P02: COMPREHENSIVE ANALYSIS OF FATTY ACIDS IN OYSTERS BY GC-FID/GC-MS

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Abstract – To the analysis of fatty acids in marine biota are generally related some uncertainties. One problem is that most profile analyses reported in literature disagree about the number and type of fatty acids monitored as well as about the percentage to assign to their sum over total fatty acids. Another issue is the discriminant effect in the split/splitless injector of gas chromatographs which can lead to relevant errors in quantifying DHA (22:6 ω3). In a previous work we solved these troubles for 12 fish species. In the present one the case of bivalve mollusks (oysters, mussels) is dealt with. Oysters are part of the analytical activity for producing a new Reference Material within the H2020 Project "PRO-METROFOOD".

Keywords: Fatty Acids (FAs); Fatty Acid Methyl Esters (FAMEs); Absolute analysis; Profile analysis; Oysters; Mussels; Reference Material

1. INTRODUCTION

Quality and quantity of fatty acids (FAs) in food are of great relevance in human health, with important benefits deriving from the consumption of ichthyic products. But the analysis of FAs in marine biota may be a difficult matter. First of all a complex matrix like a fish oil may reveal up to 100 different fatty acids, or more. Generally only a limited number of them are selected by analysts and it is often observed that different laboratories may select different sets of FAs [1-3]. Another uncertainty may derive from the injector port of the gas chromatograph since a discriminant effect exists towards the long-chain PUFAs (Polyunsaturated Fatty Acids), especially towards DHA (Docosahexaenoic acid, 22:6 omega-3) one of the most important omega-3 fatty acids exclusively present in aquatic biota. The discriminant effect is well established [4, 5] but it is little known and therefore may influence accuracy of the profile analysis.

It has to be noted that many laboratories report results as profile analysis (i.e. in percentage form) but absolute analyses (mg/100g edible portion) are much more useful. When conducted, the absolute analysis is generally performed by carrying out profile analysis and then by applying a conversion factor. But the uncertainty associated with an erroneous profile analysis, as cited little above, could affect in an unpredictable way result accuracy.

In an our previous work [5] we faced these issues: a) a set of the most important FAs was selected and then compared with all FAs present in the sample in order to ascertain the true percentage over total. To our knowledge it was the first time the task was executed; b) an experimental coefficient was determined according to European Union Regulation [6] to compensate the discrimination effect of the GC injector on DHA; c) absolute analysis was conducted by using the accurate profile so obtained and then by applying the conversion factor of Weihraush et al. [7]. Of great importance in this passage was the experimental verification that such conversion factor was actually reliable.

That research focused on 12 Mediterranean fish species and the new method was validated exclusively for such species.

In the present work we develop and propose an analogous method for oysters and mussels.

Method validation was carried out by following Good Laboratory Practice guidelines. In particular were analyzed appropriate Certified Reference Materials.

Two different instrumental techniques on the same sample were used: GC-MS and GC-FID.

2. EXPERIMENTAL

2.1 Materials and Methods

2.1.1 Reagents and Chemical Standards

The 20 monitored fatty acid methyl esters (FAMEs) are listed in Table 2. Methanol, n-hexane, acetone, petroleum ether 40-60°C and chloroform were purchased from Carlo Erba Reagents®. Boron trifluoride methanol solution (14%) was purchased from Sigma Aldrich®. Analytical standards of triglycerides and individual analytical standards of the 20 FAMEs studied were purchased either from Sigma Aldrich® or from Larodan®. The Certified Reference Material FAPAS® T1475, fish oil, was from The Food and Environment Research Agency, Sand Hutton, UK.

2.1.2 Oyster and Mussel Samples

The oyster sample to be characterized within the H2020 project "PRO-METROFOOD" was delivered in lyophilized form: five aliquots of 25 g in plastic bottles (RM 003, n. 36 to 40).

The reference material "lyophilized Sea Water Mussel SQC068MUS-30G, lot LRAA9269" was from Sigma-Aldrich®.

2.1.3 Sample Preparation

Lipids were extracted from the lyophilized samples by means of an acetone-petroleum ether procedure as already detailed [8] with minor modifications. An aliquot of oil coming from the lipid extraction step was derivatized. Fatty acids were GC-analyzed as FAMEs (methyl derivatives): derivatization was carried out by means of BF_3 and methanol as already described [5]. Errors in quantitative analysis arising from considering FAMEs as FAs are negligible, as reported [5].

