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FATTY ACID METHYL ESTERS GC/FID ANALYSIS USING ICHIHARA-FUKUBAYASHI MODYFIED METHOD

Alexei Baerle, ORCID: 0000-0001-6392-9579,
Angela Gurev *, ORCID: 0000-0001-8493-5257,
Veronica Dragancea, ORCID: 0000-0002-5938-0410,
Iurie Subotin, ORCID: 0000-0002-5570-4713,
Raisa Druta, ORCID: 0000-0001-5301-6055

Technical University of Moldova, 168 Stefan cel Mare Blvd., Chisinau, Republic of Moldova
* Corresponding author: Angela Gurev*: angela.gurev@chim.utm.md*

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Abstract. The method for preparing fatty acid methyl esters (FAMEs) from lipids for gas-liquid chromatography, developed by K. Ichihara and Y. Fukubayashi, was optimized as a result of research on the influence of mild (45 °C, for 14 h) and fast (100 °C, for 1 h) methanolysis-methylation conditions on the FAMEs yield. Based on the minor impact on the final products and the good FAMEs yields, the optimized mild methanolysis/methylation method was applied to eight vegetable oil samples and two types of fish oil dietary supplements. The FAMEs compositions were analyzed by gas chromatography with flame ionization detector (GC/FID). On the recorded chromatograms, 16 types of fatty acids with majority content were identified and their mass fractions were calculated. The modified Ichihara-Fukubayashi method allowed the conversion of ester-bound fatty acids and free acids to FAMEs in a single step. The accuracy, accessibility and convenience of the method, the use of low concentrations of samples and reagents were noted.

Keywords: *coelution, fish oil, methylation, omega-3, omega-9, oily seeds, toluene.*

Rezumat. Metoda de preparare a esterilor metilici ai acizilor grași (EMAG) din lipide pentru cromatografia gaz-lichid, elaborată de K. Ichihara și Y. Fukubayashi, a fost optimizată în rezultatul cercetării influienței condițiilor de metanoliza/metilare lentă (45 °C, timp de 14 h) si rapidă (100 °C, timp de 1 h) asupra randamentelor EMAG. Reieșind din impactul minor asupra produselor finale si randamentul EMAG bun, a fost aplicată metoda optimizata de metanoliza/metilare lentă la opt tipuri de uleiuri vegetale și două tipuri de suplimente alimentare de ulei de pește. Compozițiile EMAG au fost analizate prin cromatografia gaz-lichid cu detector de ionizare cu flacără (GC/FID). În cromatogramele înregistrate au fost identificați 16 tipuri de acizi grași cu conținut major și calculate părțile de masa ale acestora. Metoda Ichihara – Fukubayashi modificata a permis convertirea acizilor grași cu legături esterice și acizii liberi în EMAG într-o singură etapă. A fost remarcata acuratețea, accesibilitatea si comoditatea metodei, folosirea de concentrații mici de probe și reagenți.

Cuvinte cheie: *coeluție, metilare, omega-3, omega-9, semințe oleaginoase, ulei de pește, toluen*

1. Introduction

Lipids are the basic components of the human diet, important sources of energy (9 kcal/g), micro- and macronutrients for the human body. Lipids are classified into simple lipids, which are fats and oils (triglycerides of fatty acids) and compound lipids, such as phospholipids, cholesterol, phytosterols, sphingolipids etc. [1].

The physical, chemical and physiological properties of lipids are largely determined by the composition of the fatty acids (FA) they contain. FA are most often found in the form of glycerol esters, such as triglycerides (TG), phospholipids (PhL), but also in free form (FFA). FA are carboxylic acids with unbranched carbon chains and can be saturated, monounsaturated or polyunsaturated (fatty acids with multiple double bonds (PUFA), also called essential acids). Depending on the position of the double bonds, unsaturated fatty acids are divided into omega-9, omega-6 and omega-3 acids, such as oleic acid C18:1, linoleic acid C18:2 and linolenic acid C18:3, respectively [2,3]. Lipids from vegetable sources (sunflower, olive, flax, rapeseed, walnut, coconut, cocoa), animals (butter, lard, beef), fish oil and others, differ in the type and variety of fatty acids they contain. Thus, the identification of lipids is based on the qualitative and quantitative analysis of the FA in their composition. For this purpose, globally, gas-liquid chromatography (GC) analysis method that can be coupled with mass spectrometry (GC-MS) [4] is predominantly applied, due to its efficiency and affordable cost [5]. GC methods are also widely used to analyze the quality of lipids and identify their contaminants [6,7].

1.1 Lipid Sample Preparation Procedures for GC Analysis

Before analyzing lipids from various food samples, the following preparation steps are required: 1. lipid extraction; 2. lipid fractionation by polarity into neutral fats (TG) and polar lipids (PhL, glycolipids, sphingolipids) including FFA; 3. derivatization or conversion of FA into FAMEs; 3. separation of FAMEs and their analysis by GC.

