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Analytical Aspects of the Flame Ionization Detection in Comparison with Mass Spectrometry with Emphasis on Fatty Acids and Their Esters

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1. Introduction

The initial development of gas chromatography (GC) is deeply interconnected with lipid analysis. This separation technique may be considered as the main contribution for most of knowledgement regarding fatty acid (FA) composition that exists today. Actually, it is possible to convert milligrams of a lipidic sample to fatty acid methyl esters (FAMEs), separate them in a gas chromatograph and quantify these fatty acids in a short time [1].

Probably, the most important identification method which is coupled with GC is mass spectrometry (MS). It is older than GC, and this fact might be surprising for many readers. However, the basic principles and the first separations of atomic masses were primarily demonstrated in the last part of 19th century, while chromatographic columns appeared during 20th century [2].

The possibility of coupling a mass spectrometer in the exit of a chromatographic column, along with the advent of modern computers, introduction of fused silica capillary columns and reduction of interference issues allowed the achievement of analytical results which are more precise/accurate. Allied to these facts, the reduction of gas chromatographs prices enforced their application as a tool for researches with lipidic materials. However, in relation to food analysis with emphasis on fatty acid composition, the flame ionization detector (FID) is the

most popular one, because it is easy to operate, possesses a wide linearity range, rapid response, and its limit of detection is of 10^{-12}g for alkanes (an almost universal answer) [3].

In the last years, it can be observed a significant increase of published works about FAs which also envolve new discoveries regarding good and bad effects of FAs for human health, new food products derived from fats and oils available for the end user, novel FAs (such *trans* FAs) which do not exist in certain foods, supplementation of FAs in food products and optimization of biodiesel synthesis.

1.1. Derivatization of functional groups

Before analyzing FAs through GC, it is necessary to convert them into compounds with greater volatility and thermal stability (esters, for example). The transformation of FAs into other organic functionalities with lower boiling points enhances their elution through a chromatographic column. Thus, if correct conditions are used, it is possible to separate compounds with close molar masses or even isomers [3].

The formation of these esters commonly occurs from reactions between lipids with short chain monoalcohols (like (m)ethanol) which provide (m)ethyl radical. The competition between use of methanol versus ethanol originated from factors such as disponibility, reaction yield, toxicity, among others.

A method which is very efficient for fatty acid methylation was proposed in works done by Joseph and Ackman (1992) [4], and it is commonly employed as a fine tool for studies of lipidic composition.

1.2. Integration of chromatographic peaks

After sample injection in a gas chromatograph (GCph) and further separation of analytes through the chromatographic column, an chromatogram of the sample of interest is obtained. Such chromatogram is produced by the integration of the signals which are collected by the detector in a measure related with the amount of each analyte which are detected. In FID case, compounds are burned. Besides, important information such as peak number/area/percentages, as well as their respective retention times, can be obtained from the chromatogram [5].

These integration measures may be related with the existing FAs or FAMEs concentrations in a food/biodiesel sample. However, in order to apply this relation between chromatographic peak area and analyte concentration, the analyst must understand the principles and execute the necessary corrections. Otherwise, the expressed results will be erroneous and will not be clearly understood [6].

1.3. Flame ionization detector (FID) and the differential response

The used of FID for quantification of fatty acid esters (FAEs) is advantageous in relation to other detector types, due to the reasons mentioned before (it is easy to operate, possesses a wide linearity range, rapid response, and its limit of detection is of 10^{-12}g for alkanes). For a

didactical approach, from now on the discussions will be centered on FAMEs, although this book chapter may be extended for every FAE (ethyl esters, propyl esters, etc.) [5].

Instead of responding to solute property, FID is sensitive to the mass flux which passes through it in a determined amount of time. It is important to point out that, despite the fact that a solution is injected, solvent and methyl esters are separated by the column during chromatographic run. Thus, a certain mass, in gaseous form, of each compound will arrive at the detector togethter with the carrier gas. Inside FID, it undergoes combustion in a flame produced by oxygen and hydrogen, producing ions which will be collected by electrodes. The amount of formed ions when a certain methyl ester is present in carrier gas is greater than the amount of ions which are formed when only carrier gas is being burned, thus the generated current is converted into a voltage, amplified and registered under the form of a chromatogram [5].

Reaction (1) demonstrates the chemical ionization that occurs in FID:

$$CH + O \rightarrow CHO^+ + e^- \hspace{4cm} \text{(Reaction 1)}$$

The CHO^+ ion is unstable and quickly reacts with water in the flame in order to produce hydronium, according with the reaction (2):

$$CHO^+ + H_2O \rightarrow H_3O^+ + CO \hspace{4cm} \text{(Reaction 2)}$$

This reaction occurs for every 100000 carbon atoms which are introduced in the flame [3]. Thus, FID response is proportional to the number of carbon atoms which are burned.

Specifically, the magnitude of the signal generated by FID is proportional to the number of carbon atoms which are bonded to hydrogen atoms (active carbon, C*). However, there are intrinsic factors of a FAME molecule that alters FID response, such as presence of oxygen, which diminishes such response. The carbon atom of carboxylate group (COO) is not ionized in a proper manner during combustion, thus it is not considered as active carbon.

1.4. Mass spectrometric detector

Mass spectrometry is one of the most versatile and sensitive analytical methods which is employed in many studies, ranging from medical to technological science. This method allows the molecular mass/structure determination of unknown compounds, and to quantify small molecules to biomolecules such as proteins, lipids and oligonucleotides [7].

