

QUANTITATIVE DETERMINATION OF FATTY ACIDS FROM FISH OILS USING GC-MS METHOD AND $^1\text{H-NMR}$ SPECTROSCOPY

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Seven species of fish (salmon, mackerel, catfish, two species of phytophagous fish, cod liver, bream) were studied for their oil fatty acids composition. Crude fish oils were obtained by standard Soxlet extraction method. Identification and quantitative measurement of fish oils fatty acids were carried out by gas chromatography coupled with mass spectrometry and by $^1\text{H-NMR}$ spectroscopy. Fourteen individual fatty acids were identified using GC-MS technique. GC-MS was applied on fatty acids methyl esters. Identification of fatty acids from oil samples was made using a standard mixture of 37 fatty acid methyl esters. $^1\text{H-NMR}$ spectroscopy gives information about classes of unsaturated and saturated fatty acids. By this method can also determine the ω -3 and docosahexaenoic fatty acid content. Using both analytical methods, polyunsaturated fatty acids important for human health (eicosapentaenoic and docosahexaenoic acids) were identified and quantified.

Keywords: fish oil, GC-MS, $^1\text{H-NMR}$, fatty acids composition

1. Introduction

In the last years the interest for food quality, dietary fats and their influence on human health has greatly increased. It is known that a low fatty acids diet is generally healthier, but for growing and proper development and function, the human body needs a certain amount of fats. Consumption of foodstuff that contains a large amount of saturated fatty acids is associated with heart disease, diabetes, cancer; therefore, the diet must contain unsaturated fatty acids.

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Polyunsaturated fatty acids (PUFA), especially ω -3 fatty acids (DHA-docosahexaenoic acid, EPA-eicosapentaenoic acid) are essential dietary nutrients for human health; they are defined “essential” fatty acids since they cannot be synthesized by the human body and consequently they must be provided from the diet [1]. PUFAs play important roles in the human body, such as in the synthesis of specific active compounds, in the brain and eye development of infants or in reducing the „bad” cholesterol and thus in the prevention of the coronary heart disease [2, 3, 4].

Marine organisms (fish, seafood, algaes) are the main natural sources of essential fatty acids in human diet (mainly EPA and DHA). Fish oil is considered to have the highest amounts of ω -3 PUFA [5, 6, 7].

In this study, we report the use of gas-chromatography coupled with mass spectrometry (GC-MS) and ^1H -NMR spectroscopy for the determination of the fatty acids composition in fish oils. Fourteen individual fatty acids were identified and measured using the GC-MS method. By using ^1H -NMR spectra two classes of fatty acids (unsaturated and saturated), ω -3 and DHA have been quantified.

2. Experimental part

The fish oils samples subjected to this study were extracted according to the Soxhlet protocol [8] from seven species of fish (salmon, mackerel, catfish, two species of phytophagous fish, cod liver, and bream).

The standard mixture of 37 fatty acids methyl esters (SupelcoTM 37 Component FAME Mix) used for the gas-chromatographic analyses was purchased from Supelco.

Fatty acid methyl esters (FAME) were prepared by transesterification of oils with methanol, using $\text{BF}_3\text{-MeOH}$ complex as catalyst, according to the standard method [9].

The gas-chromatograms of the fatty acid methyl esters mixtures were recorded on an Agilent Technologies model 7890A instrument coupled with an Agilent Technologies model 5975 C VL MSD mass detector with Triple Axis Detector and Agilent auto-sampler. The separation into components was made on a capillary column especially designed for the fatty acids methyl esters (FAME) analysis (Supelco SPTM 2560, with the following characteristics: 100 m length, 0.25 mm inner diameter, 0.2 μm film thickness). The ready for injection solutions were prepared in CH_2Cl_2 of HPLC purity grade. Fatty acids identification was made by comparing for each peak the retention time with those of a standard mixture of 37 fatty acid methyl esters (SupelcoTM 37 Component FAME Mix). In the standard mixture the exact concentration of each component is known. Both standard mixture and each of the fatty acid methyl esters of the analyzed fish oils were chromatographically separated under the same conditions, using the same

temperature program (oven initial temperature 140 °C to final temperature 240 °C, heating rate 4 °C/min.), injection volume 1 μL , split rate 100:1, carrier gas He according to the Supelco specifications. The calibration of the signals was made by taking into account the concentration of each component of the standard mixture, correlated with the detector's response.