Vegetable oils do not require many preparation steps, if they contain moisture, they are dried with anhydrous sodium sulfate (1 g of Na_2SO_4 per 10 g of sample) at room temperature or in vacuum ovens (at 45…50 °C) [8]. In the case of food products, the fatty matter is extracted with different solvents, depending on the properties and composition of the lipids. It is important that the food samples are well dried at low temperatures in a vacuum oven or by freeze-drying, to prevent lipid oxidation [9]. After drying, the samples are finely ground to increase the contact surface with the solvent. The method of extraction of the ground sample with the appropriate solvent follows, the fatty matter will be entrained by the solvent. Triglycerides and other non-polar compounds can be extracted with *n*-hexane, petroleum ether, diethyl ether and others [10,11]. Mixtures of polar solvents - methanol, ethanol, *n*-butanol, isobutyl alcohol, with non-polar solvents - chloroform, diethyl ether, *n*-hexane, petroleum ether will extract all types of lipids, polar and non-polar, fatty acids, *etc.* from the matrix [12-14]. Folch and Bligh methods [15] and the Dyer method [16] use the chloroform: methanol : water mixture in various proportions.

1.2 Derivatization of Fatty Acids

FA in their free form cannot be analyzed by GC because they are polar compounds, form hydrogen bonds, and have high boiling points. Typically, fatty acid methyl esters, which are much more volatile, are analyzed by GC [17]. Several processes are known for derivatizing or converting FA to FAMEs, which include cleavage of ester bonds from lipids under alkaline (saponification) or acidic conditions, followed by methylation of the fatty acids [18,19].

Derivatization in basic media is most often performed with sodium methoxide ($NaOCH_3$) or potassium methoxide ($KOCH_3$). Typically, 0.5 M $NaOCH_3$ in anhydrous methanol is added to the lipids, and the mixture reacts at 45 °C for 5 min. Sodium hydrogen sulphate, $NaHSO_4$ (15%) is added to neutralize the mixture. Finally, the FAMEs are extracted with an organic solvent and analyzed by GC [17,20]. The advantages of the method are short time, low rate of isomerization of double bonds from *cis*- to *trans*-, fewer oxidative reagents [21]. The disadvantage is that FFA are not converted to FAMEs under these conditions [22]. Acid derivatization is suitable for both esterified and free fatty acids. This method uses the reagents: hydrochloric acid (HCl), acetyl chloride (CH_3COCl), sulfuric acid (H_2SO_4), and boron trifluoride (BF_3) [23]. Hydrochloric acid (HCl) is the most widely used catalyst for lipid derivatization reactions, as it is a milder reagent and gives very good yields. However, the use of anhydrous methanolic HCl, prepared by mixing acetyl chloride with methanol, is a harmful process [17,24]. The disadvantage of the method is the danger that can be caused by the exothermic reaction with acetyl chloride; in addition, some polyunsaturated fatty acids are unstable at the high temperatures (90-95 °C) applied [4]. Sulphuric acid H_2SO_4 is a strong oxidizing agent and is not recommended in the analysis of PUFA [25]. Although the BF_3 method provides efficient derivatization, its instability and artifact formation have been concerns in several studies [26].

1.3 Ichihara-Fukubayashi method

Japanese researchers K. Ichihara and Y. Fukubayashi developed an accessible and efficient method for preparing FAMEs from FFA, TG, PhL and cholesterol for GC analysis [27]. The method uses a reagent mixture composed of commercial concentrated HCl, methanol and toluene, these reagents being superior in terms of convenience, safety, and cost. Researchers developed two processes for derivatization: methanolysis/methylation by slow reaction under mild conditions (45 °C) and methanolysis/methylation by rapid reaction (100 °C) [27].

The mild methanolysis/methylation conditions at 45 °C and the duration of up to 14 h ensure the conversion of almost all fatty acids from different types of lipids into methyl esters, both those resulting from the cleavage of ester bonds and FFA. FAMEs are formed at a rate of 98-99%. Researchers Ichihara, K. and Fukubayashi, Y. determined that FFA are methylated rapidly, at a concentration of 1.2% HCl, being converted to FAMEs after 20 min, almost quantitatively. TG and PhL follow, which were converted to FAMEs after 8 h under the same conditions (HCl 1.2%, 45 °C). Cholesterols and phytosterols need 14 h to be converted to methyl esters.

1.4 Rapid methanolysis/methylation at 100 °C

The reaction conditions of HCl 1.2% at 100 °C for 90 min are required to convert all fatty acids and FFA from sterol-containing lipids to FAMEs, while 30 min is a sufficient reaction time to give good yields of FAMEs for lipid samples that do not contain sterol esters. FFA are methylated after 15 min, and triglycerides are converted to FAMEs and glycerol after 30 min. The FAMEs yield is almost 97% [27]. The authors report that ester-linked fatty acids and free acids from all lipid types were converted almost quantitatively to FAMEs in a single step by both methods. The mild methanolysis/methylation conditions were applied to blood lipids that contain considerable amounts of cholesterol, and FAMEs were prepared from a drop of blood spotted on a filter paper [27].

The aim of the research was to optimize Ichihara-Fukubayashi method for derivatization of lipids and fatty acids into methyl esters for GC/FID analysis, by elucidating the influence of methanolysis/methylation conditions on the yield of FAMEs, as well as identifying the qualitative and quantitative composition of FA from simple lipids.

2. Materials and Methods

2.1. Materials

Commercial vegetable oils: coconut oil (CoO), avocado oil (AvO), chia seeds oil (ChO), quinoa seeds oil (QuO), flaxseeds oil (FSO), refined, deodorized sunflower oils: "ordinary" (SFO) and "high oleic" (SFHO), olive oil (OO). Dietary supplements from enriched fish oil EFO_1 („Solgar Inc.", NJ-07605, USA) and EFO_2 („Balkan Pharmaceuticals Ltd.", Republic of Moldova) fortified with ethyl esters of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids. HCl (35%, *w/w*), *n*-hexane, toluene, methanol – all of GC grade ("Merck", USA).

