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Recent developments in fatty acids profile determination in biological samples - a review

Progrese recente în determinarea acizilor grași din probe biologice - un review

Ioana Tiuca*, Katalin Nagy, Radu Oprean

University of Medicine and Pharmacy "I. Hatieganu", Cluj-Napoca, Romania

Abstract

The present paper is a literature review of the recent years dealing with the most important separation techniques of fatty acids in biological samples. Our aim was to make a synthesis of the analytical methods used, to note the most used ones, but also to mention other methods that are less utilized, which can have important advantages (such as less time consuming, greener reagents, etc.). Gas-chromatographic separation methods were described and compared to liquid chromatographic separations of fatty acids in different types of biological samples. In the same time, the importance of determining fatty acids profiles in biological samples was revealed, pointing out the possible implications in diagnostics of different types of disorders or remarking different profiles compared to healthy states.

Keywords: fatty acids, gas-chromatography, liquid-chromatography, biological samples

Rezumat

Prezenta lucrare reprezintă un review al literaturii ultimilor ani în legătură cu cele mai importante tehnici de separare ale acizilor grași din probe biologice. Scopul nostru a fost de a realiza o sinteză a metodelor utilizate, de a le nota pe cele mai utilizate, dar, în același timp, de a le puncta și pe celelalte mai puțin utilizate, care ar putea avea importante avantaje (cum ar fi un consum mai mic de timp de analiză, utilizare de reactivi "verzi", etc.). Am descris și comparat metodele de separare gaz-cromatografice cu separările lichid-cromatografice ale acizilor grași pentru diferite tipuri de probe biologice. În același timp, am marcat importanța determinării profilelor acizilor grași în probe biologice, arătând posibile implicații în diagnosticul diferitelor tipuri de boli sau remarcând profile diferite la bolnavi comparativ cu voluntarii sănătoși.

Cuvinte cheie: acizi grași, cromatografie de gaze, cromatografie de lichide, probe biologice

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*Corresponding author: Ioana Tiuca, University of Medicine and Pharmacy "I. Hatieganu" Cluj-Napoca, Cluj, Romania, e-mail: tiuca.daria@umfcluj.ro

1. Introduction

In the era of search for health and well-being, fatty acids have gained an important role in human health, having a significant effect in the occurrence and prevention of cardiovascular diseases (1). In the same time, fatty acids profile has been reported to significantly influence or to be modified in a considerable way in neurologic or psychiatric diseases (2,3), endocrine disorders (4) or in metabolic syndrome related illnesses, such as diabetes mellitus (1,5,6).

Likewise, fatty acids profiles in ingested foods (1) or beverages (7,8) can influence the state of health of a certain individual. It is well known that fish oil administered in infancy can reduce risks of allergies (9) and also, due to its high content of n-3 PUFA, can reduce the inflammation biomarkers in blood (10).

For these reasons, we consider that profiling fatty acids in blood or other biological samples can predict and even prevent an eventual state of health/illness that could occur, thus it is important to have easy, fast and cost effective methods to be able to determine the profile of fatty acids with short analysis times, high efficiency and selectivity.

Gas-chromatography and high-performance liquid chromatography are the main separation techniques used nowadays. In the separation of fatty acids, they can be used as complementary techniques: a gas-chromatographic separation is very efficient and uses very small volumes of substances, but it needs derivatization, while a liquid chromatographic separation does not necessarily need derivatization, but uses higher volumes of solvents and does not give such good separations, in terms of efficiency and resolution. Moreover, if it is coupled to a UV-Vis detector, fatty acids detection can cause troubles, because they hardly absorb in this spectral range.

The methods described in the literature for the separation and/or quantification of fatty acids in different samples are mostly gas-chro-

matographic (1–3,5–8,11–22), among which most developed methods use a mass-spectrometer detector. However, liquid-chromatography (4,17,23–31) has been presented by several authors and also, there are few articles which deal with other techniques such as capillary electrophoresis or infrared (IR) and Raman spectroscopy (16,31,32).

The aim of this study was to sum up a literature synthesis of the most important separation techniques of fatty acids in bio-samples, revealing their importance in diagnosis of different disorders or noticing how their profiles can change or can be improved.

