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Characterization of Triacylglycerol Composition of Fish Oils by Using Chromatographic Techniques

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Abstract: Triacylglycerols (TAG) of two different refined fish oils from sardine and a mixture of tuna and sardine oil were separated by reverse phase high performance liquid chromatography (RP-HPLC) with a binary solvent gradient of acetone/acetonitrile. Different fractions were observed in the chromatogram and TAG species were tentatively identified by subsequent analysis of the fatty acid (FA) profile in each fraction by capillary Gas Chromatography (GC). Peak identities were assigned on the basis of a multiple linear regression analysis by using factors such as carbon number, number of double bonds, number of monounsaturated fatty acids (MUFA) and number of polyunsaturated fatty acids (PUFA) in the molecule as predictors for TAG retention time. A successful correlation was obtained between retention times and the equivalent carbon number (ECN) of triacylglycerols. Regiospecific analysis of fatty acids in the TAG has been conducted by ethanolysis of the fish oil by using an immobilized lipase. The subsequent separation of 2-monoacylglycerol (2-MAG) by TLC (thin layer chromatography) analysis showed that ethanolysis system is effective for analysis of FA composition at the 2-position in oils containing PUFA. Principal components analysis (PCA) has been also applied to establish correlations between the different fatty acids in the TAG.

Key words: fish oil, triacylglycerols, ECN, fatty acids

1 INTRODUCTION

The nutritional benefits attributed to fish oils, rich in omega-3 PUFA, have been the basis for the study of the structural composition of triacylglycerols in fish oil[1, 2]. Typical fish oil may contain more than 150 different TAG molecule species as a complex mixture. The great variety of fatty acids in fish oil, from myristic acid (14:0) to docosahexaenoic (22:6, n-3) acid, causes enormous difficulties in the approach to the TAG analysis of fish oils.

HPLC using refractive index and evaporative light-scattering detectors (ELSD), has been shown to be effective in the analysis of triacylglycerols. Other methods, such as high-temperature gas chromatography (HT-GC) with flame-ionization detector (FID) or mass spectrometry (MS), have been also reported to be effective in the separation, identification and quantitation of triacylglycerols[3]. However, analysis of highly unsaturated TAG, such as fish oils, by HT-GC cannot be recommended due to thermal degradation and polymerization of highly unsaturated molecules at high elution temperatures. The analysis of edible oils by their triacylglycerol content is also of great importance as quality control and possible origin determination[4].

In this work, relative responses of ELSD in RP-HPLC have been firstly determined by using model mixtures of standard triacylglycerols. Afterwards, analysis of TAG from two different fish oils (sardine oil and a mixture of tune and sardine oil) have been carried out by RP-HPLC technique by using gradients of acetone/acetonitrile mixtures. Fractionation of TAG was performed according to their elution time. To identify the TAG present in the fish oil samples the concept of equivalent carbon number (ECN) has been used. An approach similar to that proposed by Perona *et al*. and López-Hernández *et al*.[1, 2] has been followed. This procedure considers the retention time of a certain TAG as a function of its total carbon number (CN), its number of double bonds (DB), and the unsaturated FA (mono and polyunsaturated) present in the TAG molecule of interest.

Ethanolysis of fish oils with immobilized *Candida antarctica* lipase has been applied to determine the FA composition at the 2-position in TAG. 2-MAG were detected by TLC and separated by two different chromatographic techniques, normal phase HPLC (NP-HPLC) and TLC. 2-MAG were analyzed by GC.

Furthermore, principal components analysis, PCA, has

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been performed to confirm the most probable fatty acid association in the different fractions obtained by RP-HPLC.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

Refined sardine oil was kindly provided by Industrias Afines S.L. and the mixture of tuna oil and sardine oil was kindly supplied by AFAMSA S.A..

The triacylglycerols standards were tricaprin (CCC), trilaurin (LLL), trimyristin (MMM), tripalmitin (PPP), tripalmitolein (PoPoPo), tristearin (SSS), triolein (OOO), trilinolein (LoLoLo), trilinolenin (LnLnLn), triarachidin (AAA) and tridocosahexaenoin (DhDhDh) of purity greater than 98%. They were obtained from Sigma Aldrich. Standards of fatty acid methyl esters were also purchased from Sigma-Aldrich.

Ethanolysis of fish oils was carried out with Lipozyme 435, from *Candida antarctica* lipase immobilized on a macrosporous resine, which was kindly donated by Novozymes (Bagsvaerd, Denmark).

Chemicals used for all chromatographic analysis were HPLC grade from VWR.

2.2 Methods

2.2.1 RP-HPLC analysis

The HPLC separations were done at 30℃ on a Lichrospher 100 RP-18 (5 μm) column (25 cm × 4 mm i.d.) in a HPLC with ELSD detector (Agilent Technologies 1200 Series Model, Santa Clara CA, United States). The mobile phase was a mixture of acetone-acetonitrile at a flow-rate of 1 mL/min. The solvent gradient used is shown in Table 1. The evaporator temperature was 35℃, air pressure 3.5 bars and gain 9. Between 5 and 15 μL of a standard solution (1-10 mg/mL of each standard TAG) in hexane:acetone (1:1) were injected to calibrate the method. These measurements were performed by triplicate. Fish oil samples (100 mg/mL of hexane:acetone) injected by RP-HPLC were fractionated according to the times obtained from the TAG profile. Separations were repeated at least six times to collect enough sample for the fatty acid methyl esters (FAMEs) analysis. The fractions were stored in special flasks to evaporate the solvent under vacuum using a rotary evaporator (Heibolph VV2000, Schwabach, Germany) at 40℃. Then, the samples were transferred to screw-capped tubes to carry out the derivatization.

Table 1 Solvent gradient used to separate TAG in fish oils (mobile phase content expressed as vol %).

Time, min	% Acetone	% Acetonitrile
0	10	90
50	60	40
60	100	0
65	100	0
70	10	90

2.2.2 Ethanolysis of fish oils

Ethanolysis of TAG with immobilized *Candida antarctica* lipase has been reported to produce an accumulation of 2-MAG in the early stage of the reaction. Although this lipase is non regiospecific, it behaves as 1,3 specific with a great excess of ethanol[5]. Ethanolysis of the two oils studied in this work has been conducted as described by Shimada *et al.*[6]: A mixture of oil/ethanol (1:3, w/w) was shaken at 30℃ during 4 hours with 4% immobilized *Candida antarctica* lipase by weight of oil + ethanol. Fish oil and ethanol are not miscible[7] at the weight ratio proposed by Shimada *et al.*[6]. However, after 4 h of reaction time, the reaction mixture was a homogeneous phase due to the polarity of MAG and the ethyl esters formed during the reaction.

