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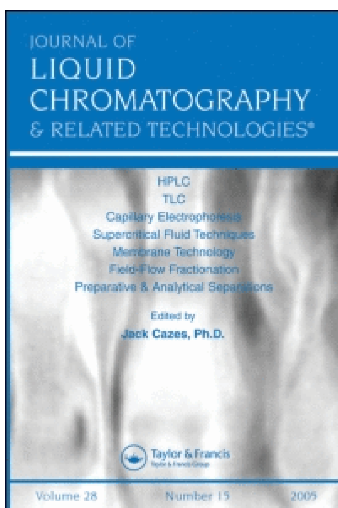

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CHARACTERIZATION OF THE TRIACYLGLYCEROL MOLECULAR SPECIES OF FISH OIL BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The nutritional benefits attributed to fish oils have been the basis for the study of the structural composition of Sardine oil triacylglycerols (TAGs). TAGs were separated reversed-phase high performance liquid chromatography (RP-HPLC) with the result of 65 chromatographic peaks resolved. The problem of identification was avoided by the use of two more chromatographic techniques. Separation of the sardine oil TAGs into fractions by silver-ion thin layer chromatography (TLC) and its subsequent fatty acid analysis by gas chromatography allowed identification of 59 of the 65 chromatographic peaks. From those peaks, the major was trimyristin (MMM), with 8.22 % of the total. Dioleoyl-acyl-glycerol (OPO, OOE), dipalmitoyl-acyl-glycerol (PPO, PPPo), dipalmitoleoyl-acyl.glycerol (PoPoO) and dieicosapentaenoyl-acyl-glycerol (EEP) species were found in important amounts.

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INTRODUCTION

Experimental evidence that dietary fat can influence carcinogenesis, coronary heart disease and inflammatory alterations has been accumulating for more than 30 years. The recent emergence of dietary polyunsaturated fat as precursor of potent metabolic intermediates has radically changed our concepts of the potential mechanisms of diet-induced effect on health and disease.[1] The observed effects of reduced mortality from coronary heart disease in people with an habitual consumption of fish oils focused important attention on the structure of these oils in the last years.[2,3] In addition, dietary fish oil has been shown to reduce the response to endotoxin and to pro-inflammatory cytokines, which are related to septic shock and chronic inflammatory diseases.[4] For these reasons there is a considerable interest in producing fish oils enriched in polyunsaturated fatty acids (PUFA) both for oral and parenteral nutrition.

The great variety of fatty acids contained, from myristic (14:0) to docosahexaenoinc (22:6, n-3) acid, causes enormous difficulties in the approach to the TAG analysis of fish oils. Due to that complexity the use of a single chromatographic technique has not been enough to elucidate their TAG profile. Silver-ion chromatography separates molecules on the basis of their degree of unsaturation. Impregnation of silver ions to thin-layer chromatography (TLC) plates or to columns has been commonly employed to partially separate fish TAGs.[5,6] This technique has also been applied to high performance liquid chromatography achieving separation of several TAG fractions, which were subsequently analyzed by gas chromatography (GC) to determine their fatty acid composition.[7-9] However, silver-ion chromatography has not been able to achieve the separation of TAGs into molecular species.

Reversed-phase high performance liquid chromatography (RP-HPLC) has been widely employed in the separation of molecular species of TAGs, mainly from vegetable sources, with considerable success.[10-13] The introduction of the evaporative light-scattering detector (ELSD) brought a great advance in the detection of TAGs upon HPLC, as it allows gradient elution systems and the use of any solvent for the mobile phase, provided it is more volatile than the sample. Since then, complex mixtures of TAGs, such as those contained in animal fats, have been analyzed by RP-HPLC coupled with ELSD.[14-17] However, little data has been published on fish oil TAGs by RP-HPLC, and moreover, identification of TAG molecular species has not been achieved yet.

The present study has the aim of complete identification of all TAG molecular species of sardine (*Sardine pilchardus),* one of the most consumed fat-rich fish in Spain. For this purpose, a combination of three chromatographic techniques was employed. TAGs were first determined by RP-HPLC and then silver-ion TLC and GC were used as helpful tools for the identification of the chromatographic peaks.