2. Gas chromatography (GC)

Gas chromatography is the first choice method for the detection and quantification of fatty acids in all types of samples. The high efficiency, high selectivity, relative low analysis times and low volume consumption make it a first selection method. However, the need for derivatization in case of fatty acids analysis can cause troubles in terms of overall reproducibility.

2.1. Sample preparation – extraction of FA and derivatization

In the majority of cases, the solvents used for the extraction of fatty acids from biological samples can be either hexane (5,6,8,11,13,18,21,22) or CHCl_3 /methanol (7,17,18–20). Before the derivatization of FA to their methyl esters (FAME), a saponification can be done, which separates FA from other lipids. For this purpose, NaOH or KOH in methanol can be used, then the solution is acidulated (5,11,16). Also, a thin layer chromatographic separation of fatty acids from the other lipids extracted can be done (1,14).

The transesterification to FAME is needed to increase the volatility of FA, which would be separated through the gas-chromatographic column. This esterification is usually done us-

ing methanolic HCl or methanolic BF₃. The last one, BF₃, seems to be preferred to be used as a catalyst for the methyl-esterification reaction, at 100°C, for 1 h (6,13,17–19). However, acid catalyzed reactions are used and H₂SO₄ has also been considered as an option for the transesterification of FA to FAME (5,19). The research group of Schmitz (21,22) have used an unusual derivatization agent, which is acetyl-chloride, the derivatization being done at 20°C, at 150 rpm, overnight. At the same time, for FA dosing, C17:0 can be added to the sample solution as an internal standard, before the derivatization process. However, any other FA with odd number of carbon atoms in the molecule can be used as an internal standard, since they are not synthesized in the human body.

After derivatization, FAME are usually extracted in hexane and the solution is concentrated or even the solvent is completely evaporated, when a solution of p-tert-butyl-phenol in hexane, as an antioxidant, can be added to the residue. Dichloromethane (DCM) has also been used for FAME extraction (16).

Main extraction, derivatization and separation procedures are listed in the Table I.

2.2. FAME separation

Fatty acids and their methyl-esters are highly non-polar compounds. In spite of this fact, most authors have chosen to use for their separation, stationary phases presenting medium to high polarity, such as Innowax columns (highly polar polyethylene glycol phase) or BPX-70 column (a stationary phase dedicated to FAME separation). These stationary phases present the advantage of giving a better resolution and efficacy, but they are less thermally stable. However, there are some studies which deal with the separation of fatty acids (derivatized to FAME) on stationary phases of low polarity, such as VF-5ms (5%-phenyl-arylene-95%-dimethylpolysiloxane phase). The advantage of using a non-polar stationary

phase would be the wider range of temperature limits. With very few exceptions (8,16,18), the mobile phase used for the separation of FAME is helium. Likewise, the detector usually coupled with a GC system is a mass spectrometer detector, but there are studies which are using the flame ionization detector (1,6–8,15,16,18,20). The MS detector is preferred due to its high selectivity towards the analyte and due to the possibility to precisely identify the analytes in a spectral mass database (NIST, Wiley etc.). Sometimes, GCx-GC can be used, for a better resolution of the analytes (11,12).

The main separation conditions concerning the GC analysis of fatty acids in bio-samples have been presented in Table I. .

3. Liquid chromatography (LC)

Liquid chromatographic methods have been developed because the extraction and derivatization processes used in GC can often be long and time consuming and can affect the reproducibility of the method. At the same time, liquid chromatographic methods can be developed for preparative purposes.

3.1. Sample preparation

HPLC with fluorescence detection is a good method used for separation and quantification of fatty acids. It offers a high sensitivity and specificity, but it requires a phase of derivatization, which can be time-consuming and, in some cases, the use of toxic reagents, such as pyridine (23,27), cannot be avoided. By using this method, FA were separated from saliva, serum samples, mice liver and different types of oils (23,27). Analyzing several fluorescent labeling reagents used for the derivatization of carboxylic acids, difluoro-boraindacene (BODIPY) have shown the best fluorescent properties, and by taking functional groups in consideration, hydrazine and amine have higher reactivity and

Table I. Derivatization, extraction of FAME and gas-chromatographic separation parameters for the analysis of fatty acids in biological samples.