Standards of methyl palmitate and methyl stearate were synthesized from the corresponding free fatty acids. The identification of other FAMEs was based on column polarity, retention time, molar mass and comparative bibliographic data – after the injections and the analysis of all samples.

2.2. Methods

2.2.1. Preparation of lipid samples

Commercially purchased vegetable oils were dried with anhydrous sodium sulfate (1 gram of Na_2SO_4 per 10 g of sample) for 2 h at room temperature. Dried and ground chia, flax and quinoa seeds were extracted with *n*-hexane in a sample:solvent ratio of 1:3 (*m/v*) at room temperature in the dark for 24 h. This was followed by filtration, evaporation of the solvent from the lipophilic extract at 55 °C and drying of the oil with anhidrous Na_2SO_4 at room temperature for 2 h. The oil layer was decanted into stoppered glass test tubes.

The contents of the fish oil dietary supplement capsules were poured into glass test tubes, from which the fatty matter was extracted with *n*-hexane in a ratio of 3:1 (*v/v*) by shaking for 2 min and left to stand for 4-5 min. The upper lipophilic extract layer was aspirated, from which the hexane was evaporated. EFO1 and EFO_2 were dried with anhydrous sodium sulfate for 2 h.

Immediately after drying, the lipid samples were weighed and subjected to methanolysis/methylation reactions as follows.

2.2.2. Preparation of fatty acid methyl esters (FAMEs)

Standard FAMEs - methyl palmitate and methyl stearate - were synthesized from palmitic and stearic acids. A total of 0.01 mol of the acid was dissolved in 1.25 mol of methanol and 0.02 mol of sulfuric acid was added as a catalyst. The reaction mixture was boiled with reflux for 1 h., then cooled and poured into 500 mL of ice-cold distilled water. The resulting esters were filtered and dried (18-20 °C). Identification of FA content in lipids was made according to FAMEs polarity, retention time, molar mass and by comparison with bibliographic data. The high content of lauric and myristic acids predominating exclusively in cocoa butter among the studied samples - facilitated their assignment.

FAMEs from lipid samples were prepared according to the bibliographic method [27], with some modifications. The concentrated hydrochloric acid reagent in methanol was prepared as follows: 9.5 mL of commercial HCl (35%, *v/v*) was diluted with 41.5 mL of MeOH, to obtain 50 mL of 8.0% (*w/v*) HCl. This HCl reagent contained 85% MeOH (*v/v*) and 15% water

(*v/V*) came from the concentrated hydrochloric acid solution. The reagent was stored in a refrigerator.

The weighed lipid (or fatty acid) sample (100 mg) was dissolved in 10 mL of toluene, from this solution 0.202 mL (202 µL) were taken with a micropipette and transferred into a screw-capped glass test tube (16.5 × 105 mm), to which another 0.200 mL of toluene was added. Thus, the mass of lipid dissolved in the toluene solution was 2 mg. To this lipid solution in toluene, it is mandatory to add in the following order: 3.00 mL of methanol and 0.60 mL (600 µL) of 8.0% HCl solution in methanol prepared above. The final HCl concentration is 1.2% (*w/v*) or 0.39 M in the 4 mL of solution in the test tube. The addition of a mixed solution of 1.2% HCl/methanol/toluene to lipid samples should be avoided due to the low solubilities of lipids in the contained water. The tubes were tightly closed, shaken for 1 min and subjected to methanolysis/methylation reactions under mild or rapid conditions. Two types of tubes were used: glass test tubes with ground glass stoppers (G-stoppers) and glass test tubes with polypropylene screw plugs (PPS-plugs). For slow methanolysis/methylation under mild conditions, the tubes were incubated at 45 °C for 14 h. Rapid methanolysis/methylation was performed at 100 °C for 1 hour. After the reaction time expired, the samples were cooled to room temperature, then 2 mL of hexane and 2 mL of water were added to the test tubes for FAMEs extraction. The test tubes were shaken for 1 min, left to stand for 2-3 min, then the upper hexane layer was carefully aspirated with a micropipette, dried with anhydrous sodium sulfate and analyzed by GC/FID.

2.2.3. Gas-Liquid Chromatography (GC)

GC analysis was carried out according to [28] with same modifications. An "Agilent 8860" instrument, equipped with autosampler, flame ionization detector (FID) and non-polar column HP-5 (30 m × 0.32 mm × 0.25 µm) was used. Conditioning gas Nitrogen (99.99%). Work gas Helium (99.99%), split mode, split ratio 4:1, flow 0.8 mL/min. Injector chamber temperature: 190 °C. Initial oven temperature: 140 °C. Temperature program: 0…4 min: plateau 140°C; 4…18 min: heating from 140 °C to 210 °C; 18…41 min – plateau 210 °C; 41…42 min – column cooling to 140 °C.

