective agents in the pancreatic beta-cell. *Prostaglandins Leukot. Essen Fatty Acids*, 2010, 82(4-6), 231-236.
6. Mozaffarian, D., Cao, H., King, I.B., Lemaitre, R.M., Song, X., Siscovick, D.S., Hotamisligil, G.S. Trans-Palmitoleic acid, metabolic risk factors, and new-onset diabetes in U.S. adults: a cohort study. *Ann. Intern. Med.*, 2010, 153(12), 790-799.
7. Teres, S., Barcelo-Coblijn, G., Benet, M., Alvarez, R., Bressani, R., Halver, J. E., Escriba, P.V. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. *Proceed. Nat. Acad. Sci.*, 2008, 105(37), 13811-13816.
8. Martin-Moreno, J.M., Willett, W.C., Gorgojo, L., Banegas, J.R., Rodriguez-Artalejo, F., Fernandez-Rodriguez, J.C., Maisonneuve, P., Boyle, P. Dietary fat, olive oil intake and breast cancer risk. *Int. J. Cancer*, 1994, 58(6), 774-780.
9. Mensink, R.P. Effects of Stearic acid on plasma lipid and lipoproteins in humans. *Lipids*, 2005, 40(12), 1201-1205.
10. Schneider, C.L., Cowles, R.L., Stuefer-Powell, C.L., Carr, T.P. Dietary Stearic acid reduces cholesterol absorption and increases endogenous cholesterol excretion in hamsters fed cereal-based diets. *J. Nutr.*, 2000, 130(5), 1232-1238.
11. Connor, W.E. α-Linolenic acid in health and disease. *Am. J. Clin. Nutr.*, 1999, 69(5), 827-828.

12. Hu, F.B., Stampfer, M.J., Manson, J.E., Rimm, E.B., Wolk, A., Colditz, G.A., Hennekens, C.H., Willett, W.C. Dietary intake of α-Linolenic acid and risk of fatal ischemic heart disease among women. *Am. J. Clin. Nutr.*, 1999, 69(5), 890-897.
13. Pan, A., Chen, M., Chowdhury, R., Wu, J.H., Sun, Q., Campos, H., Mozaffarian, D., Hu, F.B. α-Linolenic acid and risk of cardiovascular disease: a systematic review and meta-analysis. *Am. J. Clin. Nutr.*, 2012, 96(6), 1262-1273.
14. Shahidi, F. Omega-3 fatty acids in health and disease. In: Omega-3 oils: applications in functional foods, AOCS Press, 2011.
15. Namazi, M.R. The beneficial and detrimental effects of Linoleic acid on autoimmune disorders. *Autoimmunity*, 2004, 37(1), 73-75.
16. Bratu, A., Mihalache, M., Hanganu, A., Chira, N.A., Todaşcă, M.C., Roşca, S. Quantitative determination of fatty acids from fish oils using GC-MS method and 1H-NMR spectroscopy. *U.P.B. Sci. Bull. Series B*, 2013, 75(2), 23-32.
17. Mondello, L., Tranchida, P.Q., Dugo, P., Dugo, G. Rapid, microscale preparation and very fast gas chromatographic separation of cod liver oil fatty acid methyl esters. *J. Pharm. Biomed. Anal.*, 2006, 41(5), 1566-1570.
18. Jalali-Heravi, M., Vosough, M. Characterization and determination of fatty acids in fish oil using gas chromatography–mass spectrometry coupled with chemometric resolution techniques. *J. Chromatogr. A*, 2004, 1024(1-2), 165-176.
19. Petrovic, M., Kezic, N., Bolanc, V. Optimization of the GC method for routine analysis of the fatty acid profile in several food samples. *Food Chem.*, 2010, 122(1), 285-291.
20. Kris-Etherton, P.M., Harris, W.S., Appel, L.J. Fish consumption, fish oil, omega-3 fatty acids and cardiovascular disease. *Circulation*, 2002, 106(21), 2747-2757.
21. Stenson, W.F., Cort, D., Rodgers, J., Burakoff, R., DeSchryver-Kecskemeti, K., Gramlich, T.L., Beeken, W. Dietary supplementation with fish oil in ulcerative colitis. *Ann. Intern. Med.*, 1992, 116(8), 609-614.
22. Wall, R., Ross, R.P., Fitzgerald, G.F., Stanton, C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr. Rev.*, 2010, 68(5), 280-289.
23. Hegarty, B, Parker, G. Fish oil as a management component for mood disorders – an evolving signal. *Curr. Opin. Psychiatry*, 2013, 26(1), 33-40.
24. AOAC. Official methods of analysis. 17th ed Association of Official Analytical Chemists, Washington, Arlington, USA, 2000.