Quantification of the reaction products (monoacylglycerols, diacylglycerols, triacylglycerols and ethyl esters) after 4 h of reaction time has been carried out by normal phase HPLC (NP-HPLC). Separations were carried out at room temperature in a Lichrospher Diol column (5 mm, 4 mm × 250 mm) and detection was performed in an ELSD (Agilent Technologies 1200 Series Model, Santa Clara CA, United States) at 35℃ and 0.35 MPa. NP-HPLC method and calibration procedure have been previously described in detail[8]. Fraction corresponding to 2-MAG was collected in special flasks to evaporate the solvent under vacuum using a rotary evaporator (Heibolph VV2000, Schwabach, Germany) at 40℃. Then, the samples were transferred to screw-capped tubes to carry out the derivatization for conversion to methyl esters and subsequent fatty acid analysis by GC. 2-MAG fraction was also separated by TLC on silica-gel plates (Silica gel 60 F254, Merck) activated by heating at 105℃ for 30 min. 200 μL of the reaction mixture were directly spotted on the TLC plate. A mixture of standards was also spotted. The plates were then developed in chloroform/acetone/methanol (95:4.5:0.5, v/v/v). Spots of each lipid were visualized by UV lamp. The fraction corresponding to 2-MAG was scraped off from the plates and methylated according to the method of Wewer *et al.*[9].

To check that the MAG fraction does not contain 1(3)-MAG, isomers of the 2-MAG, separation of the different acylglycerols has been also performed by TLC on silica gel plates following a procedure similar to the one reported by Muñio *et al.*[5]. In this case, the plates were impregnated by immersion in a hydroethanolic solution (50%, v/v) of boric acid (1.2% in weight) activated again by heating at 100℃ for 15 min.

2.2.3 FAMEs analysis

The two fish oils considered in this work together with the different fractions obtained by RP-HPLC, and the 2-MAG fraction obtained by NP-HPLC and from the bands scraped off from the TLC, were analyzed by gas chromatography to determine the fatty acid profile by the AOAC method[10]. The fatty acid methyl esters were firstly prepared and then analyzed in a Hewlett Packard gas chromatograph (6890N Network GC System, Santa Clara CA, United States) equipped with an auto-sampler (7683B series) and a flame ionization detector (FID). A fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) was used. Most of the fatty acid methyl esters were identified by comparison of their retention times with those of chromatographic standards.

2.3 Calculation of TAG composition

In order to identify the individual triacylglycerols, the concept of equivalent carbon number (ECN) has been used. The ECN is the carbon number of a hypothetical saturated triacylglycerol, which is eluted at the same retention time as the unsaturated triacylglycerol under study[11]. In a reversed-phase HPLC system, the elution order of the acylglycerols depends on their chain length, degree of unsaturation and the presence of certain functional groups[2]. Therefore, the ECN value for each TAG is based on these parameters. Different empirical equations have been proposed to predict retention times of TAG species. In literature[1, 12, 13], the following equation has been used to correlate the dependent variable, log k', related with the retention time of each TAG:

$$\log k' = b_0 + b_1 \cdot (CN) + b_2 \cdot (DB) + b_3 \cdot (NUFA) \quad (1)$$

where CN is the total carbon number of the three fatty acids, DB is the total number of double bonds and NUFA is the number of unsaturated fatty acids of the TAG molecule. Based on equation (1) the ECN value was evaluated according to Equation 2[1, 12, 13]:

$$ECN = CN + a_1 \cdot DB + a_2 \cdot NUFA \quad (2)$$

Coefficients a_1 and a_2 were obtained from the quotient between the coefficients b_2 and b_1, and b_3 and b_1, respectively.

In this work, instead of equation (1), the equation proposed by López-Hernández *et al.*[2] has been used to correlate the dependent variable, retention time (RT), with the following independent variables CN, DB, MUFA and PUFA:

$$RT = b_0 + b_1 \cdot (CN) + b_2 \cdot (DB) + b_3 \cdot (MUFA) + b_4 \cdot (PUFA) \quad (3)$$

In equation (3) the factor NUFA of equation (1) has been replaced by two different factors: MUFA and PUFA; therefore, the effect of both types of fatty acids, present in high concentrations in fish oils, on elution time is considered. ECN values were evaluated as:

$$ECN = CN + a_1 \cdot DB + a_2 \cdot MUFA + a_3 \cdot PUFA \quad (4)$$

where a_1 is the quotient between the coefficients b_2 and b_1, a_2 between the coefficients b_3 and b_1 and a_3 between the coefficients b_4 and b_1. Retention times have been expressed as relative retention times (RRT) of the peaks with reference to tricaprin peak in the chromatogram. Parameters (b_0-b_4) of equation (3) have been obtained by multiple linear regression analyses of relative retention time for the different TAG standards by using the commercial software Statgraphics Centurion XVI.I. ECN values have been related with the RRT by a simple linear regression:

$$ECN = a + b \cdot RRT \quad (5)$$

Identification of TAG in the analysis of fish oil samples has been performed on the basis of their retention characteristics, and their ECN value was calculated through equation (5) to assign TAG to each chromatographic peak.

For each fraction recovered from RP-HPLC a number of fatty acids were obtained by GC. These fatty acids were combined three by three assuming that the three positions on the glycerol molecules were equivalent, since HPLC analysis cannot separate positional isomers. In this procedure only those fatty acids present with an area higher than 5% have been taken into account. This way, a theoretical ECN value can be calculated (eq. 4) and compared with the experimental value obtained from its characteristic retention time (eq. 5) and TAG species could be assigned to the different chromatographic peaks.

3 RESULTS and DISCUSSION

3.1 Fatty acid profile of fish oils

The results of the FA analyses of the original feedstocks by GC-FID are presented in Table 2. Both fish oils present high amounts of polyunsaturated fatty acids. The main differences between the total fatty acid compositions of both types of oils lies in the amount of eicosapentaenoic, docosahexaenoic and oleic acids. The most abundant FA in both oils was palmitic acid (around 23% mol), followed by eicosapentaenoic acid (18.3% mol) for sardine oil and by docosahexaenoic acid (21.4% mol) for the mixture of tuna and sardine oil. Perona *et al.*[1] analyzed the fatty acid profile for sardine oil and found palmitic acid as the most abundant (21.54%) followed by oleic acid (15.82%), eicosapentaenoic acid (14.83%) and docosahexaenoic acid (13.82%). On the other hand, Muñio *et al.*[5] analyzed the fatty acid profile for tuna oil and showed docosahexaenoic acid as the major fatty acid (22.1%) followed by palmitic acid (19.5%). The differences found in fatty acid and TAG composition of fish oils can be expected and are mainly due to species variations.