2.2.4. Statistical analysis of the results

The method standard deviation, SD_{method}, was calculated for three independent replicates using a sample of high oleic sunflower oil, SFHO. The P = 0.95, q = 0.05 hypothesis ($\Delta X = \pm\ 2 \cdot SD$) was accepted. Relative method error was amounted 0.043 (4.3%). The relative sample errors ε_{sample}, were calculated taking in account the number of GC-chromatogram peaks, processed as FAMEs, $n_{standard}$ and n_{sample} respectively in the SFHO and in the sample. The graduated pipette error, $\varepsilon_{pippette} = 2 \cdot (2\mu L/202\mu L) = 0.02$, also was taken in account to calculate sample relative error:

$$\varepsilon_{sample} = \sqrt{\varepsilon_{standard}^2 \frac{n_{sample}-1}{n_{standard}-1} + 0.02^2} \quad (1)$$

3. Results and discussions

3.1. Optimization of methanolysis/methylation methods

In order to investigate the influence of methanolysis/methylation conditions on the results of the experiments, two vegetable oil samples SFHO and OO were placed in glass test tubes with glass ground stoppers (G-stoppers) and in glass test tubes with polypropylene screw plugs (PPS plugs) for

mild (45 °C, for 14 h) and rapid (100 °C, for 1 h) methanolysis/methylation, according to the Ichihara-Fukubayashi method. FAMEs obtained in both experiments were GC/FID analyzed, the fatty acids with major content were identified and their mass fractions (%) of the total FA in the samples were calculated (Table 1). It was noted that heating the samples in G-stoppers test tubes at 100 °C resulted in the evaporation of the content (chemicals). The data illustrated in Table 1 confirm that PPS plugs better preserve the concentration of components during mild and rapid methanolysis/methylation, but in the chromatograms of FAMEs non-specific peaks of artifacts originating from polypropylene were detected.

Fast methanolysis/methylation at 100 °C for 1 hour led to a decrease in the yield of FA with C8-C16 carbon atoms in the chain, as a consequence, the calculated ratio resulted in higher mass fractions in favor of C18 acids (Table 1).

Under fast methylation/methanolysis conditions Ichihara, K. and Fukubayashi, Y. detected artifacts formed from cholesteryl oleate, which could not be separated from methyl oleate by silica gel column chromatography as well as by GC.

The method owners did not detect artifacts under mild methanolysis/methylation conditions, except for conjugated linoleic acid (rumenic acid), which is less stable under acidic conditions and forms artifacts in a ratio of 5% in both methods. Conjugated linoleic acid has two conjugated double bonds, separated by a single single bond, with the *cis*- and *trans*-configuration, respectively [29].

The researches [27] mentioned that in both methods, in the process of the methanolysis reaction in acidic medium, which is reversible, the presence of water derived from HCL could favor the formation of FFA. However, the results obtained by the Japanese authors demonstrate that less than 1.4% of FAMEs hydrolyzes into FFA. The researchers found that methanol containing 2.0% and 10% toluene is almost equivalent to anhydrous methanol in terms of the solubility of glyceryl trioleate. In the absence of toluene, lipid methanolysis was slow and partial. The effect of water on hydrophobic compounds was thus diminished by the addition of toluene.

The data in Table 1 are within those indicated in the literature for the mass fractions of FA in SFHO and OO, confirming the efficiency of both methanolysis/methylation regimes. However, the results obtained in these studies showed the advantage of mild methanolysis/methylation conditions, which affect less the concentration of volatile short-chain FAMEs.

It was also established that GC-FID analysis of FAMEs on the used GC column and others specified GC parameters of the given experiment (see 2.2.2) unfortunately does not allow the separation of FAMEs of oleic (C18:1 *ω*9) and α-linolenic (C18:3 *ω3*) acids, which give a common peak, Figure 1. Common elution of oleic and α-linolenic acid also correlate with bibliographical data (Tables 1 and 3).

Table 1

The influence of methanolysis/methylation conditions on fatty acid esters yield, %

No	FA	Bibliography		Mild, 45 °C, 14 h				Rapid, 100°C, 1 h	
		SFHO	OO	SFHO		OO		SFHO	OO
		[30, 31]	[32, 33]	G-stoppers	PPS-plugs	G-stoppers	PPS-plugs	PPS-plugs	PPS-plugs
1	12:0	< 0.1		0.012±0.001	0.055±0.003	0.013±0.001	0.053±0.003	0.012±0.001	0.011±0.001
2	14:0	< 0.1	< 0.1	0.050±0.002	0.062±0.003	0.028±0.001	0.025±0.001	0.055±0.003	0.021±0.001
5	15:0			0.015±0.001	0.029±0.001	0.015±0.001	0.012±0.001	0.017±0.001	0.012±0.001

Continuation Table 1

6	16:1 ω9	< 0.2	0.3 - 3.5	0.169±0.008	0.185±0.009	1.424±0.067	1.516±0.073	0.160±0.007	1.284±0.062
7	16:0	3.5 - 8	7.5 - 20	4.67±0.22	5.25±0.26	14.81±0.70	15.97±0.77	4.63±0.22	13.72±0.66
8	18:2 ω6	5.0 -17	3.5 - 21	7.00±0.33	7.05±0.34	10.50±0.50	10.40±0.50	6.94±0.33	10.40±0.50
9	18:1 ω9	75 - 90	55 - 83	82.4±3.9	81.8±4.0	66.3±3.2	64.9±3.2	80.8±3.8	65.6±3.2
10	18:3 ω3	< 0.2	< 1.0						
11	18:1 ω9		< 0.1	0.700±0.033	0.781±0.037	2.74±0.13	2.92±0.15	0.818±0.038	2.93±0.15
12	18:0	3.0 - 7.0	0.5 - 5.0	3.01±0.15	3.03±0.15	2.64±0.13	2.68±0.13	3.09±0.15	2.79±0.14

Note: FA – fatty acid; SFHO - sun flower "high oleic" oil; OO - olive oil; G-stoppers – ground-stoppers; PPS-plugs - polypropylene screwed plugs.