2.2 Gas chromatographic analysis

2.2.1 GC-FID

The hexanic solution of FAMEs coming from derivatization was injected in a 6890 Agilent gas chromatograph with flame ionization detector (GC-FID) equipped with a CP-WAX 52 CB column (60 m × 0.32 mm I.D., 0.50 µm film thickness) from Chrompack®, the Netherlands. Helium as the carrier gas was used in ramp flow mode: from 1.0 to 2.5 mL/min. Operating conditions for the oven temperature were: initial temperature 50 °C, 2-min hold, increasing to 140 °C, at a rate of 22.5 °C/min, increasing to 228 °C, at a rate of 2 °C/min, this last temperature was maintained for 44 min. Injector temperature was 220 °C, while FID temperature was 275 °C. Injections were made in split mode (40:1) with an injection volume of 1 µL. Fig. 2 shows the GC-FID chromatogram of a mussel sample.

2.2.2 GC-MS

Instrument used was a Varian 3900 gas chromatograph connected to the Mass Spectrometer Saturn 2100T (GC-MS) equipped with an Ion Trap analyser. Column installed was a CP-WAX 52 CB (60 m × 0.32 mm I.D., 0.50 µm film thickness) from Chrompack®, the Netherlands. Injections were made in split mode (100:1) with an injection volume of 0.5 ± 0.1 µL and by using 1 µL of n-hexane as a plug. Column oven temperature was settled at an initial value of 50 °C (2 min hold), increased to 140 °C (0 min hold, ramp of 22.5 °C/min), and finally increased to 228 °C (44 min hold, ramp of 2.0° C/min). Helium as the carrier gas was used at a variable flow: initial flow 1.0 mL/min, final flow 2.5 mL/min. Injector temperature was 220°C. Mass spectra were obtained in EI (Electron Ionization) mode at 70 eV. Ion trap temperature was 180°C. The selected Mass to Charge Ratio to acquire was in the 40-440 m/z range.

3. RESULTS AND DISCUSSION

Fig. 1 shows the scheme of the analysis. The procedure allows that both profile and absolute analysis are carried out.

The characterization of the oyster tissue within the PRO-METROFOOD project is currently ongoing and the definitive results will be available only when the work will be completed. However a reference material very similar to the oyster tissue under study (lyophilized Sea Water Mussel) was analyzed: it is very suitable to describe the proposed method.

3.1 Step 1, lipid extraction

Lipid extraction is exposed in section 2.1.3. The measured lipid content in lyophilized Sea Water Mussel was 12.35 % (12.35 g of lipids per 100 g of lyophilized sample).

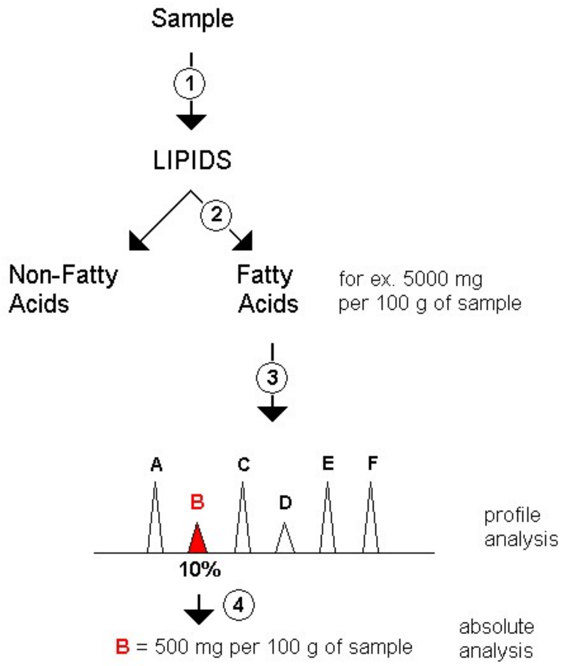

Figure 1. Scheme of the analysis

3.2 Step 2, total FAMEs (mg per 100 g of sample)

Lipids are mainly constituted by fatty acids but not entirely. For the absolute analysis as here conducted it is essential to know how much of the 12.35 g of extracted lipids is made up of fatty acids. This assessment could be conducted experimentally by means of saponification-hydrolysis of an aliquot of lipids [9]. Alternatively the same result can be obtained by applying the conversion factor of Weihrausch [7]. In the previous work [5] we have experimentally checked the factor of Weihrausch for finfish by carrying out some saponification-hydrolysis on real samples: the factor proved to be very accurate.