25. Tang, B., Row, K.H. Development of Gas chromatography analysis of fatty acids in marine organisms. *J. Chromatogr. Sci.*, 2013, 51, 599-607.
26. Thurnhofer, S., Vetter, W. A gas chromatography/electron ionization-mass spectrometry-selected ion monitoring method for determining the fatty acid pattern in food after formation of fatty acid methyl esters. *J. Agric. Food Chem.*, 2005, 53(23), 8896-8903.
27. Hadjieva, B.R., Tsvetkova, D.D., Obreshkova D.P. Development and optimization of chromatographic systems for separation of fatty acids by gas chtomatographic method. *World J. Pharm. Pharm. Sci.*, 2017, 6(4), 12-23.
28. International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human use, ICH harmonized tripartite Guideline, Validation of Analytical procedures Text and methodology Q2 (R1), 2005.
29. Zhang, H., Wang, Z., Liu, O. Development and validation of a GC-FID method for quantitative analysis of Oleic acid and related fatty acids. *J. Pharm. Anal.*, 2015, 5(4), 223-230.
30. Manzano, E., Rodriguez-Simón, L.R., Navas, N., Checa-Moreno, R., Romero-Gámez, M., Capitan-Vallvey, L.F. Study of the GC-MS determination of the Palmitic-Stearic acid ratio for the characterisation of drying oil in painting : La Encarnación by Alonso Cano as a case study. *Talanta*, 2011, 84(4), 1148-1154.
31. Ai, Y., Li, Z., Zhang, Y.F., Wang, Y.J., Li, R., Zhang, F., Yang, X.D. GC-MS determination of Palmitic acid and Stearic acid in compound hepar extractum preparations. *Chinese J. Pharm. Anal.*, 2016, 36(10), 1875-1879.
32. Benguendouz, A., Bouderoua, K., A. Bouterfa, K., Belabes, M., Bekada, A., Siori-ki, E., Zabetakis I. Fatty acid profile and assessment of heavy metals content of Sardina pilchardus captured in the Algerian coast. *Iranian J. Fisheries Sci.*, 2017, 16(3), 1021-1029.

33. Ljubojević, D., Radosavljević, V., Pelić, M., Đorđević, V., Živkov-Baloš, M., Ćirković, M. Fatty acid composition, chemical composition and processing yield of traditional hot smoked common carp (Cyprinus carpio, L). *Iranian J. Fisheries Sci.*, 2016, 15(4), 1293-1306.
34. Sajjadi, N., Mooraki, N. Determination and study the fatty acid contents and their seasonal variations by temperature of a dominant bivalve (Callista umbonella) of Haleh Creek. *Iranian J. Fisheries Sci.*, 2016, 15(3), 1134-1143.
35. Baboli J., Velayatzadeh M., Roomiani L., Khoramabadi A. Effect of sex and tissue on fatty acid composition in the meat of blue swimming crab (Portunus pelagicus) from the Persian Gulf, *Iran. Iranian J. Fisheries Sci.*, 2016, 15(2), 818-826.
36. Taheri S., Moghanjoghi, A.A.M., Taheri, T., Faraji, S., Pourashouri, P. Inhibition of fatty acids profile changes of Cobia (Rachycentron canadum) fillets during frozen storage by packaging under vacuum system. *Iranian J. Fisheries Sci.*, 2015, 14(1), 275-288.
37. Bakhtiarvandi, N.K., Kenari, A.A., Nazari, R.M., Makhdoomi, C. Ontogenetic changes in lipids, fatty acid, and body composition during larval stages of Caspian kutum (Rutilus frisii kutum). *Iranian J. Fisheries Sci.*, 2014, 13(2), 365-383.
38. Nair, P.G.V., Gopakumar, N.K. Fatty acids composition of 15 species of fish from tropical waters. *J. Food Sci.*, 1978, 43(4), 1162-1164.
39. Ugoala, C., Ndukwe, G.I., Audu, T.O. Comparison of fatty acids profile of some freshwater and marine fishes. *Internet J. Food Safety*, 2008, 10(1), 9-17.
40. Aziz, N.A., Azlan, A., Ismail, A, Alinafiah, S.M., Razman MR. Quantitative determination of fatty acids in marine fish and shellfish from warm water of straits of Malacca for nutraceutical purposes. *Bio. Med. Res. Int.*, 2013, 2013, 1-12.
41. Suseno, S.H., Saraswati, Hayati, S., Izaki, A.F. Fatty acid composition of some potential fish oil from production centers in Indonesia. *Orient. J. Chem.*, 2014, 30(3), 975-980.