3.2. Identification of the FAMEs composition from lipid samples

Based on the minor impact on the final products and the good FAMEs yield, the optimized Ichihara-Fukubayashi method of slow methanolysis/methylation (at 45 °C, for 14 h) of lipid samples, placed in G-stoppers test tubes, was applied in the research of this study. The resulting FAMEs were analyzed by GC/FID (Figures 1-3), the structural formulas of the identified FA are shown in Table 2. On the Y axis is represented the relative response of the detector (rel. Response), and on the X axis – the retention time (t_R).

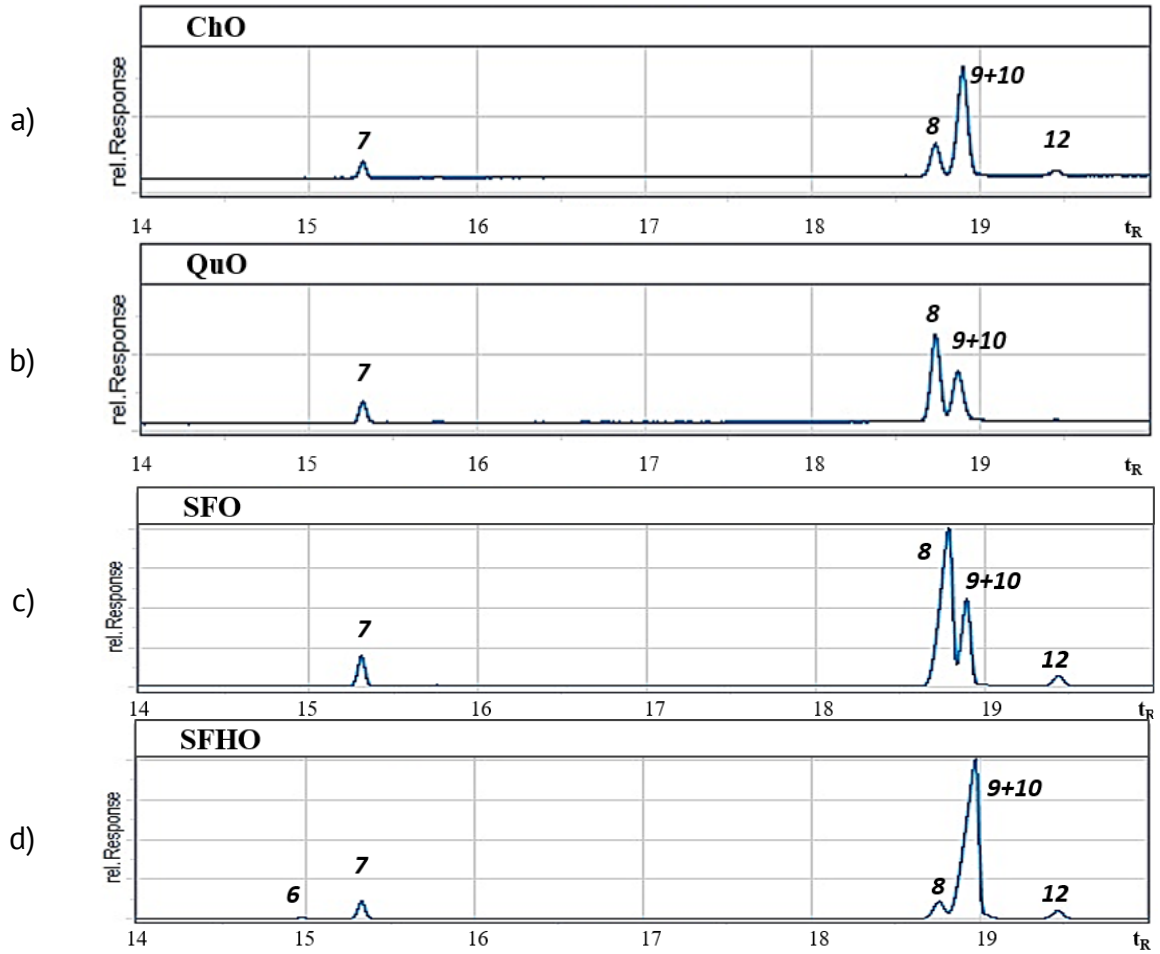


Figure 1. FAMEs C16 – C18 of a) Chia oil (ChO), b) Quinoa oil (QuO), c) Sunflower oil (SFO), d) Sunflower "high-oleic" oil (SFHO): 6 – palmitoleic; 7 – palmitic; 8 – linoleic; (9+10) – common peak of oleic and alpha-linolenic; 12 – stearic.