In the present research we apply the analogous conversion factor defined by Weihraush for mollusks [7]. It is:

$$FACTOR_{mollusks} = 0.956 - \frac{0.296}{Total\ Lipids\ (\%)} \quad (1)$$

In the present case:

$$FACTOR_{mollusks} = 0.956 - \frac{0.296}{12.35} = 0.932 \quad (2)$$

The number 0.932 is a percentage: 93.2 % of extracted lipids are constituted by fatty acids and therefore we have

$$93.2 : 100 = x : 12.35 \qquad x = 11.51 \quad (3)$$

in the lyophilized Mussel sample are present 11.51 g/100 g of total FAs (more precisely 11,511 mg/100g).

3.3 Step 3, profile analysis

The lyophilized mussel sample was deeply investigated by GC-MS. All peaks with S/N>3 were checked and integrated. A total number of 95 peaks were observed, 79 of which were FAMEs, while 16 were not FAMEs. In these last are comprised various compounds, such as for example, Diethyl Phthalate (coming very likely from the injector septum).

Among the 79 detected FAMES they were selected the most important ones for a number of 20 fatty acids analyzed as Table 2 shows.

Before calculating the percentage of each of the 20 selected FAMEs (by comparing the individual peak area with the area-sum of the 79 peaks detected) there is an important consideration to be made. If we directly relate the peak area percentage with the quantity in the sample we implicitly admit that all FAMEs have the same GC response factor. Previous works [4, 5] have demonstrated that this is not true, at least in one case: DHA (Docosahexaenoic acid, 22:6 omega-3). Due to a relevant discrimination effect in split/splitless injectors [4] the response factor of DHA significantly differs from that observed for the other FAMEs.

To face this phenomenon a coefficient was experimentally derived in our laboratory according to the EU Commission Regulation 2568/91 [6]: if an accurate profile analysis is to be achieved the DHA peak area is to be multiplied for the coefficient 1.26 to convert DHA peak area-percentage into DHA quantity-percentage, at least with the present experimental conditions [5].

In Table 1 it is reported the analysis of the Certified Reference Material FAPAS® T1475. The good agreement between the measured values and the certified ones should be noted.

Table 1. Analysis of the Certified Reference Material FAPAS® T1475 “fish oil”.

Profile analysis (%). N. of analyses = 2

FAME	measured value		certificate	
	GC-MS	GC-FID	assigned value	satisfactory range
18:3 ω-3	0.95 ± 0.04	1.03 ± 0.02	0.95	0.86 - 1.05
20:5 ω-3	11.08 ± 0.23	10.95 ± 0.18	12.93	11.64 – 14.23
22:5 ω-3	1.74 ± 0.04	1.33 ± 0.01	1.88	1.69 – 2.07
22:6 ω-3	11.97 ± 0.61	8.94 ± 0.13	10.66	9.59 – 11.73

NOTE. Only the profile analysis has been certified

3.4 Step 4, absolute analysis

Once the profile analysis is carried out without inaccuracies, as exposed above, the calculation of the absolute quantity of a given fatty acid (mg per 100 g of lyophilized sample) is easily achievable according to the equation (4):

$$\%\ FAME : 100 = mg_{FAME} : totalFAs\,(mg) \quad (4)$$

As an example we may consider the 20:5 ω-3 fatty acid (EPA) in the reference material “mussel” (see Table 2). From the GC-MS profile analysis a percentage of 13.09% was obtained. Total FAs in the sample were 11,511 mg as determined in section 3.2. Therefore:

$$13.09 : 100 = mg_{EPA} : 11{,}511 \quad mg_{EPA} = 1507 \quad (5)$$

A content of 1507 mg /100g was so determined by GC-MS for EPA.

Table 2 shows the comprehensive analysis of the reference material Sea Water Mussel SQC068MUS-30G, lot LRAA9269”, from Sigma-Aldrich®. The material was not certified for the FAME content.

The identical approach is applied for the lyophilized Oyster samples to be characterized within the H2020 project “PRO-METROFOOD”.