In MS analysis, samples may be directly inserted in the mass spectrometer (off-line analysis) or this equipment may be coupled with a separation technique such as GC, liquid chromatography (LC) or capillary electrophoresis (CE) [7].

A mass spectrometer is basically composed of an ionization source, where the analytes of a sample are ionized and/or fragmented in ions with specific values of mass/charge ratio (m/z). They are immediately accelerated towards a mass analyzer, which has the function of selecting

ions according to the desired m/z values. Such selection occurs through the application of electric and/or magnetic fields. A detector transforms the ionic (fragment) signals to electrical signals, and the magnitude of these signals in function of m/z ratio is converted by a data processor, generating a corresponding mass spectrum. The electron multipliers are among the most frequently used detectors in mass spectrometers [8].

The advanced methods in MS differ especially in sample ionization modes and mass analyzers. The most suitable ionization mode for an analysis will depend on physicochemical properties of analytes, such as polarity, molecular mass and boiling point [8], etc.

2. Methods of relative normalization and absolute quantification

Also called area normalization, this method is based on the relative area percentage of a certain FA in relation to the total area of all fatty acids which are eluted from the column. Despite the fact it is an obsolete method, a lot of works regarding FAEs analysis in foods/biodiesel still use it, where the results are expressed in relative area percentage. The disadvantages of normalization are propagation of errors due to results interdependence. Every component of a sample must be detected and, if a certain compound is omitted/estimated, the areas of other substances are affected. Besides, results obtained through this method normally are difficult to interpretand published in an erroneous form.

However, in the expression of results in form of FAMEs (biodiesel) or FAs (foods), where concentrations are expressed in FAME mass *per* raw material/sample mass, using internal standard (IS) or correction factors for FID will lead to more accurate results which can be used by professionals from different areas without major difficulties. It is noteworthy that, if correction factors are complicated for use, internal standard calibration must be used to express results as concentrations, not as area percentages [5].

3. Transformation of methyl ester percentual area in fatty acid concentration: an alternative method

In this method, conversion factors are used in the transformation of relative percentage of a chromatographic peak from a methylic ester (normalization method) in mass amounts of the corresponding FA. Results commonly are expressed in (milli)grams of FA *per* 100g of food or total lipids. Such a conversion factor is obtained from the amount in mass of FAs (tabulated values) which exists in the different classes of total lipids (triacylglycerols and phospholipids, mainly) and form their different masses in a certain food sample.

Some authors describe that the average percentual mass contribution of FAs in triacylglycerols (TAGs) and phospholipids (PLs) corresponds to 95.6% and 72.0%, respectively. This means that, upon expressing values in decimals, it can be understood that 1g of phospholipids possess

about 0.720g of FAs. Thus, the determination of F factor (decimal) may be done in the following way [6]:

F (decimal) = TAG × 0.956 + PL × 0.720

Where:

TAG = Mass amount of TAGs in total lipids.

PL = Mass amount of PLs in total lipids.

Assuming that a total lipid sample contains 65% in mass of TAG and 30% in mass of PL, the F factor with application decimal values will be:

F (decimal) = 0.65 × 0.956 × 0.30 × 0.720

= 0.84

Knowing that the mass percentage of a certain X fatty acid (relative percentage by normalization method) in sample is 26%, the amount in grams of this acid *per* 100g of total lipids (TLs) will be:

g of fatty acid X/100 g of TL = F (decimal) × % Area

g of fatty acid X/100 g of TL = 0.84 × 26

g of fatty acid X/100 g of TL = 21.76 g.

The conversion factor may be found for some specific foods. For example, Table 1 shows the F conversion factors (decimal) for different classes of TAGs and PLs in total lipids of fish.

Despite this method being easy and practical, it includes some constant values, often theoretical, that might result in errors. These constants and other interpretations will be discussed later in a practical example.

4. Internal standard calibration and correction factors

In order to obtain greater accuracy from quantification, it must be taken into account that FAEs respond to FID in a differential manner. Thus, it is necessary to use the theoretical correction factor (F_{CT}), determined through the number of active carbons (C*), and the experimental/empiric correction factor (F_{CE}). Both factors are obtained from comparisons between FAMEs and an internal standard [3].

The internal standard calibration method is considered less sensitive to injection errors and instrumental variations. Besides, the internal standard must have high degree of purity/stability, must not be component of sample, and it needs to elute separately from other analytes. It is not easy to fulfill all these requirement during a FAEs mixture analysis. However, some esters have been recommended for use as IS, such as methyl tricosanoate (23:0Me) for foods and methyl heptadecanoate (17:0Me) / ethyl oleate (18:1Et) for biodiesel, because they

% in mass of TL	% TAG	% PL	F (decimal)
0.65	-	92.3	0.66
0.70	7.1	85.7	0.68
0.80	18.8	75.0	0.72
0.90	27.8	66.7	0.75
1.00	35.0	60.0	0.77
1.25	48.0	48.0	0.80
1.50	56.7	40.0	0.83
1.75	62.9	34.3	0.85
2.00	67.5	30.0	0.86
2.50	74.0	24.0	0.88
3.00	78.3	20.0	0.89
3.50	81.4	17.1	0.90
4.00	83.8	15.0	0.91
4.50	85.6	13.3	0.91
5.00	87.0	12.0	0.92

% in mass of TL = percentage in mass of total lipids per 100g of food; %TAG and %PL= percentage in mass of triacylglycerols and phospholipids, respectively; F (decimal)= decimal factor. Source: Kinsella [9] (apud EXLER, 1975, p. 154).