Analyzed sample	Derivatization agent(s)	Extraction solvent(s)	Capillary column(s)	Carrier gas	Temperature gradient (°C)	Detector	Ref.
INS-1 cells		hexane	Rxi®-1msx Rxi®-17Sil-MS	He	40-160-260	TOF-MS	(11)
colorectal cancer cells	MeOH/BF ₃ 100°C	hexane	DB-WAX	He	120-205-250	MS	(13)
plasma	MeOH/BF ₃ 100°C	CHCl ₃ /MeOH, hexane	Omegawax 250	He	180-206-240	MS	(14)
maternal milk, powder milk	MeOH/HCl, 75°C, over night	CHCl ₃ /MeOH, hexane	Ultra-Alloy 5	He		FID	(7)
erythrocyte membranes	MeOH/BF ₃ 100°C	CHCl ₃ /MeOH, hexane	DB-23	He	140-240	FID	(15)
plasma	MeOH/H ₂ SO ₄ 70°C	KOH/MeOH (sapon.) hexane	VF-5ms	He	90-212-219-290	MS/MS	(5)
plasma	MeOH/BF ₃ 100°C	CHCl ₃ /MeOH, DCM	DB-225	N ₂		FID	(16)
human hair	MeOH/HCl DMOX 150°C		DB-WAX DB-WAXetr			MS	(17)
plasma erythrocytes	MeOH/NaOH 100°C, 5 min	hexane	DB-225ms CP-select	H ₂		FID	(18)
colon tissue	MSTFA 70°C, 30 min	CHCl ₃ /MeOH/H ₂ O toluene	DB-1 x Rxi-17	He	60-125-270 (+100)	TOF-MS	(12)
serum	MeOH/H ₂ SO ₄ 62°C, 2 h	ethyl acetate, hexane	DB-WAX	He	50-200-220	MS	(19)
serum/ plasma	MeOH/HCl 90°C, 4 h	hexane	SP 2560	He	50-190-230	FID	(6)
plasma	BF ₃ /MeOH 100°C, 1 h	heptane/diethyl ether/acetic acid, hexane	VF-23ms	He	50-170-212	FID	(1)
milk, oil, animal serum	various		RTx-65TG	He	120-220-350	FID	(20)
human milk	MeOH/HCl 100°C, 1 h	hexane	CP-Sil 88	H ₂	60-165-195-215	FID	(8)
plasma, cell homogenate	MeOH/AcCl	hexane	BPX-70	He	50-155-210-250	MS	(21)
plasma/ serum	MeOH/AcCl 20°C, 150 rpm	hexane	BPX-70	He	50-155-210-250	MS	(22)
blood	MeOH/KOH 50°C	hexane	Elite-1 GC	He	100-140-240	MS	(34)

milder derivatization conditions. Combining hydrazine and BODIPY, a new derivatization reagent has been developed (27), and by using a primary aliphatic amine as the labeling group and BODIPY as the fluorophore, another, very similar derivatization agent has been developed (23). The efficiency of the derivatization is influenced by five main factors: the amount of labeling reagent, other reagents dosage, reaction time and temperature. It has been shown that the dosage of the reagent affects the efficiency of the derivatization more considerably than the other factors (23,27).

Not only human serum can be analyzed by using a HPLC method, but different lipid species were separated from human meibum samples. In this case lipids were dissolved in a chloroform/methanol mixture, and the extracts were dried by using a centrifugal concentrator (25).

Oxygenated metabolites of PUFAs (30) and fatty acid ethanolamides (FAE) (24) can also be separated by using the HPLC-MS/MS method, preceded by a solid-phase extraction. The extraction and/or a pre-concentration step is needed because the oxygenated PUFA metabolites show low physiological levels and a large number of isomers, which can harden the analytical determination (30). Because of their low concentration, the identification and separation of FAE remains problematic (24). Dasilva and co-workers compared two different cartridges, 60 mg Oasis-HLB and 100 mg C18 and also analyzed the effects of the elution solvent, sample pH and temperature. By using HLB cartridges better recovery results were obtained (70 to 98%), than in the case of the C18 sorbent (42 to 82%). Other studies showed that the highest extraction efficiency was obtained for ALEA, while the extraction efficiencies for AEA, DHEA and LEA were lower. Liquid-phase extraction (LPE) can be used in the case of tissue samples: liver or small intestine, but the FAE showed a less efficient extraction compared to SPE results (24).