The $^1\text{H-NMR}$ spectra of the fish oils extracted were recorded on a Varian INOVA 400 spectrometer, operating at 9.4 Tesla, corresponding to the resonance frequency of 399.95 MHz for the ^1H nucleus, equipped with a direct detection four nuclei probe head and field gradients on z axis. Samples were analyzed in 5 mm NMR tubes (Norell 507). The chemical shifts are reported in ppm, using the TMS as internal standard. Typical parameters for $^1\text{H-NMR}$ spectra were: 45° pulse, 2.05 s acquisition times, 6.4 KHz spectral window, 32 scans, 26 K data points. The FID was not processed prior to Fourier transform. The average acquisition time of the $^1\text{H-NMR}$ spectra was approximately 2 minutes. The sample preparation was simply reduced to the dilution of 20 μL of fish oil in 80 μL of CDCl_3 .

3. Results and discussions

Determination of fatty acids composition of fish oils samples using GC-MS spectrometry

Gas-chromatography coupled with mass spectrometry was used to identify and measure the composition of fatty acids present in fish oils. Fig.1 illustrates the chromatogram of mackerel oil fish and Table 1 presents the fatty acids composition of the analyzed fish oils.

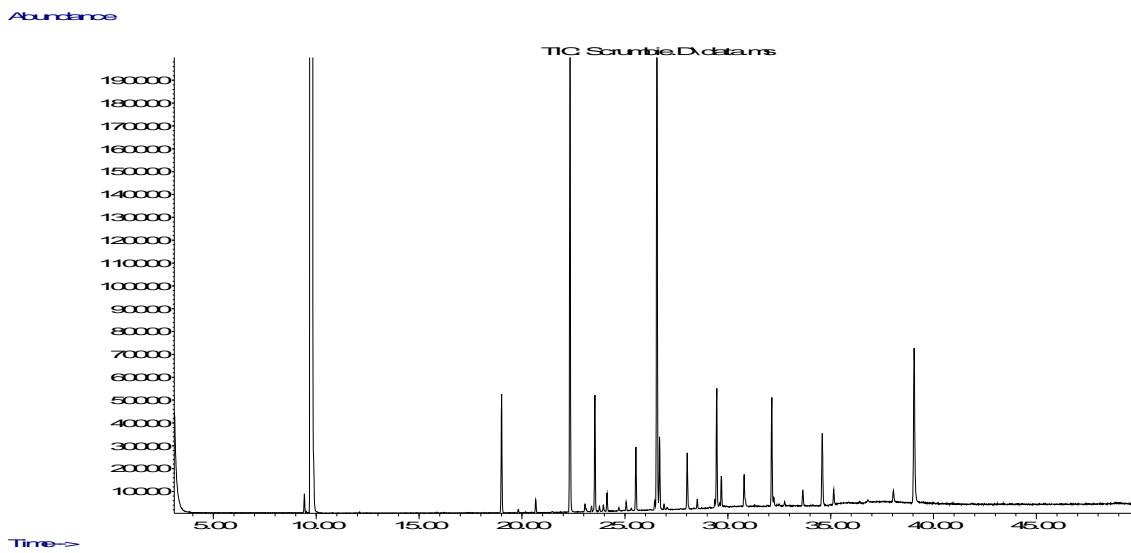


Fig.1. Chromatogram of mackerel oil

Table 1

Fatty acids composition of fish oils obtained by GS-MS technique

Fatty acids composition (% mol)	Retention times (min)	Cod liver oil	Catfish oil	Mackerel oil	Phytophagous 1	Phytophagous 2	Salmon	Bream
Myristic C14:0	18.9	5.4	4.5	6.5	4.6	3.7	7.0	2.9
Palmitic C16:0	22.3	13.0	21.4	22.0	18.6	19.2	14.7	20.8
Palmitoleic C16:1	23.5	9.3	13.7	6.0	12.5	10.7	7.5	5.2
Stearic C18:0	25.5	2.9	4.1	2.5	3.4	4.1	2.1	10.6
Oleic C18:1	26.5	16.9	27.9	27.0	21.9	22.5	20.2	19.4
Linolic C18:2	28.04	1.9	5.1	3.0	3.8	2.4	6.8	5.7
cis-11-eicosenoic C20:1	29.4	10.8	0.0	4.7	0.0	0.0	12.8	4.0