Table 1. Conversion factors based on the total lipid content.

are not naturally found in the samples cited above, possess good stability, and do not contain unsaturation in their carbonic chains[9].

4.1. Theoretical correction factor (F_{CT})

The determination of F_{CT} is based on two facts: (1) that FID proportionally responds to the relative mass percentage of a FAME carbonic chain and (2) this detector does not respond, during combustion, in a proper way to carbon of carboxylate group (COO^-). Thus, randomly assigning a $F_{CT}=1$ for a fame, for example, methyl stearate (18:0Me), the correction factors of other methyl esters can be calculated, according to Table 2. Thus, FID`s F_{CT} is a constant and it can not be modified due to instrumental errors [3].

4.2. Theoretical correction factor (F_{CT}) determination and calculus of active carbon (C*) percentage

In order to show the application of active carbon (C*) percentage determination to FID`s $F_{CT}$, methyl stearate ($C_{19}H_{38}O_2$, molar mass=298.5080g) was used as internal standard to calculate methyl decanoate`s factor ($C_{11}H_{22}O_2$, molar mass=186.2936g). It is important to remind that the

	Methyl esters of reference as internal standard						
*Methyl ester	18:0 Methyl stearate	12:0 Methyl dodecanoate	17:0 Methyl heptadodecanoate	18:1 Methyl oleate	19:0 Methyl nonadodecanoate	21:0 Methyl heneicosanoate	23:0 Methyl tricosanoate
4:0	1.5396	1.4294	1.5257	1.5501	1.5522	1.5742	1.5930
5:0	1.4009	1.3006	1.3883	1.4105	1.4123	1.4324	1.4495
6:0	1.3084	1.2147	1.2966	1.3174	1.3191	1.3378	1.3538
7:0	1.2423	1.1534	1.2311	1.2508	1.2524	1.2702	1.2854
8:0	1.1927	1.1073	1.1820	1.2009	1.2024	1.2195	1.2340
9:0	1.1542	1.0716	1.1438	1.1621	1.1636	1.1802	1.1942
10:0	1.1233	1.0429	1.1132	1.1310	1.1325	1.1486	1.1622
11:0	1.0981	1.0195	1.0882	1.1056	1.1071	1.1228	1.1361
12:0	1.0771	**1.0000**	1.0674	1.0845	1.0859	1.1013	1.1144
12:1	1.0670	0.9906	1.0574	1.0743	1.0757	1.0910	1.1040
13:0	1.0593	0.9835	1.0497	1.0666	1.0680	1.0831	1.0960
14:0	1.0441	0.9694	1.0347	1.0512	1.0526	1.0676	1.0803
14:1	1.0354	0.9613	1.0261	1.0425	1.0439	1.0587	1.0713
15:0	1.0308	0.9570	1.0215	1.0379	1.0392	1.0540	1.0665
15:1	1.0227	0.9495	1.0135	1.0297	1.0311	1.0457	1.0581
16:0	1.0193	0.9463	1.0101	1.0263	1.0276	1.0422	1.0546
16:1	1.0117	0.9393	1.0026	1.0186	1.0200	1.0345	1.0468
16:2	1.0041	0.9322	0.9951	1.0110	1.0123	1.0267	1.0389
16:3	0.9965	0.9252	0.9875	1.0033	1.0046	1.0189	1.0310
16:4	0.9989	0.9181	0.9799	0.9957	0.9970	1.0111	1.0232
17:0	1.0091	0.9369	**1.0000**	1.0160	1.0173	1.0318	1.0440
17:1	1.0019	0.9302	0.9929	1.0088	1.0101	1.0244	1.0366
18:0	**1.0000**	0.9284	0.9910	1.0068	1.0082	1.0225	1.0347
18:1	0.9932	0.9221	0.9842	**1.0000**	1.0013	1.0155	1.0276
18:2	0.9865	0.9195	0.9776	0.9933	0.9946	1.0087	1.0207
18:3	0.9797	0.9096	0.9709	0.9864	0.9877	1.0017	1.0137
18:4	0.9730	0.9034	0.9642	0.9797	0.9809	0.9949	1.0067
19:0	0.9919	0.9209	0.9830	0.9987	**1.0000**	1.0142	1.0263
20:0	0.9846	0.9141	0.9757	0.9913	0.9926	1.0067	1.0187
20:1	0.9785	0.9085	0.9697	0.9852	0.9865	1.0005	1.0124
20:2	0.9724	0.9028	0.9636	0.9791	0.9803	0.9943	1.0061
20:3	0.9663	0.8971	0.9576	0.9729	0.9742	0.9880	0.9998
20:4	0.9603	0.8916	0.9516	0.9669	0.9681	0.9819	0.9936
20:5	0.9542	0.8859	0.9456	0.9607	0.9620	0.9757	0.9878
21:0	0.9780	0.9080	0.9692	0.9847	0.9860	**1.0000**	1.0119
22:0	0.9720	0.9024	0.9632	0.9787	0.9799	0.9939	1.0057
22:1	0.9664	0.8972	0.9577	0.9730	0.9743	0.9881	0.9999
22:2	0.9609	0.8921	0.9522	0.9675	0.9687	0.9825	0.9942
22:3	0.9554	0.8870	0.9468	0.9619	0.9632	0.9769	0.9885
22:4	0.9499	0.8819	0.9413	0.9564	0.9577	0.9713	0.9828
22:5	0.9443	0.8767	0.9358	0.9508	0.9520	0.9655	0.9770
22:6	0.9388	0.8716	0.9303	0.9452	0.9465	0.9599	0.9713
23:0	0.9665	0.8973	0.9578	0.9731	0.9744	0.9882	**1.0000**
24:0	0.9615	0.8927	0.9528	0.9681	0.9694	0.9831	0.9948
24:1	0.9564	0.8879	0.9478	0.9629	0.9642	0.9779	0.9896