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EXPERIMENTAL

Sample Preparation

Atlantic sardines were purchased from the local market, the day after being caught. They were preserved in ice until processed, in order to avoid the risk of oxidation problems. Extraction of total lipids was performed the same day, following the method described by Folch et al.[18] TAGs were isolated by thin layer chromatography (TLC) on silica gel 60 plates (Kieselgel 60 F_{254}, Merck) using an elution system of hexane/diethylether/acetic acid (80:20:1,v/v/v), as described by Ruiz-Gutiérrez et al.[19]

RP-HPLC Analysis

TAG fraction, vacuum-evaporated to dryness, at temperature below 30ºC, was redissolved in n-hexane and passed through a filter with a pore size of 0.2 µm (Millipore). The chromatographic system consisted of a model 2690 Alliance liquid chromatograph (Waters), a Spherisorb ODS-2 column (250x4.6 mm) with a particle size of 3 µm (Phase Separations). The column dead time (t_o) was 1.99 minutes, as provided by the supplier.

The liquid chromatograph was coupled to a light-scattering detector model DDL31 (Eurosep). The system was controlled by a computer through Millenium System (Waters).

The mobile phase consisted of an initial elution gradient of 20% of acetone in acetonitrile raising the percentage of acetone 45% in 12 minutes and finally to 80% at 70 minutes, and held to the end of the analysis. The flow rate was 1.0 mL/min.

Quintupled runs of 10 µL of n-hexane solution containing 0.5 mg/mL of pure TAGs (Sigma Grade, 99% pure) tritridecanoin, 1,3-dioleoyl-2-palmitoyl-glycerol, trimyristin, 1,3-dioleoyl-2-stearoyl-glycerol, 1,3-dioleoyl-2-linoleoyl-glycerol, 1,3-dilinoleoyl-2-oleoyl-glycerol, tripalmitin, triolein and trilinolein were analyzed in order to establish the capacity factor (k´) of the system. All the solvents used, both in dissolving sample TAGs and in the mobile phase, were HPLC grade (Merck).

In order to calibrate the detector, tripled runs of five concentrations of the standards, between 0.25 and 2.5 mg/mL, were injected. Various regression models were tested and finally those that completely fitted the curve (r^2=1.0000) were chosen, generally cubic or fourth regression, although linear regression was also calculated.

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Silver Ion-TLC

TLC plates were impregnated with a solution of 5% silver nitrate in ethanol. The TAG fraction was applied to the plates and developed with benzene as described by Ruiz-Gutierrez and Barrón.[20] TAGs were separated in four bands, which were detected and scrapped from the plates in order to be analyzed.

GC Analysis

Total TAGs and the fractions obtained through silver-ion TLC, were transmethylated and the resulting fatty acid methyl esters (FAME) analyzed by GC as described by Ruiz-Gutierrez et al.[19] using a model 5890 series II gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector (FID) and a capillary silica column Supelcowax 10 (Sulpelco) of 60 m length and 0.25 mm internal diameter. Both injector and detector temperature was 250ºC.

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Calculation of TAG Composition

For each band resulting from silver-ion TLC a number of fatty acids were obtained by GC. These fatty acids were combined, three by three, to calculate all possible TAGs remaining in the stereoespecific positions in the glycerol molecule as equivalent, since HPLC analysis cannot separate positional isomers,[21] considering only those fatty acids present with a content higher than 2%.

The equivalent carbon number (ECN) of each TAG was calculated according to the following equation:

$$ECN = CN - a_1'*DB - a_2'*NUFA \quad (1)$$

where CN is the total carbon number of the three fatty acids, and DB the total number of double bonds and NUFA the number of unsaturated fatty acids of the TAG molecule.

The values of the constants a_1' and a_2' were calculated by multiple linear regression analysis of the experimental values of the dependent variable, log k', and the independent variables CN, ND, and NUFA for the TAG standards ($\log k' = q' + b'\ CN + c'\ ND + d'\ NUFA$), where a_1' is the quotient between the coefficients c' and b', and a_2' between the coefficients d' and b'. a_1' was 2 and a_2' was 0.2.