Linolenic C18:3	29.7	1.0	2.8	1.7	7.8	8.8	2.2	2.0
cis-11,14-eicosadienoic C20:2	30.8	2.6	0.0	0.0	2.4	2.7	2.3	3.8
Tricosanoic C23:0	32.7	0.4	1.2	0.0	1.3	0.9	0.0	0.0
cis-13,16-docosadienoic C22:2	33.6	0.7	1.2	0.9	2.7	3.9	1.7	2.8
EPA C20:5	34.6	15.5	6.5	5.9	12.5	13.4	7.7	8.6
Nervonic C24:1	35.1	0.4	0.0	0.7	0.0	0.0	0.5	0.0
DHA C22:6	39.07	19.2	11.6	19.1	8.5	7.7	14.5	14.2

As it can be remarked from Table 1 fish oils have a very high amount of ω -3 polyunsaturated fatty acids (especially docosahexaenoic fatty acid). Fish species such as cod, mackerel, salmon and bream have the highest values for the most polyunsaturated fatty acid (DHA), while the phytophagous species have the lowest content. The mono-unsaturated fatty acids (MUFA) content in the analyzed fish oils are approximately identical. As we expected, small amounts of ω -6 fatty acids have also been determined. The saturated fatty acid present in all the investigated samples in larger amount is palmitic acid.

There is plenty of scientific research dealing with ω -3 and ω -6 fatty acids. It is well known that for human health a ω -6/ ω -3 unsaturated fatty acids ratio of

5:1 or less is desired. Because nowadays diet is characterized by a high consumption of junk food the ω -6/ ω -3 ratio is up to 25:1 [See for example a recent review [5] and the literature cited], this is why it is very important to consume products rich in ω -3 such as fish, some vegetable oils (nuts, rapeseed, soybean oils) or ω -3 fatty acids enriched products. Thus, ω -6/ ω -3 ratio for the analyzed fish oils was calculated (see Table 2).

Table 2

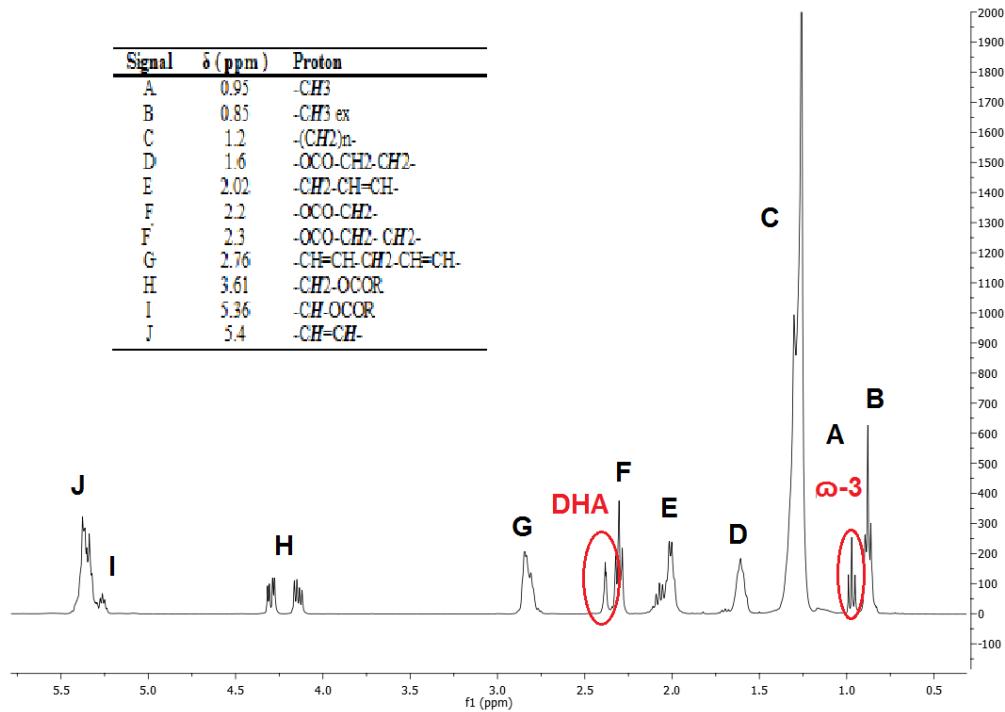
ω -6/ ω -3 ratio calculated in fish oils	
Fish oil sample	ω -6/ ω -3 ratio
Cod liver oil	1/7
Catfish oil	1/3
Mackerel oil	1/8
Phytophagous 1	1/3
Phytophagous 2	1/3
Salmon	1/2
Bream	1/2