3.2. LC separation of FA

High or ultra-performance liquid chromatographic methods have been developed for the separation of fatty acids in reversed-phase mode, due to the low polarity of their molecules. The most used stationary phase has been C18, in different isocratic or gradient elution types of mobile phases, the gradient elution being preferred (Table II). In recent years, we have noted a preference towards the use of a mass spectrometer as a detector, this being a powerful tool in precisely identifying the analytes, offering excellent detection limits, good selectivity and, in the same time, being able to be applied to a broad range of compounds.

FA were separated from saliva, serum samples or different types of oil by using a HPLC system with a fluorescence detector (490/510 nm) and a reversed-phase C18 column with a gradient elution. The most frequent eluents were methanol and double-distilled water (27), or methanol and citrate buffer (23). The methanol content has to be lower than 65% and the pH between 6-7.5, otherwise the peaks of the acid derivatives and the reagent become overlapped (27).

The same detector was used for the identification and separation of FAME after the derivatization process. This methyl derivate can be separated by a reversed-phase high performance liquid-chromatography (RP-HPLC) by using a C18 column and a gradient elution. Peaks were identified by UV detection at 205 nm (28).

FA from blood samples can also be separated and quantified by using an optimized multiple reaction monitoring (MRM) method with the usage of UPLC coupled with tandem mass spectrometry. Chromatography separation was carried out by using a non-polar column and a gradient elution. Gradient program was solvent B (acetonitrile), 70% (0 min), 90% (3 min), 100% (3.01-4 min) and 70% (4.01-8 min). The mobile phase was carried out by using acetonitrile and ammonium acetate (26,29).

Table II. Separation conditions of FA in LC

Sample type	Pre-treatment	Stationary phase	Mobile phase	Elution type	Detector	Ref.
serum	isopropanol extraction	C18	A: isopropanol: ACN:n-hexane (55:45:15) B: 0.1 mol/L ammonium formiate methanolic solution	isocratic A:B (95:5)	MS/MS	(35)
human serum (healthy, pan- creatic cancer) mice liver tissue	highly fluorescent labeling reagent (derivatization)	C18	A: 15mM citrate buffer B: MeOH	gradient	fluorescence detector (490/510 nm)	(23)
plasma (human, hamster) tissue (hamster)	SPE, LPE	C18	A: 0.1% formic acid in H ₂ O B: 0.1% formic acid in ACN	gradient	MS/MS	(24)
meibum	CHCl ₃ /MeOH extraction	C18	A: 0.1% ammonium acetate in H ₂ O B: 0.1% ammonium acetate in MeOH	gradient	Orbitrap	(25)
blood	hexane extraction	C18	A: ammonium acetate 10mM in H ₂ O B: ACN	gradient	ESI-MS	(26)
saliva (smoker, non-smoker)	fluorescent derivatization reagent	C18	A: MeOH B: H ₂ O	gradient	fluorescence detector (490/510 nm)	(27)
human hair	DMOX derivatization	C18	A: MeOH:H ₂ O (50:50) + 0.1% for- mic acid B: MeOH + 0.1% formic acid	gradient	fluorescence detector (350/450 nm)	(17)
cell cultures (cystic fibrosis)	FAME derivatization	C18	A: H ₂ O +0.02% fosforic acid B: ACN	gradient	UV (205 nm)	(28)
plasma (sickle cell disease)	hexane extraction	C18	A: ammonium acetate 10mM in H ₂ O B: ACN	gradient	ESI-MS	(29)
plasma (rat)	SPE	C18	A: H ₂ O +0.02% formic acid B: MeOH +0.02% formic acid	gradient	QqQ-MS LIT-MS	(30)
plasma	LPE	C18	A: H ₂ O + 0.1% trifluoroacetic acid B: ACN + 0.1% trifluoroacetic acid	gradient	MS	(31)
plasma	SPE	C18	A: ACN B: H ₂ O	gradient	MS/MS	(36)
cell culture	CHCl ₃ /MeOH extraction	C18	A: ACN:MeOH: H ₂ O (19:19:2)+ 0.1% acetic acid+ 0.028% NH ₃ B: isopropanol+ 0.1% acetic acid+0.028% NH ₃	gradient	ESI-MS	(4)

Reversed phase liquid chromatography can be coupled along with direct electron impact mass spectrometry (EI-MS). This method has the advantage of not requiring derivatization of the sample and not showing matrix effect (31). According to this, the time of analysis can be reduced, however, when dealing with a complex matrix, such as plasma, the separation is anticipated by a liquid-liquid extraction, which can take about 30 minutes. This method can be used for the detection of all NEFAs in plasma with a satisfactory sensitivity and selectivity, because the direct EI-MS response is not affected by co-eluting endogenous plasma components (31).