Table 1

Fatty Acid Composition (%) of the Total Triacylglycerols and the Fractions Obtained by Silver-Ion TLC from Sardine

Fatty Acid	Total	Fraction 1	Fraction 2	Fraction 3	Fraction 4
14:0	6.90	2.87	3.84	6.05	8.81
14:1 n-5	0.77	0.29	0.43	0.71	0.99
16:0	21.54	9.36	14.33	19.11	28.37
16:1 n-7	6.96	4.38	5.94	7.53	8.33
18:0	5.41	2.16	3.78	4.31	6.69
18:1 n-9	15.82	9.76	16.69	17.57	22.94
18:1 n-7	3.45	1.86	2.50	3.43	2.74
18:2 n-6	1.42	0.75	2.14	1.76	1.83
18:3 n-3	0.13	0.90	1.25	0.62	0.65
20:0	1.76	3.19	2.41	1.43	---
20:1 n-9	2.85	0.87	1.93	2.33	4.62
20:5 n-3	14.83	33.15	22.36	16.66	6.52
22:5 n-3	2.97	4.41	4.01	2.85	1.31
22:6 n-3	13.82	25.58	17.81	14.93	5.87
Saturated	35.61	17.58	24.36	30.90	43.87
MUFA	29.85	17.16	27.49	31.57	39.62
PUFA	33.65	64.79	47.57	36.82	16.18
Others	0.89	0.47	0.58	0.71	0.33

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A simple linear regression analysis was applied to relate ECN with log k' of the pure TAGs. The result was the equation:

$$NEC=4,3133+29,7759*\log k' \quad (2)$$

Including log k´of each chromatographic peak, their ECN was obtained and was employed to assign TAGs to chromatographic peaks.

RESULTS

Silver-Ion TLC

Table 2 shows the results of the RP-HPLC analysis of the four fractions obtained by silver-ion TLC. TAGs were separated according to their degree of unsaturation. The first fraction was composed of TAGs with ECN<38, that is

Table 2

Triacylglycerol Composition (%) of the Total Oil and the Fractions Obtained by Silver-Ion TLC from Sardine

Peak	Tag	Total	F1	F2	F3	F4
1	DDD	0.01	0.78	---	---	---
2	EED	0.06	0.78	---	---	---
3	EED	0.01	0.70	---	---	---
4	EEE	0.08	0.49	---	---	---
5	DpDD	0.04	0.34	---	---	---
6	EDpD	0.02	---	---	---	---
7	EEDp	0.04	---	---	---	---
8	PoDD	0.03	0.56	0.15	---	---
9	PoED	0.02	---	1.19	---	---
10	PoEE	0.07	---	2.32	---	---
11	ODD	0.46	6.57	0.93	---	---
12	OED	0.75	9.26	0.71	---	---
13	OEE	0.39	3.38	4.25	---	---
14	PoDpD	0.17	0.35	7.25	---	---
15	PoEDp	3.22	0.02	---	---	---
16	MDpD/MEDp	0.37	4.45	20.42	---	---
17	PDD	0.96	10.35	13.18	0.05	---
18	PED	2.98	29.83	5.70	0.87	---
19	PEE	2.72	20.59	7.24	2.44	---
20	PoPoD	0.41	4.72	8.04	1.75	---
21	PoPoE	0.91	---	3.82	---	---
22	MME/MMD	1.40	1.24	3.44	0.47	---
23	PoOE/PoOD	1.56	2.04	---	0.86	---
24	PPoD	0.25	0.62	3.35	4.68	0.23
25	PPoE	0.56	---	2.71	---	---
26	MPD	0.81	1.53	7.19	---	---
27	MPE	1.12	---	4.14	---	---
28	OOD	1.82	1.45	---	16.62	0.36
29	OOE	5.04	---	---	10.59	0.24
30	POD	2.23	---	---	2.64	0.61
31	POE	1.83	---	---	0.91	0.67
32	MPoPo	0.62	---	---	1.80	0.80
33	MMPo	0.85	---	2.30	5.24	1.68
34	PPD	1.22	---	1.66	21.23	1.01
35	PPE	3.07	---	---	15.74	0.88
36	MMM	8.22	---	---	4.58	1.49
37	PoPoO	4.96	---	---	1.50	3.80

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Table 2 (continued)