The symbology represents the principal chain of the FAME in question. The following atomic masses were used: C = 12.0110; H = 1.0079; O = 15.9994. FAMEs positional and geometrical isomers, as well as the ramified methyl esters, possess the same F_{CT} since they show the same C number.

Table 2. Theoretical correction factor (F_{CT}) for FAMEs

COO⁻ group (molar mass=44.0098g) from FAMEs shows negligible response in FID and, only considering the active carbons (C*) in FAMEs molecules. The following calculations may be done:

Methyl stearate = 18 C* × 12.0110 = 216.1980;

Mehtyl decanoate = 10 C* × 12.0110 = 120.1100.

Thus, the relative percentage for every FAME will be:

Methyl stearate 298.5080 (100%) and 216.1980 (% of C*) = 72.4262% of C*;

Mehtyl decanoate 186.2936 (100%) and 120.1100 (% of C*) = 64.4735% of C*.

Dividing methyl stearate`s C* percetage by methyl decaniate`s C* percetage:

F_{CT} of methyl decanoate = 72.4262% / 64.4735% = 1.1233.

Thus, the value of 1.1233 must be used as FCT in the quantitative determination of methyl decanoate (10:0Me) to correct FID`s differential response with methyl stearate as IS. Values for other FAMEs are expressed in Table 2.

It is possible to obtain, in a simplified manner, new values of FAEs F_{CT} through the use of another internal standard. Just use the obtained values for methyl stearate as IS (first column-Table 2) and values of the new IS in question. For example, to determine the new F_{CT} value of alpha-linolenic acid methyl ester (18:3Me) using methyl tricosanoate (23:0Me) as IS, just divide 18:3Me F_{CT} by 23:0Me F_{CT}, according to F_{CT} values from Table 2, the calculation is shown in equation 1.

$$F_{CT} \text{ of } 18:3 = F_{CT} 18:3 / F_{CT} 23:0 = 0.9797 / 0.9665 = 1.0137 \tag{1}$$

Table 2 shows F_{CT} for several FAMEs with different internal standards. Every entry from Table 2 is in accordance with the values published in the literature.

4.3. Experimental correction factor (F_{CE}) determination

Visentainer (2012) determined FID's experimental correction factors (F_{CE}) using a mixture of several FAME's with known amounts of methyl tricosanoate as IS, and from masses and percentual areas of FAME's and IS, the F_{CE} for every FAME was determined. Table 3 shows the average values with the respective standard deviations. The following equation is used for F_{CE} determination:

$$F_{CE} = A_P \times M_x / M_P \times A_x \tag{2}$$

where: A_P = standard area; M_x = FAME mass; M_P = standard mass; A_x = FAME mass.

	Response factor		Coefficient ©
FAMEs	Experimental (F_{CE})[a]	Theoretical (F_{CT})[b]	C = F_{CE} / F_{CT}
12:0	1.0535 ± 0.0095	1.1144	0.9454
14:0	1.0653 ± 0.0093	1.0803	0.9861
16:0	1.0491 ± 0.0092	1.0546	0.9948
18:0	1.0282 ± 0.0094	1.0347	0.9937
18:1n-9	1.0329 ± 0.0098	1.0276	1.0052
18:2n-6	1.0524 ± 0.0189	1.0207	1.0311
18:3n-3	1.0505 ± 0.0168	1.0137	1.0363
20:0	1.0274 ± 0.0083	1.0187	1.0085
20:4n-6	1.0484 ± 0.0198	0.9936	1.0552
20:5n-3	1.0443 ± 0.0239	0.9878	1.0572
22:0	0.9905 ± 0.0092	1.0057	0.9849
22:6n-3	1.0442 ± 0.0278	0.9713	1.0751
23:0	1.0000 ± 0.0000	1.0000	1.0000
24:0Me	0.9874 ± 0.0104	0.9948	0.9926

[a]Average values ± estimation of standard deviation (n = 6), [b]Visentainer *et al*., 2007. FAMEs = fatty acid methyl esters.

Table 3. FCT and FCE in relation to the internal standard methyl tricosanoate (23:0Me)

In a general manner, the coefficients © between F_{CE} and F_{CT} of saturated FAMEs (Table 3) are closer to 1.000 than that from polyunsaturated FAMEs. Thus, it is recommended the use of a saturated FAME as IS. Coefficients with values equal to 1.000 are preferred, because they indicate that the equipment is working with optimized conditions. A very high coefficient for a saturated fatty acid means that the GCph is not optimized, or there are some inconsistencies with the employed method. It is also important to remind that, every time as possible, fresh standards with proper preparation and storage procedures should be used.