The FAMEs components identified on the chromatograms (Figure1a-d) are: 6 – palmitoleic acid (C16:1), visible in the SFHO sample; 7 – palmitic acid (C16:0), present in all samples, but with significant variations in intensity; 8 – linoleic acid (C18:2); 9+10 – co-eluent mixture of oleic acid (C18:1) and linolenic acid (C18:3); 12 – stearic acid (C18:0), detected in ChO, SFO and SFHO (Figure 3a, c, d), but less visible in QuO (Figure 3b). The common peak 9+10 is dominant in all samples, reflecting the high content of unsaturated fatty acids (oleic and/or linolenic).

All chromatograms of the seed oils show a moderate content of saturated acids and an unsaturated composition dominated by linoleic, oleic and α-linolenic acids. Although C18:1 and C18:3 acids give a common peak (9+10), the retention time of this peak is influenced by the ratio of the FAMEs concentration - linoleic:oleic:α-linolenic (Figure 1).

According to bibliographic sources, oil from different sunflower hybrids is classified into three groups: „ordinary" low oleic acid (10-29%), medium oleic acid (30-59%) and high oleic acid (60-90%) [34]. It is also known that sunflower oil contains a small amount (less than 0.2-0.5%) of α-linolenic acid [30,31], from which it follows that the major peak in the SFHO chromatogram belongs to oleic acid. These results are in accordance with the data indicated on the label of "high oleic" sunflower oil by the manufacturer, containing over 68.92 g of oleic acid per 100 mL of oil.

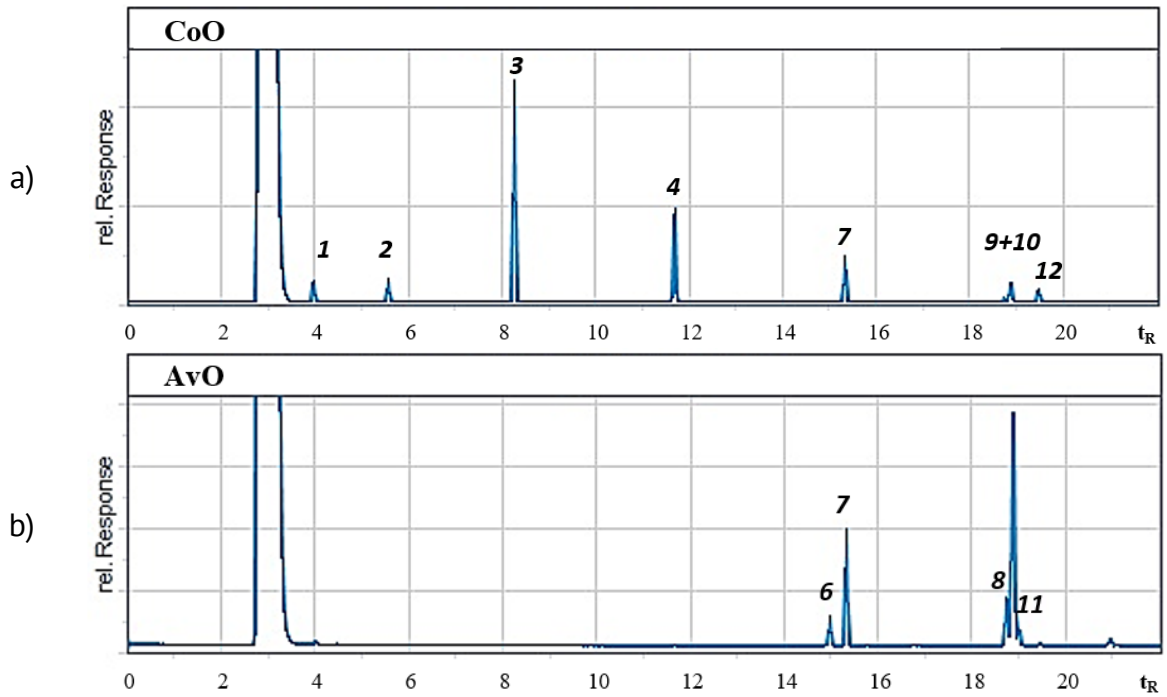


Figure 2. Nuts' FAMEs of: a) Cocoa oil (CoO), b) Avocado oil (AvO): 1 – caprylic; 2 – capric; 3 – lauric; 4 – myristic; 6 – palmitoleic; 7 – palmitic; 8 – linoleic; (9+10) – mixture of oleic and alpha-linolenic (common peak); 11 - elaidic; 12 – stearic.

The chromatograms in Figure 2 show the compositions of the CoO and AvO samples in methylated fatty acids. For coconut oil (Figure 2a), a profile dominated by short and medium chain FA results: caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitoleic acid (C16:1) and palmitic acid (C16:0). The majority peak is 3 (lauric acid) – typical for coconut oil [35]. In avocado oil (Figure 2b), the series of short and medium chain fatty acids is completely absent, the profile is rich in unsaturated FA: oleic/linolenic

(9+10) and linoleic (8) are dominant. The content of palmitoleic acid (6) is also notable, a signal specific for avocado oil [36,37].

The characteristic peaks of methyl esters of long-chain essential fatty acids with retention times more than 20 minutes were found in fish oil EFO_1 and EFO_2 (Figure 3a, b) The intensity of peaks 14 and 16 confirms the presence of increased amounts of EPA and DHA, respectively, which goes beyond the characteristics of regular fish oil. A typical fish oil provides 180 mg of EPA and 120 mg of DHA per 1000 mg of fish oil, but dosages can vary widely [38,39].

