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FATTY ACIDS DETERMINATION IN TROUT PLASMA AND MEAT BY GC-MS

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ABSTRACT. The aim of this study was to develop a simple and reliable GC-MS method to compare the fatty acids in trout plasma and meat. The lipids were extracted from 0.5 mL of plasma and 1 g of meat using chloroform: methanol 1:2 (v:v) and then derivatized into fatty acids methyl esters (FAMEs) by esterification with methanol: acetyl chloride 4:1 (v:v). For FAMEs quantitation, undecaenoic acid (C11:1) was used as internal standard. High proportions of unsaturated fatty acids (UFAs) were found both in plasma and in meat samples. The highest proportion of UFAs in meat samples (53%) was represented by the ω-3 fatty acids.

Keywords: *DHA, EPA, essential fatty acids, FAME, GC-MS, SFA, UFA.*

INTRODUCTION

Gas chromatography coupled to mass spectrometry (GC-MS) is the method of choice for fatty acids identification and quantitation [1-3]. Fatty acids methyl esters (FAMEs) are the most widely used derivatives for GC analysis due to their easy derivatization procedure, volatility and good chromatographic separation [4].

In the vast family of fatty acids, only the polyunsaturated fatty acids (PUFAs) are essential nutrients. They were named *essential fatty acids* (EFA) and divided in two main categories: ω-6 fatty acids and their homologous and ω-3 fatty acids. Unlike the linear and rigid chemical structure of the saturated fatty acids (SFA), the structure of PUFAs is bended, twisted and flexible [5].

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The ω-3 fatty acids get into our diet through a food chain that starts from the algae – the richest ω-3 fatty acids sources – which are consumed by small aquatic animals, and ends with the fish, which eat them. Other sources of dietary ω-3 fatty acids, but with a shorter carbon chain, are the flaxseeds and the walnuts. The sources of ω-6 fatty acids in our diet are mostly the vegetable and seed oils [6].

Once consumed and absorbed into the body, the ω-3 and ω-6 fatty acids are incorporated into the cell membranes. Then, they are converted into intermediate molecules and finally, into hormone-like substances named *eicosanoids*. Among the *eicosanoids*, the most important are the *prostaglandins* – cellular signaling molecules that mediate the inflammatory process, fighting against infections and performing multiple roles within the immune and cardiovascular systems, and even within the brain [5, 6]. Recent studies have confirmed that mankind has evolved due to a balanced diet in ω-3 and ω-6 fatty acids. The ideal ω-3/ω-6 ratio, established by nature, is 1:1, while, in the modern world, in the typical American diet, this ratio has reached 1:20 [6].

Docosahexaenoic acid (4,7,10,13,16,19-DHA; $C_{22}H_{32}O_2$) is an ω-3 PUFA. Highest body concentrations of DHA are found in retinal membranes [5].

Eicosapentaenoic acid (5, 8, 11, 14, 17 -EPA; $C_{20}H_{30}O_2$) is the other major dietary ω-3 PUFA. EPA is present in blood components [5] and when working in tandem with DHA, the EPA eicosanoids derivatives maintain control over DHA eicosanoids derivatives [6].

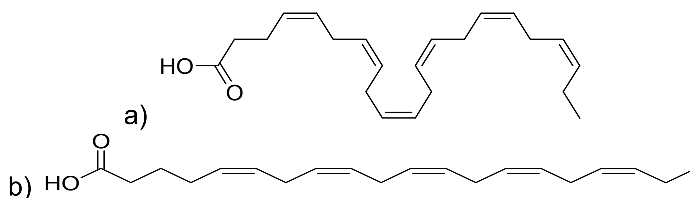

Figure 1. The ω-3 fatty acids chemical structures: a) DHA (C22:6 ω-3); b) EPA (C20:5 ω-3).

The nutritional quality of fish species can be evaluated from the fatty acids profile and by determining the EPA and DHA proportions [7].

The purpose of this research was to develop and validate a GC-MS method in order to investigate the essential fatty acids composition of rainbow trout (*Oncorhynchus mykiss*) plasma and meat.

RESULTS AND DISCUSSION

The fatty acids profile of freshwater fish is unique in variety and degree of unsaturation [8-16]. Their nutritional role is recognized. The two main omega-

3 fatty acids eicosapentaenoic acid and docosahexaenoic acid appear to decrease the risk of cancer [17-18]. Seasonal variation of these nutrients study is also very much studied [19-21].

Therefore, it is essential to have a simple and rapid method for qualitative and quantitative characterization of less common fatty acids (e.g. long-chain polyunsaturated fatty acids). The sensitivity and selectivity of GC-MS make it a powerful tool for the analysis of FAMEs [3].