The analyses of plasma samples collected from genetically obese, spontaneously hypertensive female rats were carried out on two LC-MS systems; one was coupled to a triple quadrupole MS, and the other was coupled to a LIT mass spectrometer. The operating conditions of the ESI source in both cases were negative ion mode, and acceptable precision was achieved by both instruments. The LIT system had the advantages of lower matrix effect and higher global recovery than in the case of the QqQ system. Although the QqQ assays were highly sensitive, when applied to rat plasma samples, they gave several false identifications, because many isomers with similar MRM transitions were co-eluted (30).

By using an Orbitrap Fourier transform mass spectrometer as detector, FAs and OAHFAs were separated by using a negative ion mode because OAHFAs, like FAs, possess a carboxylic group. As well as in the case of the QqQ system, this method also identified a number of molecular species in one chromatographic peak, demonstrating co-elution of isomers of OAHFA molecular species. The main advantage of this method is that long chain fatty acids could be separated without a derivatization process. Mori and co-workers succeeded identifying 61 OAHFA molecular species with 34-56 carbon atoms by using this method. The species with the high-

est abundance were C18:1/32:1, C18:1/30:1, C16:1/32:1 (25).

Human skin fibroblasts from X-ALD or Zellweger patients were analyzed by using the same LC-ESI-MS/MS method. For acidic phospholipids MS/MS analysis was performed in negative ion mode, while phospholipids with choline were detected in positive ion mode. The lipids were eluted from the reversed-phase column in accordance with their hydrophobicity and exhibited molecular ion signals dependent on their mass/charge (m/z). The longest fatty acyl chains detected were C36, and the highest unsaturated bond number observed was 6.

Separation of fatty acid ethanolamides by UPLC-MS/MS was performed on a C18 column by using a gradient condition: over the first 2 min mobile phase B (acetonitrile with 0.1% formic acid) was increased from 70% to 72% and in the subsequent 6 min increased to 74%. The tandem MS equipped with an atmospheric pressure ionization (API) probe was operated in positive electrospray ionization mode (+ESI), because of the structure of the ethanolamides (24).

4. Alternative analytical techniques

Capillary electrophoresis (CE) represents an alternative to chromatographic methods which gives high efficiency, low separation times and high reproducibility. However, there are few studies which deal with electrophoretic separation of fatty acids (32) compared to GC-MS studies or HPLC-MS studies and even fewer dealing with biological samples. Similar to HPLC, capillary electrophoresis presents the disadvantage of the UV detection, because fatty acids hardly absorb in this spectral range. However, in addition to the MS detector which can be coupled to the CE system, other types of detectors can be used, such as the contactless conductivity detectors (37).

Alternatively to the separation methods, spectral techniques such as ATR-FT-IR and Ra-

man spectroscopy (33,38) can be used for the analysis of fatty acids. These methods, coupled with the multivariate analysis of spectral data can give rapid quantitative information about the analyzed sample (38). These complementary spectroscopic methods provide very fast analysis times (seconds to few minutes), specificity towards the analyte through the fingerprint spectral area and very low consumption of solvents.

5. Potential clinical utility of fatty acids profile analysis

There are two main directions in which the fatty acids composition in biological samples have been studied: the presence of a malignant state and the presence of a disorder related to the metabolic syndrome. In the first case, authors (12,13,15) have separated FA from colorectal cancer tissue and healthy adjacent cells as control obtained postoperatively, and from erythrocyte membranes in multiple myeloma patients, after derivatization to FAME. Results showed, in all cases, an increase in the profile of some saturated fatty acids (SFA) and also a decrease in the profile of mono-unsaturated fatty acids (MUFA) in the cancerous tissue compared to the normal cells. In the same time, a decrease in the n-3/n-6 PUFA ratio has been observed in the malignant tissue, compared to the normal cells. Moreover, Mal and co-workers (12) have realized an entire biochemical metabotyping of colorectal cancer, using GCxGC-TOF-MS, where they have remarked not only different profiles of FA, but also of amino-acids and saccharides, with important roles in biochemical cell stages.