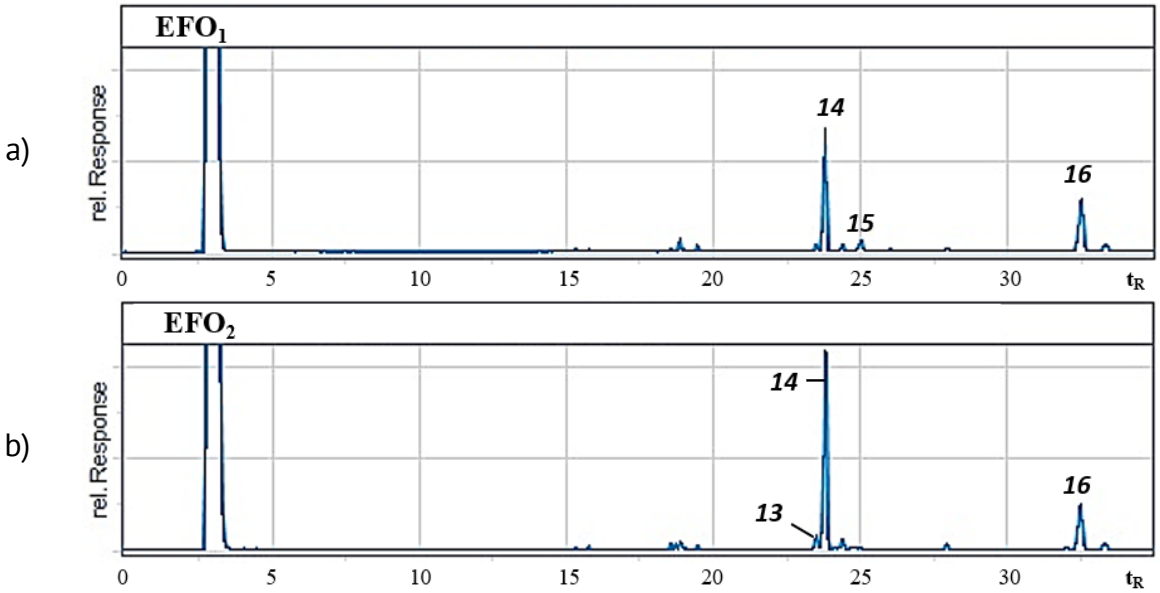
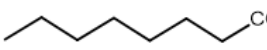
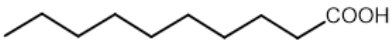
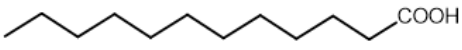
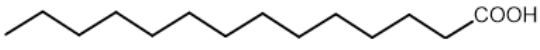
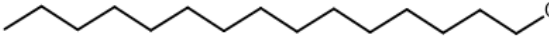
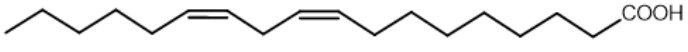
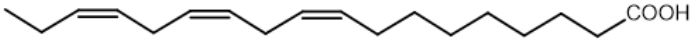
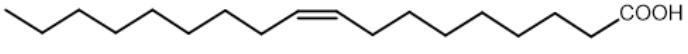


Figure 3. Fish Oil FAMEs C20 – C22 of: a) Dietary supplements from enriched fish oil "Solgar Inc" (EFO_1), b) Dietary supplements from enriched fish oil "Balcan Pharmaceutical Ltd" (EFO_2): 13 – arachidonic; 14 – eicosapentaenoic; 15 – eicosenoic; 16 – docosahexaenoic.

Table 2 represents the skeletal structural formulas of FA which were identified in all investigated samples. Only one trans-acid, C18:1-t ω9, called elaidic (or *trans*-oleic,) was detected in all of samples studied.

Table 2

Fatty acids detected by GC/FID

No	Fatty Acid	Code	Structure
1	Caprylic	8:0	
2	Capric	10:0	
3	Lauric	12:0	
4	Myristic	14:0	
5	Pentadecanoic	15:0	
6	Palmitoleic	16:1 ω9	
7	Palmitic	16:0	
8	Linoleic	18:2 ω6	
9	Linolenic	18:1 ω9	
10	Oleic	18:3 ω3	

Continuation Table 2

The calculated FA mass fractions (%) of the total fatty acids contained in the analyzed samples are within the bibliographic data. Table 3 presents the values determined for the major FA identified in the research. Thus, according to literature data, CoO contains up to 7% caprylic acid, 5-8% capric acid, 48-55% lauric acid, 21-16% myristic acid, 9-10% palmitic acid, 3% stearic acid, 5-6.5% oleic acid and less than 0.01% linolenic acid [35], these data are similar to those in Table 3.

The values recorded for AvO and ChO fit into bibliographic data, which show that AvO contains 13-28% palmitic acid, 16% linoleic acid, 55-60% oleic acid and less than 1% α -linolenic acid [36,37]. ChO is rich in 6-8% palmitic acid, 18-21% linoleic acid, 4-10% oleic acid and up to 63% α -linolenic acid [40].

The recorded data correspond to those in the literature for linseed oil, containing 5-8% palmitic acid, 12-17% linoleic acid, 2-5% stearic acid, 17-19% oleic acid and 40-60% α-linolenic acid [41,42]. Also, the results in Table 3 are in line with those recorded by other researchers for QuO, rich in 60% linoleic acid, 20.5% oleic acid and 6.5% α-linolenic acid [43]. SFO contains 48-74% linoleic acid, 2.7-6.5% stearic acid, 14-39% oleic acid and less than 0.5% *α*-linolenic acid, according to the literature data [30,31].