4.4. Determination of fatty acid concentration

For determination of FAME mass in milligrams, equation 2 should be used. However, in order to increase the degree of accuracy in fatty acid/FAME quantification, it is recommended to use F_{CT}. The instrumental and chemical parameters must be optimized to minimize possible errors from these sources. It has been reported that, due to oxidative instability of polyunsaturated fatty acids (PUFAs), it is virtually impossible to obtain and keep standards of this kind with high degrees of purity, and they recommend the use of F_{CT} as a good approach for PUFAs analysis. Transforming equation 2 in function of a mass from a fatty acid X, equation 3 is obtained:

$$M_x = M_P \times A_x \times F_{CT} / A_P \tag{3}$$

where: M_x = FAME mass; M_P = standard mass; A_x = FAME area; F_{CT} = theoretical correction factor; A_P = standard area.

4.5. Final equation and FAME conversion factor for foods

In food analysis, results must be expressed in mass of fatty acids, unlike biodiesel, in which results must be expressed in FAME. Thus, sample mass (M_A) and the conversion factor of methyl ester to fatty acid (F_{CEA}) must be added to equation 3, and this new equation (shown below) is called "final equation for determination of fatty acids in mg/g of oil or fat":

$$M_x = M_P \times A_x \times F_{CT} / A_P \times M_A \times F_{CEA} \tag{4}$$

where: M_X = mass of fatty acid in mg/g of oil or fat; M_P = internal standard mass in mg; A_X = FAME area; F_{CT} = theoretical correction factor; A_P = internal standard area; M_A = sample mass (oil or fat) in g; F_{CEA} = conversion factor of methyl ester to fatty acid.

The F_{CEA} is determined through division of a FAME molecular mass (MM) by the MM of its corresponding fatty acid, according to equation 5:

$$F_{CEA} = MM \text{ of FAME} / MM \text{ of the corresponding fatty acid} \tag{5}$$

Fatty acids	MM of FA	MM of FAME	F_{CEA}
Tetradecanoic- 14:0 ($C_{14}H_{28}O_2$)	228	242	1.061
Hexadecanoic- 16:0 ($C_{16}H_{32}O_2$)	256	270	1.055
Octadecanoic- 18:0 ($C_{18}H_{36}O_2$)	284	298	1.049
9-Octadecenoic- 18:1n-9 ($C_{18}H_{34}O_2$)	282	296	1.050
9,12-Octadienoic- 18:2n-6 ($C_{18}H_{32}O_2$)	280	294	1.057
9,12,15-Octadecatrienoic- 18:3n-3 ($C_{18}H_{30}O_2$)	278	292	1.050
Eicosanoic- 20:0 ($C_{20}H_{40}O_2$)	312	326	1.045
5,8,11,14-Eicosatetraenoic- 20:4n-6 ($C_{20}H_{32}O_2$)	304	318	1.046
5,8,11,14,17-Eicosapentaenoic- 20:5n-3 ($C_{20}H_{30}O_2$)	302	216	1.044
Docosanoic- 22:0 ($C_{22}H_{44}O_2$)	340	354	1.041
4,7,10,13,16,19-Docosahexaenoic- 22:6n-3 ($C_{22}H_{32}O_2$)	328	342	1.042

The following atomic masses were taken in account: H = 1; C = 12 e O = 16. MM = molecular mass, FA = fatty acid, FAME = fatty acid methyl ester.

Table 4. Conversion factor of methyl ester to fatty acid F_{CEA}

The FAME of a determined fatty acid possesses methyl group (CH_3) in substitution of an atom of hydrogen. Thus, the methyl ester will show greater response in FID in relation to its corresponding FA, because the CH_3 contributes to increase C* number. Table 4 shows MM and FCEA values for some methyl esters and their respective FAs.

In case of biodiesel determination (FAMEs), F_{CEA} must be equal to 1, because in biodiesel case results are expressed in FAME and not in fatty acids.

5. Exemplifying the fatty acid quantification using gas chromatography

Experimental design, sample preparation, interpretation and processing of generated data are the intrinsic components of a successful GC analysis. In order to assist especially beginner scientists to get started smoothly, the fatty acid quantification of a sardine sample will be demonstrated in a practical manner, using all of the previously described methodologies: normalization, internal standard calibration, use of F_{CT}, F_{CE} and F_{CEA} [3].

5.1. Lipidic extraction (total lipids)

There are several methods of extraction and determination of total lipids in oils and fats from foods. It must be considered that some fatty acids derived from them are unstable, especially the unsaturated ones. The steps that come before FAs analysis must be attended with care, in order to protect the lipidic constituents. Such measures include: (1) the avoidance of metal utensils during sample preparation, (2) packing under vacuum and in a dark place to reduce interactions of light and oxygen with analytes, (3) storing samples in frozen state and (4) use of lipid extraction methods at room temperature.

Among methods that may be used for extracting lipids without system heating, the Bleigh and Dyer method (1959) [10] is one of the most recommended. In this method, three solvents are employed: chloroform ($CHCl_3$), methanol (H_3COH) and water (H_2O). The obtained extract is more reliable for evaluating FAs compositions.