Table 2 Fatty acid composition of fish oils (% mol) and of *sn*-2[a] of TAG in oils studied in this work.

Fatty acid		Sardine oil		Tuna-sardine oil	
		TAG	*sn*-2	TAG	*sn*-2
Myristic (M)	14:0	12.4	41.8	4.7	41.4
Palmitic (P)	16:0	22.9	41.9	23.2	27.9
Palmitoleic (Po)	16:1n-7	12.5	38.1	6.9	32.3
Stearic (S)	18:0	3.6	6.7	6.1	8.4
Oleic (O)	18:1n-9	9.8	16.6	18.4	19.4
Vaccenic (V)	18:1n-7	3.7	10.1	3.1	16.2
Linoleic (Lo)	18:2n-6	2.5	32.7	2.4	33.8
Linolenic (Ln)	18:3n-3	1.0	29.0	0.7	36.5
Steriadonic (St)	18:4n-3	3.3	26.4	1.6	30.8
Eicosenoic (G)	20:1n-9	nd	nd	1.9	12.9
Eicosatrienoic (Et)	20:3n-3	1.3	19.6	1.8	35.0
Eicosapentaenoic (Ep)	20:5n-3	18.3	12.1	6.5	30.2
Docosapentaenoic (Dp)	22:5n-3	1.8	76.4	1.6	44.4
Docosahexaenoic (Dh)	22:6n-3	7.0	82.8	21.4	61.0

(a) % *sn*-2 = [mol % *sn*-2 fatty acid / mol % fatty acid in TAG·3]·100
nd: non detected

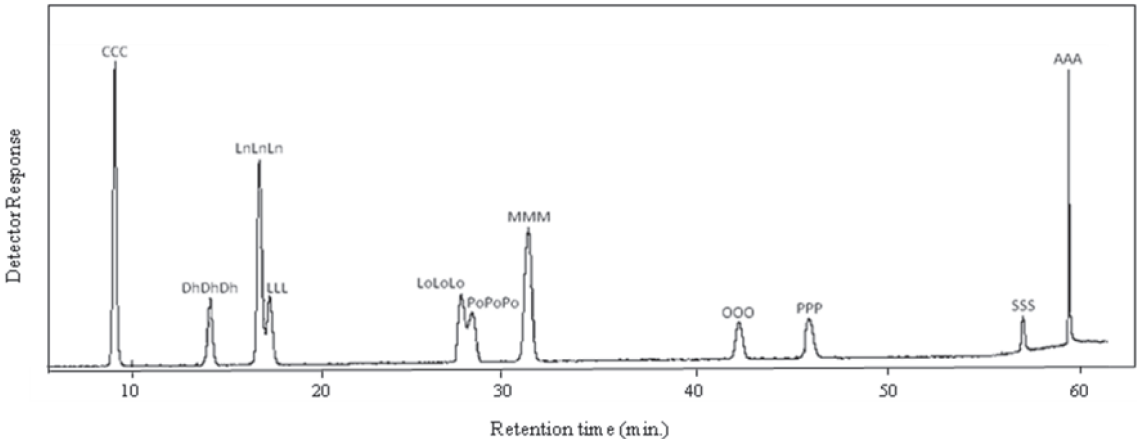

Fig. 1 RP-HPLC of TAG standards.

3.2 Reverse-Phase Liquid chromatographic analyses

3.2.1 Mixture of standards TAG

The mixture of TAG standards was clearly separated in the order CCC＜DhDhDh＜LnLnLn＜LLL＜LoLoLo＜PoPoPo＜MMM＜OOO＜PPP＜SSS＜AAA (Fig. 1).

Detector response: The same standard solutions used to determine retention times of the pure TAG were employed to obtain calibration curves for all the standards used in this work. Perona *et al.*[1] proposed the use of cubic or fourth regression curves. That means the determination of four or five coefficients in the regression analysis. In this work the approach followed by Ruiz-Sala *et al.*[14] has been preferred and linearity has been reached by plotting log area versus log amount injected. Parameters of the regression for all the standards used in this work can be found in Table 3. Figure 2 shows the calibration curve of DhDhDh. In general, a good behavior has been observed for the light-scattering detector (ELSD), taking into account its non-linear response[1, 15].

3.2.2 Fish oil samples

The chromatograms of sardine oil and a mixture of tuna and sardine oil are showed in Fig. 3 and Fig. 4 respectively. Forty four peaks and thirty six peaks have been detected respectively.

As it has been explained in section 2.2 different fractions were collected from RP-HPLC chromatogram to obtain the

Table 3 Parameters of linear regression curves obtained for calibration of standards in RP-HPLC.
log area = a·(log μg injected) + b.

TAG standard	a	b	r^2
CCC	1.4522	2.524	0.9980
LLL	1.9837	2.3262	0.9954
MMM	1.6518	2.4137	0.9910
PPP	2.8336	1.9691	0.9975
PoPoPo	1.6548	2.4287	0.9998
SSS	3.7912	1.957	0.9997
OOO	1.812	2.1294	0.9972
LoLoLo	2.0601	1.9225	0.9998
LnLnLn	1.7775	2.1997	0.9956
AAA	1.0191	0.6597	0.9916
DhDhDh	1.7192	2.3328	0.9919

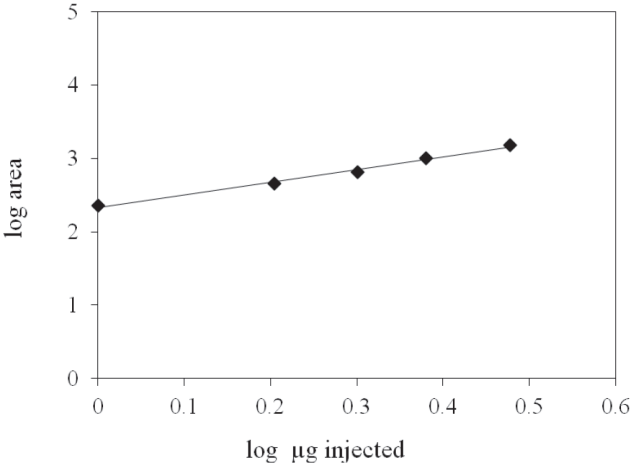

Fig. 2 Calibration curve for DhDhDh standard analyzed by RP-HPLC. The continuous line corresponds to the linear regression. Log area = 1.7192·(log μg injected) + 2.3328.

different FAME composition of each fraction by GC. Based on Fig. 3 and Fig. 4, a total of seven fractions were collected from HPLC. The fatty acid profiles of these fractions are presented in Table 4 for sardine oil and in Table 5 for the mixture of tuna and sardine oil. In both cases, it can be observed that the first fractions (1-3) contain most of the highly unsaturated FAME species as eicosapentaenoic, docosapentaenoic and docosahexaenoic acids. In the last fractions, the content of PUFA continuously decreases in each fraction. The highly saturated FAME species, especially palmitic and stearic acids, appear mainly in the last fractions (5-7). Concentration of linolenic and eicosatrienoic acids was lower than 2% in the FAME analysis of sardine oil; for this reason these fatty acids were nearly absent in the analysis of fractions. The same result is observed for steriadonic and linolenic acids in the mixture of tuna and sardine oil.