Figure 2 presents the total ion current chromatogram of a mixture of trout plasma fatty acids. The FAMEs were identified using the NIST Mass Spectral Library.

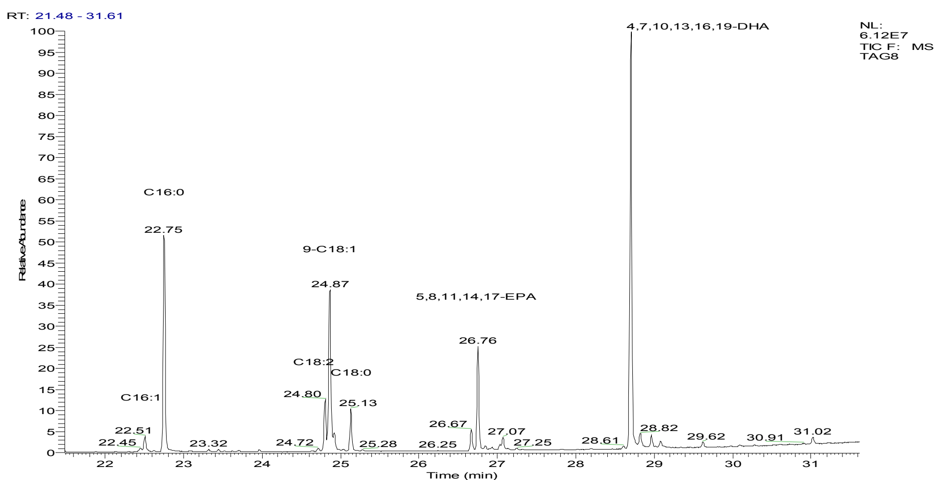

Figure 2. TIC chromatogram of trout plasma FAMEs obtained from GC-MS analysis.

The method was validated using fatty acids standards of 20 µg/ml. The limit of detection (LOD) was 1ng and precision and accuracy gave values lower than 20%.

Table 1 shows the fatty acids composition (weight% of total fatty acids) of trout plasma and meat. Saturated fatty acids represent only 25.61% (in plasma) and 31.04% (in meat) of the total fatty acids, palmitic acid (C16:0) having the highest concentration. Stearic acid (C18:0) is present in relatively smaller proportions (5.42% and 6.27%).

The unsaturated fatty acids (UFAs) constitute more than half of the total fatty acids found in plasma and meat samples (74.39% and 68.95%, respectively). The major monounsaturated fatty acids (MUFAs) were: C16:1, C18:1n-7, C18:1n-9, oleic acid (C18:1n-9) being the most abundant. Linoleic acid (C18:2 ω-6) represents 19% (in plasma) and 11.3% (in meat) of the total UFAs. The ω-3 PUFAs (the sum of EPA and DHA) represent approx. 26% and 36.6% respectively, of the total FAs found in trout plasma and meat.

Table 1. Fatty acid concentrations (%) in trout plasma and meat n = 5
(R_t – retention time; SFA – saturated fatty acids, UFA – unsaturated fatty acids;
SFA = C16:0 + C18:0; UFA = C16:1 + C18:2 + 9-C18:1 + C18:1 + EPA + DHA)

		Fatty acids (%)	
	R_t (min)	Plasma	Meat
hexadecenoic acid (C16:1)	22.51	2.86	2.19
hexadecanoic acid (C16:0)	22.75	20.18	24.78
9,12 octadecadienoic acid (C18:2)	24.80	14.13	7.81
9-octadecenoic acid (C18:1)	24.87	27.79	18.91
octadecenoic acid (C18:1)	24.93	3.62	3.43
octadecanoic acid (C18:0)	25.13	5.42	6.27
5,8,11,14,17 eicosapentaenoic acid (C20:5)(EPA)	26.76	5.41	8.51
4,7,10,13,16,19 docosahexaenoic acid(C22:6)(DHA)	28.71	20.59	28.11
SFA		25.61	31.04
UFA		74.39	68.95
EPA		5.41	8.51
DHA		20.59	28.11

The representatives mass spectra of DHA and hexadecanoic acid (palmitic acid) as FAMEs are shown in figures 3 and 5.

In the mass spectrum of DHA methyl ester the molecular ion is missing but it presents the specific ions M-15 (m/z 313), M-101 (m/z 227) of small intensity, indicating the molecular mass (M=328) and also high intensity ions, specific for alkyl group with double bonds, as m/z 55, 67, 79, 91 etc.