As it can be noted from Table 2, cod liver and mackerel have the best value for the ω -6/ ω -3 ratio.

Determination of fatty acids composition of fish oils samples using $^1\text{H-NMR}$ spectroscopy

Another method used to determine the fatty acid composition of the fish oils samples was $^1\text{H-NMR}$ spectroscopy; in this respect, the spectra of fish oils were recorded. The spectra were integrated in triplicate, the mean integral being used in the following computation.

Fig. 2 presents the $^1\text{H-NMR}$ spectrum of mackerel oil, the chemical shifts and pick assignment of the spectrum (according to the literature [10, 11]).

Fig. 2. ^1H -NMR spectrum of mackerel oil

Based on the integral values from the ^1H -NMR spectra the composition of fish oils was estimated on two classes of fatty acids: unsaturated and saturated. From the ^1H -NMR spectra ω -3 fatty acids and DHA (docosahexenoic acid) can be also calculated.

For the chemometric equations the following notation were adopted:

- n , s , ω -3, h represents the molar ratio of unsaturated, saturated, omega-3 fatty acids and docosahexenoic acid;
- x represents the number of double bonds from the polyunsaturated fatty acids;
- I_A , I_B , I_C , etc. represent the integral values of the signals;
- k is a coefficient which correlates the signal integral with the number of protons that signal is due to [12,13].

Fatty acids composition from ^1H -NMR spectra was determined on the basis of the proton balances accounting for different signals in the spectrum.

$$\text{balance on signal A: } I_A = k \cdot 3 \cdot \omega_3 \quad (1)$$

$$\text{balance on signal B: } I_B = k \cdot 3 \cdot (s + n - \omega_3) \quad (2)$$

$$\text{balance on signal D: } I_D = k \cdot 2 \cdot (s + n - h) \quad (3)$$

$$\text{balance on signal E: } I_E = k \cdot 4 \cdot n \quad (4)$$

- balance on signal F: $I_F = k \cdot 2 \cdot (s + n - h)$ (5)

- balance on signal F': $I_{F'} = k \cdot 4 \cdot h$ (6)

- balance on signal G: $I_G = k \cdot 2 \cdot n \cdot (x - 1)$ (7)

- balance on signal H: $I_H = \frac{4 \cdot k}{3}$ (8)

- balance on signal J: $I_J = k \cdot 2 \cdot x \cdot n$ (9)

Another equation is: $s + n = 1$ (10)