The fatty acid profiles of each fraction would help to predict the most probable TAG composition for each fish oil studied in this work, without regard of the regiospecificity of each fatty acid in the glycerol molecule.

3.3 Qualitative analysis of triacylglycerols composition of fish oils

3.3.1 Calculation of TAG composition

Multiple linear regression analysis has been performed to obtain the parameters of equation (3) using the relative retention time of TAG standards. When all the standards were included, the correlation was not very successful. To improve data correlation, TAG species of little relevance in fish oil, in this case trilaurin and triarachidin were excluded in the fitting procedure. The same behavior in the fitting procedure has been observed by López-Hernández[2] in the analysis of the TAG composition of reaction mixture of hydrogenation of fish oil with hydrogenated soybean oil. Nonlinear multiple regression analysis leads the following values of the model parameters:

$$RRT = -64.228 + 2.138 \cdot CN - 3.360 \cdot DB - 1.877 \cdot MUFA - 3.571 \cdot PUFA \quad (6)$$

As it has been described in section 2.3, using the parameters from equation (6), the following equation for the ECN value has been obtained:

$$ECN = CN - 1.572 \cdot DB - 0.878 \cdot MUFA - 1.670 \cdot PUFA \quad (7)$$

Table 6 shows the ECN values obtained with equation (7) for the different TAG standards used in this work. It can be

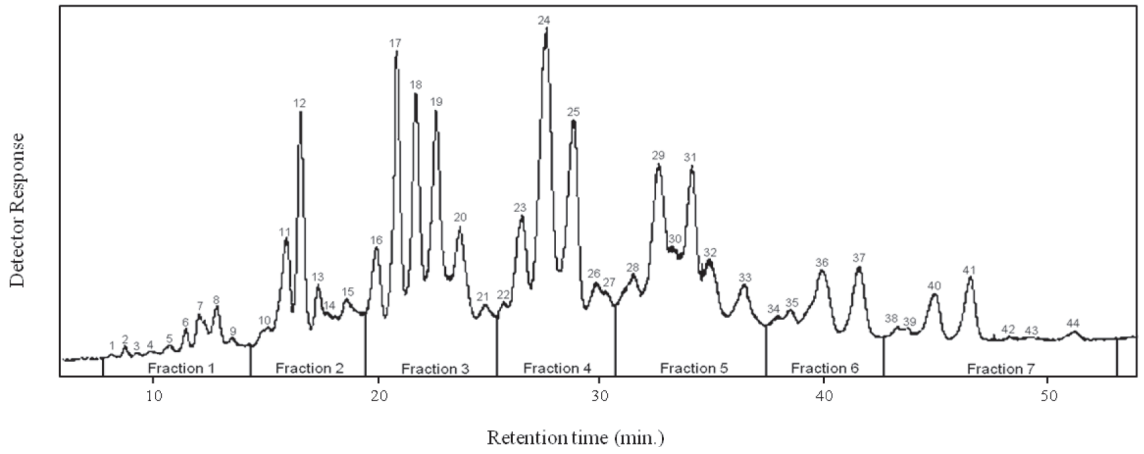

Fig. 3 TAG analysis of sardine oil using the gradient solvent RP-HPLC technique. Numbers correspond to the peaks of Table 8.

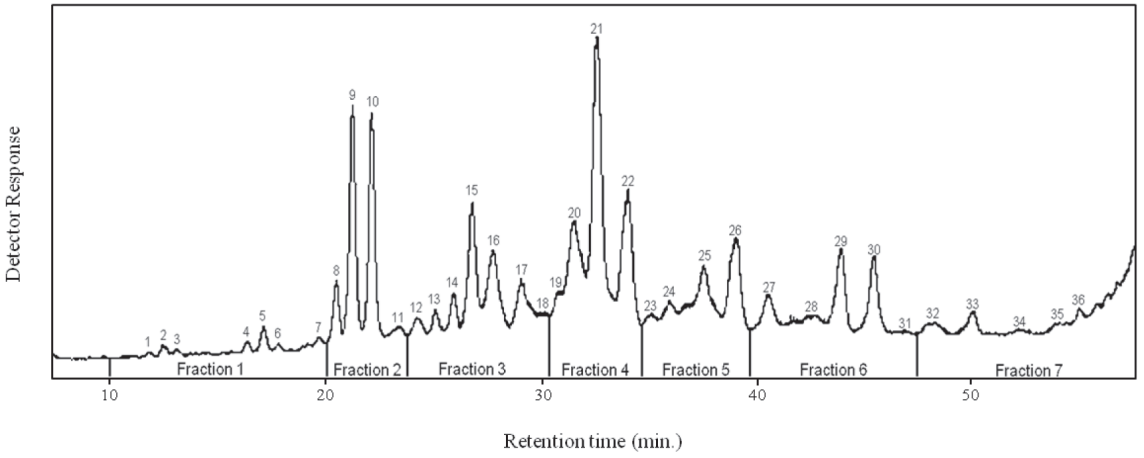

Fig. 4 TAG analysis of tuna-sardine oil using the gradient solvent RP-HPLC technique. Numbers correspond to the peaks of Table 9.

Table 4 Fatty acid composition (% mol) of sardine oil fractions.