The most important disorder related to the metabolic syndrome is diabetes mellitus, usually because of its complications. Authors have studied FA in plasma from patients with this disease (1,5,6), but also FA from insulin secreting cells INS-1 have been separated using GCxGC-TOF-MS (11) and plasma from other disorders related

to insulin resistance, such as polycystic ovary syndrome (PCOS) (14). In the last case, results have showed that insulin resistance has a bigger influence on the fatty acids profile than the presence of PCOS. Nervonic acid (C24:1 n-9) was identified as a biomarker for the presence of PCOS and dihomo- γ -linolenic acid (DGLA) was identified as a biomarker for insulin resistance, after multivariate interpretation of data. Moreover, authors have noticed, as in case of malignant diseases, a decreased ratio of n-3/n-6 PUFA in case of PCOS with insulin resistance, compared to control or with non-insulin resistant PCOS, but also a modified profile of MUFA in PCOS. In the same time, Han et al. (5) have identified the arachidonic acids (C20) to be biomarkers in the plasma of diabetic patients, with or without different stages of nephropathy, as a diabetic complication. Moreover, they have noticed, compared to control, an increase in the EFA profiles and a decrease in the NEFA profiles in the plasma of diabetic patients. This can be related to insulin resistance and/or lack of insulin. At the same time, Sertoglu and co-workers (6) have noticed that the n-3/n-6 ratio is the lowest in plasma of diabetic patients compared to control or to end-stage renal disease patients, but the n-3/n-6 ratio is the highest when determined from the erythrocyte membranes of the same groups. When analyzing plasma samples from obese patients by GC-FID, Tremblay-Franco et al. (39) discovered that the n-9 FA ratio decreased and n-7 ratio increased in case of obese females, compared to control.

All these findings can be explained in all cases due to the anti-inflammatory, cardioprotective, neuroprotective effects of n-3 PUFA and the opposite effects of n-6 PUFA, thus the pathological effects are reflected in the n-3/n-6 PUFA ratio. Arakawa et al. (40) have pointed out the importance of n-3 PUFA intake, their cardioprotective properties and have showed that the total serum levels of DHA and EPA can be pre-

dictor of ischemia immediately after myocardial reperfusion, thus can predict a myocardial damage. Moreover, Nishi et al. (1) have shown that a daily ingestion of n-3 PUFA through different types of supplements (e.g. nuts) can improve the FA profile in diabetic patients, with decreasing their coronary heart disease risk. For the same reason, authors (16) have studied recovery from patients with periodontitis, after administering supplements with n-3-PUFA, considering the anti-inflammatory effects of this type of FA. Results have shown a better recovery at patients having n-3-PUFA supplements than controls. In the same time, n-3 PUFA have proved their cardioprotective properties and the total serum levels of EPA and DHA can be used as predictors for the level of ischemia after myocardia reperfusion (40).

The fatty acids profile seems to be modified even in infectious diseases. Khedr et al. (34) have identified several EFA as biomarkers for the early febrile stage of the dengue fever disease; among them: the esterified C14:0, C16:0, C18:0, C20:4-n6, C22:6-n3, which, in all cases, had a decreased profile in the blood of infected patients compared to control.

It has been demonstrated that the same saturated fatty acids, C16:0 and C18:0 are present in lower amounts in the meibum of meibomian gland dysfunction patients than control. Meibum lipids are a highly complex mixture of lipids from various classes, including OAHFAs. These species decrease as the severity of dry eye disease increase, so they can be considered as a potential biomarker in meibomian gland dysfunction. It is important to elucidate the relationship between odd number FA and bacterial infections in meibomian gland dysfunction patients, because bacteria can produce an odd number of FAs (25). On the other hand many hydroxyl-derived compounds of EPA and DHA are involved in the regulation of vascular tone, arteriosclerosis and are considered markers of lipid peroxidation (30).

The level of propionic acid in the smokers' saliva is higher than in the nonsmokers', and the level of heptanoic acid in the smokers' saliva is lower than in the case of nonsmokers (27). The level of C16:0, C18:1, and C18:0 in the serum of patients dealing with advanced pancreatic cancer is higher than the dosage found in the serum of healthy patients (23).