5.2. Bligh and Dyer (1959) method – Simplified

Transfer 100g of homogenized sardine meat to a becker with capacity of 500mL. Add a precise amount of 100mL of chloroform and 200mL of methanol. The contribution of food's water content must be 80%, in order to keep the proportions of chloroform:methanol:water at (1:2:0.8). If it is needed, correct the water content. Stir vigorously during 5 minutes. Add 100mL of chloroform and 100mL of distilled water. Return to vigorous stirring for 5 minutes. The final proportion of solvents, as mentioned above, must be of 1chloroform:2methanol:0.8water. Filter in a Büchner funnel, transfer the filtrate to a separatory funnel and let the aqueous and organic phases to separate. Collect the lower layer (organic phase-chloroform), transfer it to a previously weighed flat-bottomed flask, remove the chloroform in excess through rotary evaporation at 33-35^{0}C and determine the remaining total lipids through gravimetry. After such

determination, transfer the total lipids from the flask to a amber-type recipient, and cover the new recipient into aluminium foil. Samples must be stored at -18 °C.

5.3. Preparation of internal standard solution

The first step of analysis is to prepare a solution of the ester that will be used as internal standard. Regardless of FA type, the steps described below always will be the same.

In this moment the analyst must know, through bibliographic research, which FAs will be present in this sample, because it must not contain the ester that will be used as IS. For example, animal fat possesses 17:0Me and 19:0Me FAs, thus these two compounds cannot be used for IS calibration. In this case, 23:0Me is used instead.

The internal standard must be of analytical grade (high purity degree) in order to avoid presence of impurities that might compromise future results.

The IS mass must be measured in an analytical balance and transferred to a volumetric flask (for instance, 50 mL for 50 mg IS). A bit of solvent should be added to the flask. Alkanes such as n-hexane, n-heptano and iso-octane are recommended to be used as solvents, although other alkanes may also be employed. In this case, iso-octane is chosen for use. The final solution should be stored in amber flask and under refrigeration. Other volumes might be prepared, according to the number of samples to be analyzed.

### 5.4. Transesterification of total lipids

After extraction of total lipids from the sample, they must be derivatized for further FAs analysis through GC. There are several methods for this purpose. For the example of this chapter, the most recommended methodology for fish samples is the one reported by Joseph and Ackman (1992) [4]. This method originated from a collaborative study which involved 21 international laboratories.

In a screw-capped glass tube, 1 mL of the previously prepared IS solution is added. Soon after, solvent is removed by a gentle flow of gaseous N_2. Then, approximately 25 mg of total lipids are weighed in the same tube, and to it 1.5 mL of a methanolic solution of NaOH 0.5 mol/L is added. The entire system is double-boiled to 100 ^{0}C for five minutes, and cooled to room temperature.

After cooling, 2.0 mL of solution which corresponds to 12% of BF_3 in methanol is added, and the system is once again double-boiled to 100 ^{0}C for thirty minutes and rapidly cooled with running water to room temperature. Immediately after this step, 1 mL of iso-octane is added to the tube, followed by vigorously shaking for 30 seconds and, finally, 5 mL of a saturated NaCl solution is added. The esterified sample is left to rest in a fridge, to allow a better separation of phases. The supernatant is removed and transferred to a 5 mL amber flask. An additional iso-octane amount is further added to the tube. After shaking, the new supernatant is removed and united with the previous fraction in the amber flask, and this final mixture is concentrated to a final volume of approximadetely 1 mL, with the help of gaseous N_2.