Table 3

The composition of fatty acids in the form of methyl esters, %, determined in lipid samples by the modified Ichihara-Fukubayashi method

FA	CoO	AvO	ChO	FSO	QuO	SFO	EFO_1	EFO_2	t_R
8:0	4.98±0.19								3.97
10:0	5.05±0.19								5.57
12:0	47.2±1.8	0.20±0.01							8.26
14:0	20.72±0.76	0.71±0.03	0.05±0.01	0.06±0.01	0.14±0.01	0.09±0.01			11.69
15:0			0.02±0.01	0.03±0.02	0.07±0.01	0.02±0.01			13.50
16:1 ω9	0.21±0.01	5.31±0.19	0.09±0.01	0.16±0.01	0.23±0.02	0.12±0.01	0.21±0.02	0.04±0.01	14.96
16:0	10.56±0.39	20.06±0.71	7.09±0.38	6.41±0.32	10.02±0.53	6.93±0.34	0.45±0.03	0.35±0.02	15.33
18:2 ω6	1.18±0.05	11.62±0.41	18.66±0.98	16.16±0.80	49.5±2.6	61.4±3.1	0.38±0.03	1.12±0.07	18.77
18:1 ω9 18:3 ω3	6.06±0.23	55.2±2.0	68.9±3.6	70.8±3.5	32.2±1.7	26.0±1.3	2.82±0.18	1.88±0.11	18.94
18:1-t ω9	0.07±0.01	4.23±0.15			0.68±0.04	0.68±0.04	0.14±0.01	0.17±0.01	19.05
18:0	4.01±0.15	1.02±0.04	3.48±0.19	5.12±0.26	0.69±0.04	3.22±0.16	1.74±0.11	0.72±0.05	19.45
20:4 ω6							2.32±0.14	3.94±0.23	23.50
20:5 ω3							41.3±2.4	58.4±3.3	23.81
20:1 ω9				0.11±0.01	1.59±0.09		4.40±0.26	0.59±0.04	25.00
22:6 ω3							27.8±1.7	20.22±1.6	32.49
Others		1.62±0.06	1.71±0.09	1.12±0.06	4.86±0.26	1.56±0.08	18.5±1.1	5.11±0.29	4-42

Continution Table 3

N_{id}/N_{proc}	10/10	8/9	7/23	8/20	9/23	8/20	11/29	11/27	---
ε_{sample}	0.0367	0.0352	0.0522	0.0491	0.0522	0.0491	0.0580	0.0562	---
Bibliographic data about contain of Oleic and *α*-Linolenic acids [34-42]									
18:1 *ω*9	5 – 6.5	55 – 60	4 – 10	17 – 19	20.5	14 – 39	< 15.1	< 15.1	---
18:3 *ω*3	0.01	< 1.0	< 63	40 - 60	6.5	< 0.5	n/d	n/d	---
Sum	5 – 6.5	55 – 61	< 73	57 – 79	27	14 – 40	< 15.1	< 15.1	---

Note: FA – fatty acid; CoO - coconut oil;AvO - avocado oil; ChO - chia oil; QuO - quinoa oil; FSO - flax seed oil; SFO - sunflower oil; EFO_1 and EFO_2 - dietary supplements from enriched fish oil "Solgar Inc" and "Balcan Pharmaceutical Ltd"; t_R- retention time, min; N_{id} – identified FAMEs; N_{proc} – processed peaks; ε_{sample} – relative error for each oil sample.

Traditional fish oil typically contains 9.6% palmitoleic acid, 17.1% palmitic acid, less than 15% oleic acid, 2.7% stearic acid, 2.1% arachidonic acid, up to 18.6% EPA, and on average 14% DHA [38,39]. The EFO_1 and EFO_2 fish oil supplements analyzed in this study have increased EPA (41.3 and 58.4%) and DHA (27.8 and 20.22%), respectively, values indicated by manufacturers for fish oil supplements fortified with ethyl esters of the mentioned acids.

4. Conclusions

A convenient modification of Ichihara-Fukubayashi analysis method is pre-dilution of the oil sample with toluene in a ratio of 0.1:10.0 and further analysis of 202 microliters of the resulted solution, now containing 2 mg of oil. Heating the samples in test tubes with glass ground stoppers at 100°C result analysis failure. Test tubes with polypropylene screw plugs preserve the components better, but non-specific peaks or artifacts, probably originating from polypropylene, were detected in the FAMEs chromatograms. At the same time, fast methanolysis/methylation (100 °C, 1 h) led to a decrease in the yield of FA with C12-C16 chains. Therefore, only mild slow methanolysis/methylation at 45 °C for 14 h is optimal for FAMEs analysis. Because of the minor impact on the final products and the good yield of FAMEs, the optimized mild methanolysis/methylation method was applied to 8 types of vegetable oils and two types of fish oil dietary supplements. In the analyzed lipid samples, 16 fatty acids were identified qualitatively and quantitatively (from which linolenic and oleic as a sum, due to used column parameters) obtaining good correlation with bibliographical data. The modified Ichihara-Fukubayashi method is accessible and convenient because it uses reasonable quantities of samples and reagents.

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Submission of manuscripts: jes@meridian.utm.md