Fatty acid		1	2	3	4	5	6	7
Myristic (M)	C14:0	14.09	27.72	13.11	18.51	11.85	12.58	10.56
Palmitic (P)	C16:0	18.27	14.94	17.73	26.28	34.76	32.11	28.52
Palmitoleic (Po)	C16:1n-7	12.95	25.31	12.93	18.62	11.37	12.56	8.29
Stearic (S)	C18:0	7.36	–	1.49	3.91	4.06	7.69	12.50
Oleic (O)	C18:1n-9	13.75	6.95	4.29	8.43	13.59	16.23	22.02
Vaccenic (V)	C18:1n-7	–	–	2.45	4.57	5.81	4.86	8.54
Linoleic (Lo)	C18:2n-6	10.32	4.12	1.85	3.84	3.49	3.26	9.56
Linolenic (Ln)	C18:3n-3	–	–	1.51	–	–	–	–
Steriadonic (St)	C18:4n-3	4.62	3.40	4.57	1.75	1.70	–	–
Eicosatrienoic (Et)	C20:3n-3	–	–	2.03	–	–	1.44	–
Eicosapentaenoic (Ep)	C20:5n-3	11.36	15.41	25.46	8.84	8.30	3.99	–
Docosapentaenoic (Dp)	C22:5n-3	2.91	–	2.83	1.67	1.29	1.73	–
Docosahexaenoic (Dh)	C22:6n-3	4.37	2.15	9.75	3.57	3.78	3.55	–
% area of each fraction (RP-HPLC)		3.6	8.1	19.5	23.8	19.0	12.8	13.2

Table 5 Fatty acid composition (% mol) of mixture of tuna and sardine oil fractions.

Fatty acid		1	2	3	4	5	6	7
Myristic (M)	C14:0	4.63	5.66	10.41	4.93	5.55	3.90	4.92
Palmitic (P)	C16:0	17.83	24.51	21.55	31.68	28.00	31.24	36.42
Palmitoleic (Po)	C16:1n-7	6.86	8.95	14.14	6.67	8.85	6.14	4.95
Stearic (S)	C18:0	4.26	2.01	6.35	4.36	11.98	9.31	16.07
Oleic (O)	C18:1n-9	44.76	13.96	15.57	22.72	24.09	33.66	28.11
Vaccenic (V)	C18:1n-7	2.29	2.61	2.83	3.42	3.09	3.92	–
Linoleic (Lo)	C18:2n-6	4.41	2.01	4.22	2.93	3.42	2.03	–
Eicosenoic (G)	C20:1n-9	–	–	–	2.12	2.57	3.55	6.42
Eicosatrienoic (Et)	C20:3n-3	–	1.66	2.67	1.71	2.07	–	–
Eicosapentaenoic (Ep)	C20:5n-3	5.35	9.63	5.57	4.30	2.13	0.58	–
Docosapentaenoic (Dp)	C22:5n-3	–	1.46	1.98	1.26	–	–	–
Docosahexaenoic (Dh)	C22:6n-3	9.60	27.54	14.71	13.90	8.26	5.67	3.11
% area of each fraction (RP-HPLC)		2.6	14.5	11.9	30.7	14.5	18.2	7.6

Table 6 ECN values of TAG standards obtained with different equations.

TAG standard	ECN (eq.7)	ECN (eq.8)
CCC	30.00	30.00
DhDhDh	32.70	35.08
LnLnLn	34.84	37.22
LoLoLo	39.56	41.94
PoPoPo	40.65	40.65
MMM	42.00	42.00
OOO	46.65	46.65
PPP	48.00	48.00
SSS	54.00	54.00

observed that the elution order agrees with the ECN value of each TAG.

When equation (1) was used to correlate the experimental retention times of standards TAG, equation (8) was obtained to evaluate the ECN values:

$$ECN = CN - 1.572 \cdot DB - 0.878 \cdot NUFA \quad (8)$$

In this expression the effect of MUFA and PUFA on retention time is not distinguished. Table 6 also shows the ECN values obtained with equation (8). In this case, the order of the ECN values does not correspond with their retention times. Therefore when no addition factor is considered for the long chain polyunsaturated fatty acids, ECN values do not longer correlate with the elution time.

From the ECN values for standards TAG, it is possible to derive partial ECN values for each individual acid component of triacylglycerol, since these partial values seem to be additive and it is possible to calculate by addition the ECN values for unknown triacylglycerols[16]. The partial ECN values for fatty acids are presented in Table 7 for the standards used in this work. This table also shows ECN values found in the literature for these fatty acids[11, 16–18]. The ECN value for all the fatty acids present in fish oils could not be directly determined since no triacylglycerol standards were available in our laboratory. These ECN values were obtained from equation (7) and are also presented in Table 7. Therefore, these values are, to some extent, uncertain. Nevertheless these values will be used to calculate the ECN values from the unknown triacylglycerols of fish oil.

For each chromatographic peak obtained in the analysis of fish oils, experimental ECN values were calculated from their relative retention times by using the linear regression obtained for the ECN standard TAG and their relative retention times (Fig. 5):

$$ECN = 0.465 \cdot RRT + 30.111 \quad (9)$$

To determine a tentative composition of TAG in the fish oils studied in this work, experimental ECN values for each chromatographic peak (eq. 9) have been compared with theoretical ECN values of TAG. This theoretical ECN value has been obtained combining three by three the different fatty acid composition obtained by GC analysis of each fraction collected by RP-HPLC. The ECN error was estimated ± 0.3 (from three chromatograms). Usually, two or even more TAG can be assigned to a single chromatographic peak since different combinations of fatty acids can give the same ECN value. In these cases, TAG formed with fatty acids with less than 5% area percentage in the GC analysis of each fraction have not been included in the tentative composition of fish oil. When TAG peaks were not identified, fatty acids with more than 4% and 3% area, for

Table 7 Partial ECN values for the unsaturated fatty acids obtained with equation (7) together with others values found in the literature.

Fatty acid	ECN1, eq. (7)	ECN2 [11]	ECN3 [16]	ECN4 [17]	ECN5 [18]
Palmitoleic	13.55	13.12	–	13.12	–
Oleic	15.55	15.00	15.60	15.05	15.42
Linoleic	13.19	12.38	13.10	12.73	12.84
Linolenic	11.61	10.75	11.10	10.81	10.40
Docosahexaenoic	10.90	–	–	–	–
Steriadonic	10.04				
Eicosenoic	17.55				
Eicosatrienoic	13.61				
Eicosapentaenoic	10.47				
Docosapentaenoic	12.47				

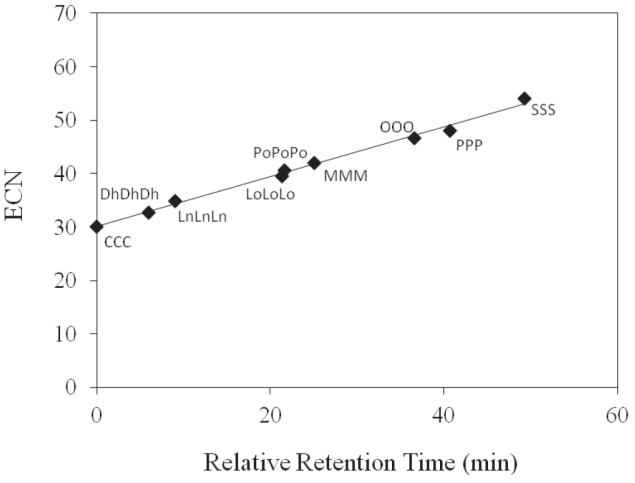

Fig. 5 Correlation between ECN (equation 7) and RRT of TAG standards. ECN = 0.4647·(RRT) + 30.111.

sardine and the mixture of sardine and tuna oils, respectively, were also taken into account. Table 8 and Table 9 present the compositional results of predicted TAG species of the seven fractions obtained by RP-HPLC. From these tables it can be observed that, for both type of oils, around 60% of TAG molecular species were bound to one or more polyunsaturated fatty acids.