Chemometric equation for the composition determination

The molar ratio of the ω -3 fatty acids can be determined from equation (1):

$$\omega_3 = \frac{I_A}{3 \cdot k} \quad (11)$$

The molar ratio of docosahexaenoic fatty acid can be calculated from equation (6):

$$h = \frac{I_{F'}}{4 \cdot k} \quad (12)$$

Another equation which molar ratio of DHA [10] can be calculated is:

$$h = \frac{I_{F'} / 2}{I_{F'} / 2 + I_F} \cdot 100 \quad (13)$$

The molar ratio of unsaturated fatty acids is obtained from equation (4):

$$n = \frac{I_E}{4 \cdot k} \quad (14)$$

The molar ratio of saturated fatty acids is obtained from equation (2), (3) or (10):

$$s = \frac{I_B}{3 \cdot k} - n + \omega_3 \quad (15)$$

$$s = \frac{I_D}{2 \cdot k} - n + h \quad (16)$$

$$s = 1 - n \quad (17)$$

The number of the double bonds can be obtained using equation (9):

$$x = \frac{I_J}{2 \cdot k \cdot n} \quad (18)$$

From equation (8):

$$k = \frac{3 \cdot I_H}{4} \quad (19)$$

Based on these equations the composition of fish oil samples was calculated on two classes: saturated and unsaturated fatty acids as total. From unsaturated fatty acids we determined the ω -3 fatty acids content and from this we quantified the DHA fatty acid. The results are presented in Table 3.

Table 3
Fatty acids composition determinate from $^1\text{H-NMR}$ spectra

Fatty acids	Sample (%mol)						
	Cod liver	Catfish	Mackerel	Phytophagous 1	Phytophagous 2	Salmon	Bream
Unsaturated	79.05	68.33	69.95	71.66	72.98	76.35	66.21
Saturated	20.95	31.67	30.05	28.34	27.02	23.65	33.79
ω -3	34.44	22.22	26.30	27.38	28.00	23.87	24.00
DHA	19.18	12.00	18.80	7.81	7.00	13.98	14.00

The results obtained from the methods previously presented were compared (see Table 4).

Table 4
Comparison of fatty acids profiles from fish oil between $^1\text{H-NMR}$ and GC-MS methods

Fish oil sample	$^1\text{H-NMR}$				GC			
	Unsaturated fatty acids	Saturated fatty acids	ω -3	DHA	Unsaturated fatty acids	Saturated fatty acids	ω -3	DHA
Cod liver	79.0	21.0	34.4	19.2	78.3	21.7	35.7	19.2
Catfish	68.3	31.7	22.2	12.0	68.8	31.2	20.9	11.6
Mackerel	69.9	30.1	26.3	18.8	69.0	31.0	26.7	19.1
Phytophagous 1	71.7	28.3	27.4	7.8	72.1	27.9	28.8	8.5
Phytophagous 2	73.0	27.0	28.0	7.0	72.1	27.9	29.9	7.7
Salmon	76.4	23.6	23.9	14.0	76.2	23.8	24.4	14.5
Bream	66.2	33.8	24.0	14.0	65.7	34.3	24.8	14.2

The two methods give similar results with attached advantages and disadvantages. GC-MS method has the advantage that offers information about individual fatty acids present in fish oils, but it is very laborious and time-consuming because the fats must be turned into fatty acid methyl esters (FAME).

However, $^1\text{H-NMR}$ spectroscopy is an alternative method, the advantages being that it is fast, accurate and it can be applied directly on triglycerides, without any sample preparation. By using $^1\text{H-NMR}$ spectroscopy the fatty acid composition can be determined only on classes of fatty acids.

4. Conclusions

The fish oil samples were extracted according to the Soxhlet method from seven species of fish (salmon, mackerel, catfish, two species of phytophagous fish, cod liver, and bream).

For determination of fatty acids from oil samples, fats were converted into corresponding methyl esters by transesterification with methanol, using $\text{BF}_3\text{-MeOH}$ complex as catalyst, according to the standard method. The GC-MS chromatograms were recorded.

The $^1\text{H-NMR}$ spectra of fish oil samples were recorded on triglycerides.

Using GC-MS technique fourteen individual fatty acids present in fish oil samples were identified and quantified, while based on $^1\text{H-NMR}$ spectroscopy the composition of fish oils was determined on two classes of fatty acids (unsaturated as total-of them ω -3 and DHA individually- and saturated).

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