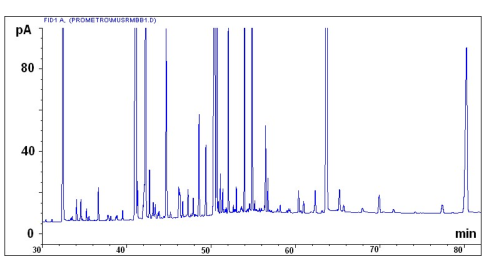

Figure 2. GC-FID chromatogram of a mussel sample

Table 2. Analysis of the reference material “lyophilized Sea Water Mussel”.

N. of analyses = 1

FAME	profile analysis (%)		absolute analysis (mg/100g)	
	GC-MS	GC-FID	GC-MS	GC-FID
14:0	8.13	6.55	936.39	754.02
15:0	0.34	0.24	39.63	27.58
16:0	13.14	15.93	1512.04	1833.48
17:0	0.06	0.06	6.99	7.26
18.0	1.12	0.95	129.20	109.36
14:1 ω-5	0.20	0.12	23.05	13.55
16:1 ω-7	6.11	6.27	703.14	721.97
18:1 ω-9	14.06	14.96	1617.97	1721.71
18:1 ω-7	8.17	8.66	940.99	996.87
20:1 ω-9	1.23	0.96	141.30	110.49
22:1 ω-9	0.76	0.43	87.13	49.91
22:1 ω-11	0.00	0.00	0.00	0.00
18:2 ω-6	2.03	2.04	233.80	235.27
18:3 ω-3	2.07	2.23	238.21	256.57
18:4 ω-3	4.77	5.87	549.05	675.16
20:2 ω-6	0.11	0.09	12.29	9.89
20:4 ω-6	0.38	0.32	43.93	37.14
20:5 ω-3	13.09	13.15	1507.30	1513.44
22:5 ω-3	0.43	0.24	49.98	27.82
22:6 ω-3	10.85	7.24	1248.55	833.86

4. CONCLUSIONS

Fatty acids deriving from the consumption of fish, mollusks and crustaceans bring noticeable benefits to human health. The analysis of FAs is a very important matter since such benefits strongly depend from the quality and quantity taken daily with diet. Historically, this type of analysis made use of more or less large approximations given the complexity of the matter.

The method here presented introduce some new procedures that make it possible to analyze FAs in lyophilized mollusks with great accuracy.

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REFERENCES

[1] D. Pacetti, F. Alberti, E. Boselli, N.G.B. Frega, Characterisation of furan fatty acids in Adriatic fish, Food Chem. 122, (2010) pp. 209-215.

[2] M. Jalali-Heravi, M. Vosough, Characterization and determination of fatty acids in fish oil using gas chromatography–mass spectrometry coupled with chemometric resolution techniques, J. Chromatogr. A 1024, (2004) pp. 165-176.

[3] F. Jabeen, A.S. Chaudhry, Chemical compositions and fatty acid profiles of three freshwater fish species, Food Chem. 125, (2011) pp. 991-996.

[4] M. Schreiner, Quantification of long chain polyunsaturated fatty acids by gas chromatography. Evaluation of factors affecting accuracy, J. Chromatogr. A 1095, (2005) pp. 126-130.

[5] T. Nevigato, M. Masci, E. Orban, G. Di Lena, I. Casini, R. Caproni, Analysis of fatty acids in 12 mediterranean fish species: advantages and limitations of a new GC-FID/GC–MS based technique, Lipids 47, (2012) pp. 741-753.

[6] European Union Regulation, Commission regulation (EEC) No 2568/91 (1991) Analysis by gas chromatography of methyl esters of fatty acids: use of correction factors. Official Newspaper of the European Community 1991R2568:59–60.

[7] J.L. Weihrauch, L.P. Posati, B.A. Anderson, J. Exler, Lipid conversion factors for calculating fatty acids contents of foods, J. Am. Oil Chem. Soc. 54, (1977) pp. 36-40.

[8] M. Masci, E. Orban, T. Nevigato, Organochlorine pesticide residues: An extensive monitoring of Italian fishery and aquaculture, Chemosphere 94, (2014) pp. 190-198

[9] J.E. Kinsella, J.L. Shimp, J. Mai, J. Weihrauch, Fatty acid content and composition of freshwater finfish, J. Am. Oil Chem. Soc. 54, (1977) pp. 424-429.