3.3.2 Distribution of FA in sn-2 and sn-1 (or sn-3) positions

In order to predict in a better way the positional distribution of fatty acids, ethanolysis of both fish oils has been carried out under the experimental conditions described in section 2.2. After 4 h a maximum in the 2-MAG production has been described[6]. The amount of 2-MAG for sardine oil and the mixture of tuna and sardine oil was 22.0% and 20.9% respectively. FAME analysis for 2-MAG separated by TLC and NP-HPLC has been conducted to determine FA bound to sn-2 position. Both techniques exhibited similar findings without significant differences. Table 2 shows the results obtained in the regiospecific analysis. Results of FA distribution in sn-2 position are expressed as[19]:

$$\%\ sn\text{-}2 = [\text{mol}\ \%\ sn\text{-}2\ \text{fatty acid/mol}\ \%\ \text{fatty acid in TAG}\cdot 3]\cdot 100 \quad (10)$$

sn-1 (or sn-3) positions can be easily calculated as:

$$\%\ sn\text{-}1\ (\text{or}\ sn\text{-}3) = 100 - [\%\ sn\text{-}2] \quad (11)$$

From Table 2, it can be observed that for both oils docosahexaenoic acid is mostly found at sn-2 position, especially for sardine oil. This result agrees with recent studies in the regiospecific analysis of fish oil triacylglycerols[19, 20].

Furthermore for both oils, myristic, palmitic, palmitoleic, linoleic, linolenic and steriadonic acids are randomly distributed in sn-1 (or sn-3) and sn-2 positions (26.4–41.9% mol). On the other hand, stearic, oleic and vaccenic acids and also eicosenoic acid for the mixture of sardine and tuna oil are hardly bound to the sn-2 position (6.7–19.4% mol).

The main difference in the regiospecific analysis for both types of oils has been found for docosapentaenoic acid. This fatty acid is mainly found at 2 sn-position in sardine oil (76.4 % mol); however it is randomly distributed in the mixture of sardine and tuna oil (44.4% mol). Additionally, an important percentage of eicosatrienoic and eicosapentaenoic acids (35.0 and 30.2% mol) are bound to sn-2 position for the mixture of sardine and tuna oil compared to sardine oil (19.6 and 12.1% mol).

3.3.3 Principal component analysis

Seven fractions (objects) have been collected in the analysis of fish oils by RP-HPLC and their corresponding fatty acid composition has been considered the chemical descriptors.

To visualize the trends of the data, the scores for samples and the loadings for variables were represented in the space of the two principal components (PCs) obtained from PCA[21]. Figure 6 and Fig. 7 show the loading and

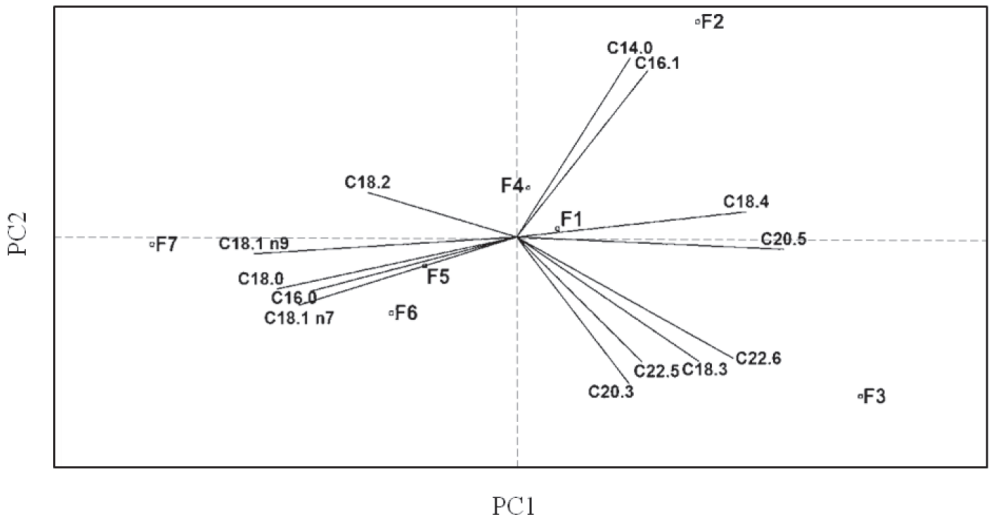

Fig. 6 Loadings and scores plot from PCA of data about fatty acids of fractions for sardine oil, in the PC1-PC2 planes.

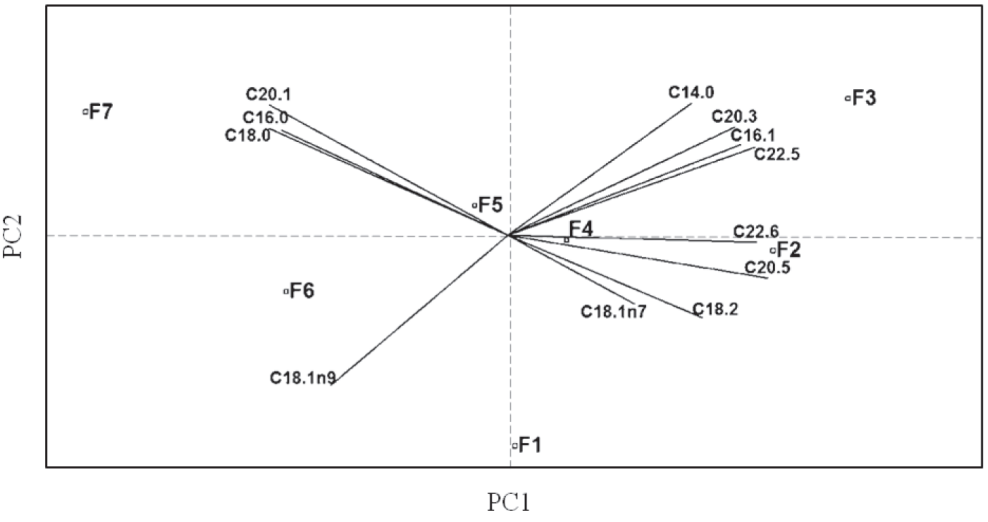

Fig. 7 Loadings and scores plot from PCA of data about fatty acids of fractions for mixture of tuna and sardine oil, in the PC1-PC2 planes.

score plots of the two principal components for the differences between the fatty acid compositions of the fractions for sardine oil and the mixture of tuna and sardine oil respectively. In both fish oils, loading plots in the planes PC1–PC2 and PC1–PC3 reveal that all of the fatty acids variables give their variance to PC1. For this reason only planes PC1–PC2 are plotted.