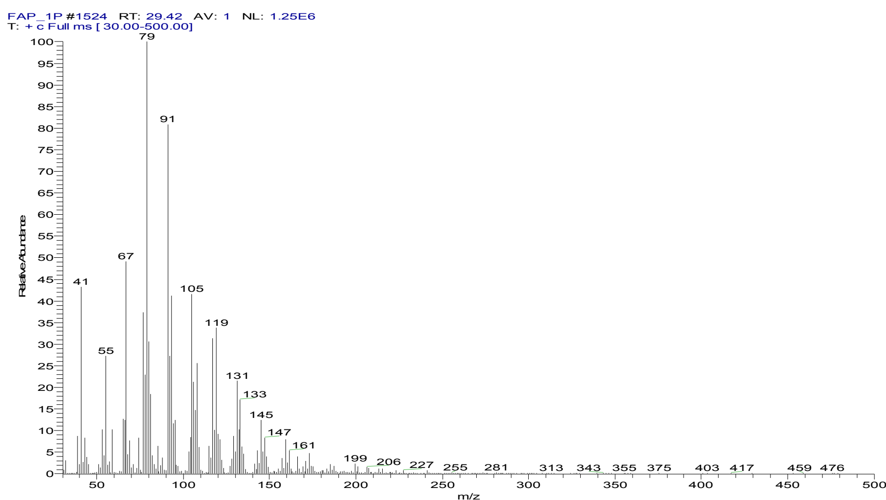

Figure 3. Representative mass spectrum of 4,7,10,13,16,19 DHA methyl ester (M=328) in trout meat.

In figure 5, the ion m/z 270 represents the molecular ion peak of palmitic acid methyl ester. The ion m/z 239 $[M-31]^+$ representing loss of a methoxy group confirming a methyl ester compound. *m/z* = 227 $[M-43]^+$ represents loss of a C_3 unit (carbons 2 to 4), via a complex rearrangement, and *m/z* = 74 is the McLafferty rearrangement ion, also a specific ion confirming that the spectrum is that of a methyl ester. The series of ions *m/z* = 87, 101, 115,129, 143, 157, 199, etc., of general formula $[CH_3OCO(CH_2)_n]^+$, is a series of related ions formed by losses of neutral aliphatic radicals14 amu, of which that at *m/z* = 87 is most abundant.

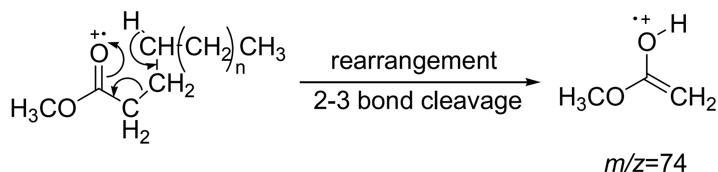

Figure 4. McLafferty rearrangement ion m/z 74 [9]

In the rearrangement ion m/z 74, a hydrogen atom from position 4 of the aliphatic chain migrates to the carbo-methoxy group, through a six-membered transition state, which is sterically favoured. If one of the hydrogen atoms on carbon 4 is substituted, the McLafferty ion will be lower in intensity, as appears in the mass spectra of derivatives of unsaturated fatty acids with increasing numbers of double bonds. The ion *m/z* = 227 $[M-43]^+$ is formed by a loss of a propyl radical. The ion at $[M-29]^+$ results by a cleavage between carbons 3 and 4 [9].

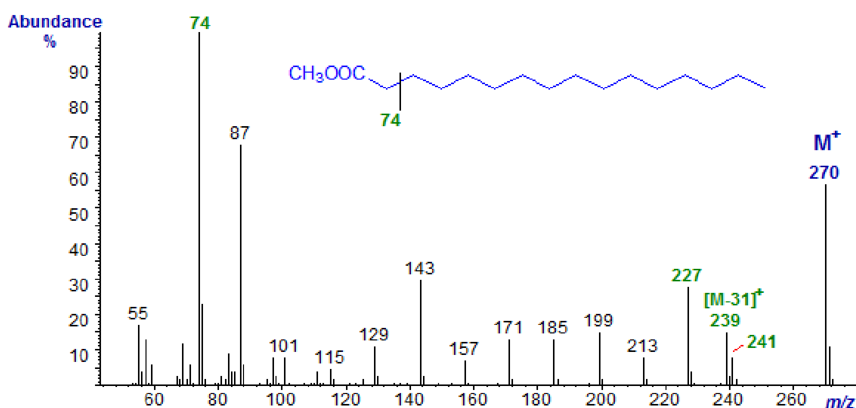

Figure 5. The mass spectrum of hexadecanoic acid methyl ester (palmitic acid, M=270) [9]

Fig. 6 shows the considerable proportion of UFAs in comparison with SFAs, both in plasma and in meat. It should be noted that in meat samples, the ω-3 PUFAs represent 53% of the total UFAs.