5.5. Chromatographic analysis

In the sequence, a GCph is needed for a proper chromatographic run. For the example of this book, 2µL of sample were injected and separated in a GCph from Thermo brand, model 3300, equipped with a flame ionization detector, automatic injector and a capillary column of fused silica CP-7420 SELECT-FAME (100 % bonded cyanopropyl, 100m length, 0.25mm internal diameter and 0.39 µm of stationary phase). Injector temperature was 230°C. Initially, the column temperature was maintained at 165°C for 18 minutes. Then, it was raised to 235°C, at a rate of 4°C/min. The flow rates for the carrier (H_2), auxiliary (N_2) and detector flame (H_2 and synthetic air) gases were 1.2 mL/min, 30 mL/min, 30 mL/min and 300 mL/min, respectively. Sample split ratio was 1/80. Figure 1 is an illustrative chromatogram for the sardine oil sample that was esterified. It can be observed in the chromatogram a peak regarding solvent, which must be removed. After this removal, only the peak areas from esters will compose the chromatogram.
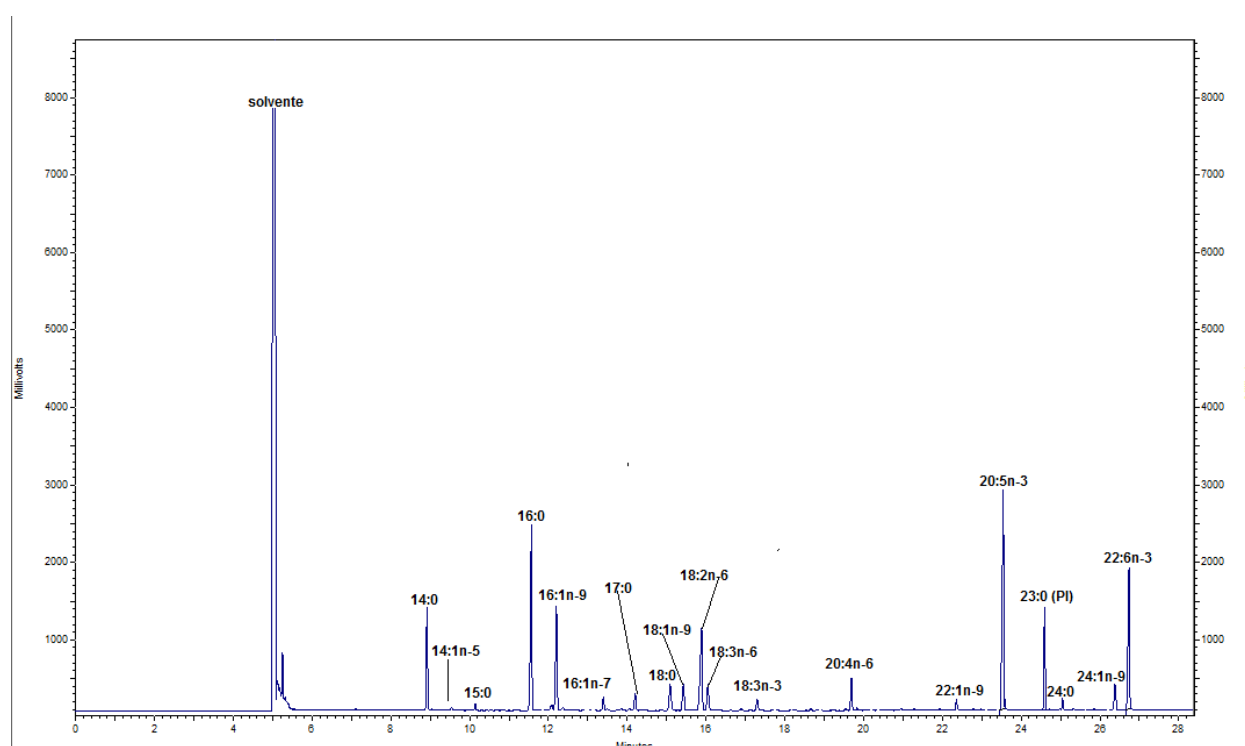
Figure 1. Ilustrative chromatogram of the obtained esters from sardine oil sample with a known amount of internal standard (23:0Me).

Table 5 shows the obtained areas for the different esters (these areas are provided by the software which is used for peak integration).

The values in percentage area (%) were calculated through the normalization method, which will be detailed below:

Assuming to calculate the percentage of 22:6n-3 (DHA) in sample, the following formula can be used:

Methyl esters (ME)	Area	Area in percentage (%)
14:0	21169396	7.32
14:1n-5	1061405	0.37
15:0	3288731	1.14
16:0	80167690	27.71
16:1n-9	3076733	1.06
16:1n-7	13561700	4.69
17:0	3607101	1.25
18:0	16666471	5.76
18:1n-9	23694148	8.19
18:2n-6	6308497	2.18
18:3n-6	4883567	1.69
18:3n-3	1984215	0.69
20:4n-6	4888882	1.69
22:1n-9	1774930	0.61
20:5n-3	28582886	9.88
23:0 (IS)	10889779	-
24:0	2684844	0.93
24:1n-9	3789879	1.31
22:6n-3	68164925	23.56
Total	289356000	100.00

Table 5. Methyl esters which were identified in the chromatogram as well as their respective areas and relative area percentages.

X [%] = Area of 22:6n-3 (DHA) × 100 / Total area (with exception of IS

= 23.56 [%] of DHA.

The procedure is repeated for every peak, and the final sum of percentages is equal to 100%.

The problem of this method is that it considers that every compound present in sample was eluted through the column and detected. This is not always true because some compounds might stay retained on the column. Besides, as mentioned before, the detector must have the same response for every compound. However, this does not happen with FID, because it shows differential responses between the different carbonic chains of methyl esters. Thus, it is not correct to affirm that, in this sardine oil sample, the DHA concentration corresponds to 23.56%.

In order to correct this problem, it is recommended to use conversion factors, as mentioned before. Thus, now it will be exemplified for the same fatty acid (DHA), which would be its percentage after using the correction factor for fish.

For a fish meat sample with 3% of total lipids, the conversion factor is 0.89 (Table 1). Thus, the % of DHA in sample is obtained:

% DHA = (% area of DHA) × (conversion factor).

= (23.56) x (0.89) = 20.97%.

This calculation may be repeated for the remaining FAs, through use of the equation above, but with their respective values of area percentage and correction factors.

This method is very practical and easily done to express results in amount of FAs *per* amount of food. However, it has some critics:

1. In the application, FID's differential response for the different FAs are not taken tinto account;

2. The different total lipid fractions are dependent of many variables, and the tabulated values might not correspond to the real values. A way of minimizing this problem is the empirical determination of the desired fractions;

3. In many foods, total lipids were not fractionated. In this case, the percentage of each fraction must be determined, as mentioned in item II. However, a proper method for fractionating should be employed;

4. Calculations involve mass percentage, through the use of normalization method. Thus, all the sample constituents must be present.

Although the FA quantification with use of internal standard calibration is labor-intensive, especially in relation to its implementation in a laboratory, the obtained results compensate for the spent time, because they are reliable and of easy interpretation, allowing comparison of composition with works which use modern quantification methods.