It has been demonstrated that by increasing the concentration of substrates from the parallel n-3 and n-6 polyunsaturated fatty acid pathways, the formation of the products in that pathways also increases and metabolism in the parallel pathway is reduced. DHA and EPA suppress the expression of both $\Delta 5$ and $\Delta 6$ -desaturases and normalize LNA and AA levels (28). Because of the pro-inflammatory and immunoactive functions of the AA, the increase of this fatty acid in serum and AA/EPA ratio can be considered a biomarker in many diseases which include a pro-inflammatory state like sickle cell disease, obesity or cystic fibrosis.

Elevated plasma AA levels may thus be a source of pro-aggregatory substances in case of sickle cell disease patients (29). On the other hand, AA deficiency may predispose human organism to developing type 2 diabetes mellitus, because a significant decrease of the principal n-6 PUFA and AA has been observed in diabetic patients (26). Moreover, NEFA's concentrations were found to be higher in plasma samples of diabetic patients (31).

Cystic fibrosis is the most common lethal genetic disease in Caucasian population, which presents an alteration of plasma levels of polyunsaturated fatty acids. The major ones are decreases in linoleate and docosahexaenoate and increases in palmitoleate and mead acid. In case of cystic fibrosis the AA increases only at the highest LNA:ALA ratio, and the production of AA is influenced by ALA, the initial substrate of the parallel n-3 pathways. Also EPA levels were higher and DHA levels lower in cystic fibro-

sis compared to healthy patients. Katrangi and co-workers also demonstrated that the serum ratio of LNA to ALA has a greater impact on LNA to AA metabolism than on ALA to EPA metabolism. The increased production of AA plays a significant role on cystic fibrosis related inflammation (28).

Levels of FAE have been shown to vary between species, in hamster plasma samples the mean levels of OEA and PEA are higher compared to human plasma samples. Compared to plasma samples, levels of AEA were higher in the intestine and in hamster liver. The concentration gradient for hamster liver and small intestine was shown to be $PEA > OEA > AEA$, but further investigation is required in order to fully understand FAE distribution patterns in case of different tissues. Levels of OEA can be influenced by the volunteers' physiological state and also by the type of dietary treatments (24).

Very long chain fatty acids (VLCFA) are minor constituents and are difficult to detect and separate. They are biosynthesized from fatty acids by elongase and they are degraded by β -oxidation in peroxisomes. In genetic peroxisomal diseases, such as Zellweger syndrome and X-linked adrenoleukodystrophy (X-ALD), VLCFA are known to accumulate and increase intracellular calcium concentrations and decrease mitochondrial respiration, which can lead to cell death in oligodendrocytes and astrocytes. The accumulation of VLCFA, including C26:0 has been accepted as the principal biochemical abnormality in X-ALD and Zellweger syndrome. The proportions of sphingomyelin with C26:0, C26:1 and C26:2 were significantly increased in peroxisomal disease (4). New molecular species with VLCFA were found in the phospholipids classes of phosphatidyl-ethanolamine and phosphatidyl-serine, which can be used as new biomarkers for peroxisomal diseases. The main disadvantage was the observed differences in the molecular species between patients (4).

Milk is the primary food and also comes as a biological fluid (7,8). Authors have revealed by GC-FID analysis that the FA profile in human milk is different from that in infant formulas (7), where essential fatty acids and their eicosanoids had less concentrations in the commercial formulations than in human milk. However, Cruz-Hernandez and co-workers (8) showed an inter-individual variability in fatty acids profile in human milk, which is to be taken in consideration. But a supplementation with DHA at lactating mothers can increase this fatty acid's profile in milk and plasma and also, can improve the n-3/n-6 PUFA ratio in these biological fluids (41).

All of the up-mentioned clinical implications of fatty acids profiles are listed in Table III.

6. Conclusions

This paper has presented an extensive literature review of the recent years, regarding the most important techniques for the analysis of fatty acids in biological samples. We have presented the main utilized methods in each case, but also have noted unusual methods which can be successfully used. GC-MS is the main chromatographic technique applied in clinical lab for the analysis of fatty acids from serum, plasma and other biological fluids or tissues, after their methyl-transesterification. In the same time, HPLC or CE coupled with different types of detectors can be successfully used for the analysis of fatty acids in these bio-samples. Alternative spectral methods, like infrared and Raman spectroscopy, have been used for the analysis of fatty acids, mostly coupled with multivariate analysis of data.