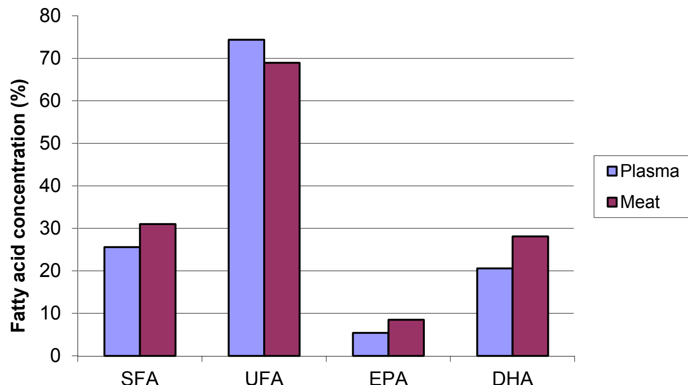

Figure 6. Fatty acids content of plasma and meat trout samples.

In plasma, the ω-3/ω-6 ratio was 1.84, while in meat, 4.68. The DHA/ EPA ratio was 3.8 in plasma and 3.3 in meat. This result can be explained by the fact that EPA is not found in great amounts in tissues as it is quickly used in DHA and eicosanoids biosynthesis [5].

CONCLUSIONS

The GC-MS method developed here for determining the fatty acid profile of trout plasma and meat samples is simple and reliable. Good validation parameters were obtained.

The PUFAs concentration found in trout plasma and meat was higher than that of the other FAs, in the following order: PUFA>MUFA>SFA. Our results proved that trout meat is a valuable source of essential fatty acids.

EXPERIMENTAL SECTION

Materials and methods

Acetyl chloride was purchased from Fluka (Germany) while all the other chemicals were from Merck (Darmstadt, Germany).

To quantify the FA concentrations by GC-MS, undecaenoic acid (C11:1) was used as internal standard.

The fatty acids (FA) were determined from trout plasma and meat samples. The FA were extracted from 0.5 mL of plasma by adding 0.5 mL chloroform: methanol 2:1 (v:v). The solution was shaken vigorously for 30 s, at room temperature.

1 g of trout meat was crushed with 1 g of quartz sand in a ceramic dish and homogenized with 5 mL distilled water. After a 5 min centrifugation, the supernatant was collected and the FAs were extracted by using the same solvent extraction conditions as for plasma. The samples were centrifuged for 5 min (5800 rot/min) and the upper methanol - water phase was removed. The lower chloroform phase containing the extracted fatty acids was then dried in a nitrogen flow, at 60°C.

The lipids were converted to corresponding FAMEs (fatty acids methyl esters) by esterification of the carboxylic functions with 200 µL methanol: acetyl chloride 4:1 (v:v), 20 min, 80°). The derivatives were evaporated to dryness by a nitrogen stream, at 60°C, and then dissolved in 500 µL dichloromethane. 10 µg of C11:1 was added to each sample for GC-MS quantitation.

GC – MS apparatus

The fatty acids were separated and identified using a Gas chromatograph Trace GC equipped with an Rtx-5MS capillary column (30m x 0.25mm I.D., 0.25 µm film thickness) and coupled to a quadrupole mass spectrometer Trace DSQ (Thermo Finnigan). The temperature program for FAMEs separation was: 50°C for 2 min rising with a rate of 8°C/min at 310°C (8 minutes). Helium was used as carrier gas at a flow rate of 1 mL/min. 1µL of each sample was injected into the GC-MS using the split mode (10:1) and a TriPlus autosampler. The mass spectrometer operated in electron impact (EI) mode at 70 eV. The following conditions were ensured: the transfer line temperature was set at 250°C, the injector temperature, at 200°C and the ion source temperature, at 250°C. The emission current was 100µA. The qualitative analysis was carried out in the 50-500 a.m.u. mass range.

GC – MS quantitation

The quantitative analysis was performed with respect to the internal standard (C11:1), by using the following formulas:

$$F_i = \frac{\frac{A_i}{A_j}}{\frac{m_i}{m_j}} \qquad (1)$$

$$m_i(\mu g) = m_j(\mu g)\frac{A_i}{F_i \cdot A_j} \quad (2)$$

where F_i is the response factor of the compound *i*, m_i is the quantity of compound i, m_j is the internal standard quantity; A_i and A_j are the peak areas of the compounds (*i* and *j*).

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