For this same sardine oil sample, the percentage of DHA will be calculated with this method:

Knowing that DHA area from the chromatogram (Figure 1) is 68164925, in this analysis a 23:0Me 1 mg/mL solution was used as PI, with application of 1 mL (mass 1mg and area of 10889779) from this, the 0.024g of total lipids were used for esterification, the F_{CT} for DHA (Table 3) is 0.97 and the F_{CE} for DHA is 1.04. Thus, through the use of the equation cited below:

$$M_{DHA} = A_x \times M_p \times F_{CT} / A_p \times F_{CE} \times M_A \qquad (6)$$

Where:

M_x = Mass of DHA in mg/g of total lipids; M_P = Mass of internal standard in milligrams; M_A = Mass of total lipids in grams; A_X = Area of DHA; A_P = Area of internal standard; F_{CT} = theoretical correction factor; F_{CEA} = conversion factor of methyl ester to fatty acid.

Thus :

M_{DHA} = (68164925).(1).(0.97)/(10889779).(1.04).(0.027)

Finally:

M_{DHA} = 215.61 mg/g of total lipids.

Performing the same calculation for the remaining FAs, it can be obtained the composition of the esterified sardine oil.

Fatty acids	GC-FID			GC-MS
	N (%)	FC (%)	PI	PI
14:0	7.32	6.51	73.29 ± 0.034	73.33 ± 0.031
14:1n-5	0.37	0.33	3.64 ± 0.014	3.62 ± 0.013
15:0	1.14	1.01	11.31 ± 0.017	11.02 ± 0.016
16:0	27.71	24.66	273.95 ± 0.114	271.14 ± 0.103
16:1n-9	1.06	0.95	10.41 ± 0.010	10.78 ± 0.009
16:1n-7	4.69	4.17	45.91 ± 0.016	45.20 ± 0.014
17:0	1.25	1.11	12.13 ± 0.015	9.11 ± 0.014
18:0	5.76	5.13	55.66 ± 0.018	54.91 ± 0.016
18:1n-9	8.19	7.29	79.05 ± 0.032	82.18 ± 0.029
18:2n-6	2.18	1.94	20.84 ± 0.029	17.26 ± 0.026
18:3n-6	1.69	1.50	15.98 ± 0.256	15.28 ± 0.230
18:3n-3	0.69	0.61	6.49 ± 1.287	6.21 ± 1.158
20:4n-6	1.69	1.50	15.81 ± 1.992	14.83 ± 1.792
22:1n-9	0.61	0.55	5.71 ± 0.422	5.91 ± 0.380
20:5n-3	9.88	8.79	91.43 ± 0.288	87.50 ± 0.259
23:0 (PI)	-	-	-	-
24:0	0.93	0.83	8.71±0.139	8.08 ± 0.126
24:1n-9	1.31	1.17	12.17 ± 0.144	11.11 ± 0.130
22:6n-3	23.56	20.97	215.61 ± 0.302	214.57 ± 0.272
Total	100.00	89.02	958.10	942.04

N = values determined through the normalization method; FC = values determined through the correction factor method ; PI = values determined through internal standard calibration method with FID correction factors.

Table 6. Obtained values for sardine oil quantification in GC-FID and GC-MS.

For comparison effects, 1μL of the same esterified sardine oil sample was injected in GC-MS. In the example of this book, samples were separated in a GC-MS from Thermo brand, model Focus DSQ II, equipped with automatic injector, and a capillary column DB-5 (5% phenyl and 95% methylpolisiloxane, 30m length, 0.25mm internal diameter and 0.25 μm of stationary phase). Initially, the column temperature was maintained at 165°C for 4 minutes. It was then raised to 200°C, at a rate of 6°C/min, and kept at this temperature for 5 minutes. After this period, it was once again raised to 235°C at a rate of 7°C/min and maintained for 5 minutes. Finally, it was raised for the last time to 290°C at a rate of 20°C/min and kept at this temperature

for 8 minutes. The flow rate for carrier gas (He) was 1.0 mL/min. Injector temperature was 240°C. Sample split ratio was 1/20. Data from MS detection was obtained in full scan mode, with range of masses 50-650 m/z and 0.8170 scans per second. Temperature of ionization source was 250°C. Since the MS detector does not show differential response between the different carbonic chain of FAMEs, a very reliable quantification is obtained, and the results of this analysis can be compared with the ones from GC-FID as given in Table 6.

6. Conclusion

The illustrations and results shown here indicate that it is possible to increase accuracy in expression of fatty acids (in foods) or FAEs (in biodiesel), especially when compared with the area normalization method, which is limited and frequently used in an erroneous manner. Besides, results of analyses using internal standard calibration and correction factors, in a sardine oil sample, showed that the expression of concentration results, in mass of fatty acid *per* oil mass, must be used whenever possible, because in this quantification only relevant peaks from a chromatogram are taken into consideration. Thus, the obtained results are reliable and easily interpreted, allowing quantitative comparisons of fatty acids/FAEs in foods/biodiesel. The support of such results were obtained upon comparison of the same sample using GC-FID and GC-MS, and the results were indistinguishable, demonstrating that FID may be used for fatty acid analysis, as long as the proper correction factors are applied. Thus, using FID for such quantifications is highly recommended and, comparing to MS, it has additional advantages because it is cheaper and easier to operate, while keeping good sensitivities.

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