We have also presented the most important results obtained along with the clinical relevance of fatty acids profiles and, as applicable, biomarkers or fatty acids with importance in the presence or diagnosis of the investigated diseases. The n-3/n-6 PUFA ratio can be pointed out

as an important factor in many diseases, starting from inflammatory states and diabetes mellitus up to cancerous states.

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Abbreviations

AA (arachidonic acid);
AEA (arachidonyl-ethanolamide);
ALA (alpha-linolenic acid);
ALEA (alpha-linolenoyl-ethanolamide);
ATR-FT-IR (attenuated total reflectance Fourier transform infrared spectroscopy);
CE (capillary electrophoresis);
DCM (dichloromethane);

Table III. Potential clinical utility of FA profiles in different diseases

Diseases	Analyzed sample	Method	Biomarkers	FA profile	Ref.
colorectal cancer	colon tissue	GCXGC/ TOFMS	N/A	decrease ratio of n-3/n-6 PUFA ratio increase SFA	(12,13)
pancreatic cancer	human serum	HPLC-fluorescence detector	N/A	increase in C16:0, C18:1, and C18:0 serum levels	(23)
neurologic/ psychiatric	brain samples	TLC, GC-FID	cholesteryl esters	decrease of AA and DHA concentration	(2)
X-ALD, Zellweger	cell culture	LC-ESI-MS/MS	C26:0/C22:0 ratio	increase of VLCFA levels	(4)
sickle cell disease	human plasma	UFLC	AA/EPA ratio	increase in AA and DGLA levels decrease of EPA and DHA levels	(29)
cystic fibrosis	human bronchial epithelial cells	RP-HPLC	LNA:ALA ratio	increases in EPA, C16:1 and mead acid levels decrease in C18:2, DHA levels	(28)
meibomian gland dysfunction	human meibum	LC-FTMS	N/A	C16:0 and C18:0 are present in lower amounts	(25)
polycystic ovary syndrome	plasma	(GC-MS)	nervonic acid (C24:1 n-9)-PCOS	decreased ratio of n-3/n-6 PUFA	(14)
insulin resistance	plasma	GC-MS	dihomo- γ -linolenic acid (DGLA)	decreased ratio of n-3/n-6 PUFA	(14)
diabetes mellitus	human plasma	UFLC-MS/MS LC-EI-MS GC-MS GC-FID	arachidonic acids (C20)	increase in the EFA decrease in the NEFA and AA	(5,6,11,31)
myocardial infarction	serum	GC	total DHA+EPA profile	DHA+EPA profile significantly correlated with the size of ischemia	(40)

DHA (docohexaenoic acid);
 DHEA (docohexaenoyl-ethanolamide);
 DMOX (4,4-dimethyl-oxazoline);
 EFA (esterified fatty acid);
 EPA (eicosapentaenoic acid);
 FA (fatty acid);
 FAE (fatty acid ethanolamide);
 FAME (fatty acid methyl ester);
 GC-FID (gas chromatography coupled with flame ionization detector);
 GC-MS (gas chromatography coupled with mass spectrometer detector);
 HPLC-MS (high performance liquid chromatography coupled with mass spectrometer detector);
 LC-ESI-MS (liquid chromatography coupled with electro-spray ionization mass spectrometer detector);
 LEA (linoleoyl-ethanolamide);
 LNA (linoleic acid);
 LPE (liquid phase extraction);
 MSTFA (N-methyl-N-trifluoroacetamide);
 MUFA (mono-unsaturated fatty acid);
 NEFA (non-esterified fatty acid);
 OAHFA (O-acyl-hydroxy-fatty acid);
 OEA (oleyl-ethanolamide);
 PEA (palmitoyl-ethanolamide);
 PUFA (poly-unsaturated fatty acid);
 SPE (solid phase extraction);
 TOF-MS (time of flight mass spectrometry);
 UPLC-MS (ultra-high performance liquid chromatography coupled with mass spectrometer detector);
 VLCFA (very long chain fatty acid).

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