For both fish oils studied, the first fractions (1-4) are located on the right side of the graph, where the PC1 presents positive values. However, the last fractions (5-7) are located on the left side of the graphs, where the PC1 shows negative values. Polyunsaturated fatty acids, myristic and palmitoleic acids variables present large and positive loadings, regarding PC1 while saturated and monounsaturated fatty acids variables exhibit large and negative loadings.

For sardine oil, PC1 and PC2 were able to describe respectively 51.59% and 25.82% of total variance, while PC3 described 13.06%. Moreover, those variables whose loadings present small angles are correlated, positively or negatively, according to their position in the plot. For instance, myristic and palmitoleic acids present positive correlation. Namely, when one grows, the other grows too. Otherwise, palmitic, stearic and vaccenic acids present negative correlation; so when one increases, the others decrease. This type of correlations have been also observed when predicting a probable TAG composition of sardine oil. For instance, from Table 8, it can be observed, in the first fractions, TAG formed by combination of C16:1 and C20:5n-3 or C22:6n-3 as well as TAG with C14:0 and C20:5n-3 or C22:6n-3; that is, when C14:0 is present, C20:5n-3 or C22:6n-3 will be also present in the same TAG.

On the other hand, for the mixture of tuna and sardine

Table 8 Predicted TAG species from HPLC peaks present in area > 0.2 % of total in sardine oil.

Fraction	Peak No.	RRT	Experimental ECN (Eq. 9)	Predicted TAG	% Area	Predicted ECN (Eq. 7)
1	1	− 0.05	30.1	St-St-St	0.24	30.1
	2	0.72	30.5	St-St-Ep	0.53	30.6
	3	1.18	30.7	St-St-Ep	0.21	30.6
	4	1.53	30.8	St-St-Dh / St-Ep-Ep	0.20	31.0
	5	2.78	31.4	Ep-Ep-Ep	0.25	31.4
	6	3.29	31.6	Ep-Ep-Ep / Ep-Ep-Dh	0.38	31.4 − 31.8
	7	4.19	32.1	Ep-Ep-Dh / Ep-Dh-Dh	0.79	31.8 − 32.3
	8	4.63	32.3	Ep-Dh-Dh	0.91	32.3
	9	5.48	32.7	Dh-Dh-Dh	0.35	32.7
2	10	6.9	33.3	Lo-St-St	0.42	33.3
	11	7.73	33.7	Po-St-St / Lo-St-Ep	1.75	33.6 − 33.7
	12	8.4	34.0	Po-St-Ep	4.47	34.1
	13	9.21	34.4	Po-Ep-Ep	0.75	34.5
	14	9.46	34.5	Po-Ep-Ep	0.27	34.5
	15	10.48	35.0	M-Ep-Ep	0.95	34.9
3	16	11.69	35.5	Po-Dh-Dh / M-Ep-Dh / M-Dh-Dh	1.85	35.4 − 35.8
	17	12.53	35.9	M-Dh-Dh	7.57	35.8
	18	13.47	36.4	O-St-Ep / P-St-St / O-Ep-Ep / O-St-Dh / P-St-Ep	4.08	36.1 − 36.5
	19	14.26	36.7	P-Ep-Ep	4.09	36.9
	20	15.15	37.2	P-Ep-Ep / M-M-Dh / P-Ep-Dh	0.83	36.9 − 37.4
	21	15.59	37.4	M-M-Dh / P-Ep-Dh / Po-Po-Ep	0.61	37.2 − 37.6
4	22	16.57	37.8	Po-Po-Ep / P-Dh- Dh / Po-Po-Dh / M-Po-Ep	0.46	37.6 − 38.0
	23	18.14	38.5	M-Po-Dh / M-M-Ep	2.24	38.4 − 38.5
	24	19.11	39.0	S-Ep-Ep / O*-Lo-Ep	12.42	39.0 − 39.2
	25	20.14	39.5	Po-O*-Ep	7.54	39.6
	26	21.36	40.0	Po-O*-Dh / M-O*-Ep / P-Po-Ep	0.54	40.0
	27	21.49	40.1	Po-O*-Dh / M-O*-Ep / P-Po-Ep	0.29	40.0
5	28	22.64	40.6	M-O*-Dh / P-Po-Dh / M-P-Ep / M-P-Dh	1.90	40.4 − 40.9
	29	23.95	41.2	M-Po-Po	8.38	41.10
	30	25.15	41.8	M-M-Po / O*-O*-Ep / M-M-M / O*-O*-Dh / P-O*-Ep	0.65	41.6 − 42.0
	31	25.63	42.0	M-M-M / O*-O*-Dh / P-O*-Ep	3.23	42.0
	32	26.37	42.4	P-O*-Dh / P-P-Ep / Po-Po-O*	1.55	42.4 − 42.7
	33	27.64	43.0	P-P-Dh / M-Po-O* / P-Po-Po	3.24	42.9 − 43.1
6	34	28.86	43.5	M-M-O* / M-P-Po	0.55	43.6
	35	30.10	44.1	M-M-P / S-O*-Ep	0.87	44.0
	36	31.20	44.6	P-S-Ep / Po-O*-O*	4.83	44.5 − 44.7
	37	32.61	45.3	M-O*-O* / P-Po-O* / Po-Po-S / M-P-O* / M-Po-S / P-P-Po	6.52	45.1 − 45.6
7	38	34.06	45.9	M-M-S /M-P-P	0.64	46.0
	39	35.24	46.5	O*-O*-O* /S-O*-Lo	0.56	46.7
	40	35.99	46.8	O*-O*-O* /S-O*-Lo / P-O*-O* / Po-S-O*	3.79	46.7 − 47.1
	41	37.36	47.5	P-S-Lo / S-S-Lo / M-S-O* /P-P-O* / P-Po-S	5.93	47.2 − 47.6
	42	38.80	48.1	M-P-S / P-P-P	0.43	48.0
	43	40.30	48.8	S-O*-O*	0.48	49.1
	44	41.74	49.5	P-S-O* / Po-S-S	1.44	49.6

Fatty acids: M=myristic acid (14:0); P=palmitic acid (16:0); Po= palmitoleic acid (16:1n-7); S= stearic acid (18:0); O=oleic acid (18:1n-9); Lo= linoleic acid (18:2n-6); Ln=linolenic acid (18:3n-3); St=stearidonic acid (18:4n-3); Et=eicosatrienoic acid (20:3n-3); Ep=eicosapentaenoic acid (20:5n-3); Dp=docosapentaenoic acid (22:5n-3); Dh=docosahexaenoic acid (22:6n-3).

* Oleic acid in the corresponding TAG could be also vaccenic acid, both acids show the same carbon number, double bonds and number of MUFA; therefore it is not possible to distinguish them.

oil, PC1 and PC2 were able to describe 54.05% and 20.91% respectively of total variance, while PC3 described 13.17%. In this case, palmitoleic and eicosapentaenoic acids present positive correlation, while palmitic and stearic acids present negative correlation. These results can be also observed in Table 9.

Table 9 Predicted TAG species from HPLC peaks present in area > 0.2 % of total in tuna-sardine oil.

Fraction	Peak No.	RRT	Experimental ECN (Eq. 9)	Predicted TAG	% Area	Predicted ECN (Eq. 7)
1	1	4.5	32.1	Ep-Ep-Dh / Ep-Dh-Dh	0.29	31.8 − 32.3
	2	5.17	32.4	Ep-Dh-Dh / Dh-Dh-Dh	0.38	32.3 − 32.7
	3	5.83	32.7	Dh-Dh-Dh	0.33	32.7
	4	9.25	34.3	Lo-Ep-Ep / Po-Ep-Ep / Lo-Ep-Dh	0.44	34.1 − 34.6
	5	10.33	34.7	Po-Ep-Ep / Lo-Ep-Dh / Po-Ep-Dh	0.58	34.5 − 34.9
	6	10.63	34.9	Po-Ep-Dh / Lo-Dh-Dh	0.18	34.9 − 35.0
	7	12.61	35.9	M-Dh-Dh	0.35	35.8
2	8	13.42	36.2	O-Ep-Ep	1.10	36.5
	9	14.26	36.6	O-Ep-Ep / O-Ep-Dh	6.39	36.5 − 36.9
	10	15.25	37.1	O-Ep-Dh / P-Ep-Ep / O-Dh-Dh / P-Ep-Dh	6.56	36.9 − 37.4
	11	16.54	37.7	Po-Po-Ep / P-Dh-Dh	0.46	37.6 − 37.8
3	12	17.54	38.2	Po-Po-Dh / M-Po-Ep / M-Lo-Dh	0.62	38.0 − 38.1
	13	18.19	38.5	M-Po-Dh / M-M-Ep	0.58	38.5
	14	19.08	38.9	S-Ep-Ep	0.77	38.9
	15	20.05	39.3	S-Ep-Dh / Po-O-Ep	4.24	39.4 − 39.6
	16	21.07	39.8	Po-O-Ep / S-Dh-Dh / Po-O-Dh / M-O-Ep / P-Po-Ep	2.48	39.6 − 40.0
	17	22.23	40.3	M-O-Dh / P-Po-Dh / M-P-Ep	2.68	40.5
	18	23.79	41.1	M-P-Dh / M-Po-Po	0.49	40.9 − 41.1
4	19	24.01	41.2	M-P-Dh / M-Po-Po	0.51	40.9 − 41.1
	20	24.79	41.5	O-O-Ep	4.16	41.6
	21	26.04	42.1	O-O-Dh /P-O-Ep	17.66	42.0
	22	27.34	42.7	P-O-Dh / P-P-Ep / Po-Po-O / P-P-Dh	8.40	42.5 − 42.9
5	23	29.04	43.5	M-M-O* / M-P-Po	0.24	43.6
	24	30.04	43.9	G-Lo-Lo / M-M-P / G-O*-Dh	0.47	43.9 − 44.0
	25	31.31	44.4	S-O*-Dh / Po-O*-O*	5.84	44.5 − 44.7
	26	32.49	45.2	P-S-Dh / P-Po-O* / Po-Po-S	7.93	44.9 − 45.1
6	27	33.99	45.8	P-P-Po	2.77	45.6
	28	36.19	46.8	O-O-O / S-S-Dh	1.39	46.7 − 46.9
	29	37.38	47.4	P-O-O / Po-S-O / P-P-O / P-Po-S	7.48	47.1 − 47.6
	30	38.91	48.1	P-P-P	6.17	48.0
	31	40.60	48.9	S-O-O	0.42	49.1
7	32	41.26	49.4	S-O-O / G-P-O / P-S-O / G-P-P	2.64	49.1 − 49.6
	33	43.35	50.2	P-P-S	1.92	50.0
	34	45.95	51.4	G-G-P / G-S-O / S-S-O / G-P-S	0.98	51.1 − 51.6
	35	47.3	52.0	P-S-S	0.88	52.0
	36	48.28	52.4	G-G-G	1.20	52.7

Fatty acids: M=myristic acid (14:0); P=palmitic acid (16:0); Po= palmitoleic acid (16:1n-7); S= stearic acid (18:0); O=oleic acid (18:1n-9); Lo= linoleic acid (18:2n-6); G=eicosaenoic acid (20:1n-9); Et=eicosatrienoic acid (20:3n-3); Ep=eicosapentaenoic acid (20:5n-3); Dp=docosapentaenoic acid (22:5n-3); Dh=docosahexaenoic acid (22:6n-3).

PCA can be a useful tool to verify TAG composition calculated in Tables 8 and 9.

4 CONCLUSION

In this work, HPLC combined with GC has been used to analyze the triacylglycerol composition of two different sources of fish oils, sardine and a mixture of tuna and sardine oil.

The equation obtained through a multiple linear regression analysis, with the relative retention times of TAG standards, is able to provide good ECN values to indentify the majority of TAG molecular species present in very complex mixtures as those from fish oil. Moreover, the PCA analysis provides a versatile tool to assurance the most probable TAG contents.

As expected from the FA profile, the major TAG species present in these fish oils were rich in docosahexaenoic, eicosapentaenoic, and docosapentaenoic acids. Regiospecific analysis of FA in the TAG by ethanolysis with *Candida antarctica* shows that docosahexaenoic acid is mainly bound at sn-2 position. The present findings explore the possibilities of utilizing these oils as a PUFA-rich source in fish oil industry.

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