Peak	Tag	Total	F1	F2	F3	F4
38	PoPoP	2.23	---	---	0.50	4.11
39	MPoO	1.74	---	---	1.78	1.09
40	MPPo	1.4	---	---	1.02	2.42
41	n.i.	2.21	---	---	---	---
42	MMO	1.36	---	---	2.20	0.58
43	MMP	2.32	---	---	0.44	2.26
44	PoOO	2.02	---	---	0.47	11.58
45	PPoO	1.54	---	---	0.27	11.94
46	n.i.	0.89	---	---	---	---
47	n.i.	1.13	---	---	---	---
48	MOO	1.13	---	---	0.11	3.35
49	PoPoS	1.56	---	---	0.40	---
50	PPPo	5.95	---	---	0.29	2.97
51	MPO/MPoS	5.73	---	---	0.19	14.75
52	MPP/MMS	2.04	---	---	0.10	16.63
53	OOO	1.24	---	---	0.12	3.07
54	POO	5.81	---	---	0.03	0.95
55	PPO	6.98	---	---	0.09	3.39
56	OPO?	0.18	---	---	---	---
57	PPP	1.21	---	---	---	4.91
58	n.i.	0.14	---	---	---	0.14
59	n.i.	0.13	---	---	---	---
60	MPS	1.02	---	---	---	1.02
61	n.i.	0.05	---	---	---	---
62	SOO	1.71	---	---	---	0.60
63	PSO/PoSS	0.40	---	---	---	0.66
64	PPS/MSS	0.27	---	---	---	0.10
65	SSO	0.29	---	---	---	0.27

Fatty acid: M=myristic acid, 14:0; P=palmitic acid, 16:0; Po=palmitoleic acid, 16:1; S=stearic acid, 18:0; O=oleic acid, 18:1; L=linoleic acid, 18:2; E=Eicosapentenoic acid; Dp=Docosapentenoic acid; D=docosahexaenoic acid, 22:6. Triacylglycerols: PPP=sn-glycerol-tripalmitate; MPL=sn-glycerop-myristate-palmitate-linoleate; POL=sn-glycerol-palmitate-oleate-linoleate, etc.

the most unsaturated TAGs. The second fraction contained TAGs with ECN between 35-42. The chromatographic peaks of the fraction corresponded to ECN between 36-44. The fourth band was of those less unsaturated TAGs, with ECN between 42-52. The minor ECN value was 28.1 and the major was 50.2.

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Fatty Acid Analysis by GC

The fatty acid analysis of the total TAGs, is depicted in Table 1. The most abundant fatty acid in sardine oil was palmitic acid (16:0) with 21.54% of the total fatty acids. Similar amounts were found for oleic acid (18:1, n-9) with 15.82%, eicosapentaenoic acid (20:5, n-3) with 14.83% and docosahexaenoic acid (22:6, n-3) with 13.82%. Relevant amounts were observed for myristic, stearic and palmitoleic acids, all above 5%. Table 1 also shows the fatty acid composition of each TAG band obtained by silver-ion TLC. As it was expected, the content of eicosapentaenoic and docosahexaenoic acids were approximately 60% together in the most unsaturated fraction (fraction 1). Polyunsaturated fatty acids (PUFA) were progressively reduced in each fraction, whereas the content of saturated was increased. Fraction 4 only contained eicosapentaenoic and docosahexaenoic acid in amounts around 6% and the level of palmitic acid raised almost to 30%. Considerable amounts of myristic and palmitoleic acids were observed in all fractions, always above 3% and even reaching 9%.

TAG Analysis by RP-HPLC

The RP-HPLC analysis of the total TAGs lasted for 75 minutes, with the first peak at minute 16.85 and the last at minute 71.00. Each fraction was then injected independently until minute 75. The combination of the fatty acid obtained in the GC analysis permitted the identification of the chromatographic peaks of Figure 1, which are shown in Table 2. The identification was more accurate in the central part of the chromatograms, since the content of each TAG was better reflected. Generally, one TAG was assigned to a single chromatographic peak, although sometimes two or even three TAGs had to be assigned. Nevertheless, that occurred mainly with TAGs containing eicosapentaenoic or docosahexaenoic acids, since the ECN value of the species containing one or the other fatty acid is the same.

Fraction 1 contained species from tridocosahexaenoin (DDD) to dioleoyl-docosahexaenoyl-glycerol (OOD)/dioleoyl-eicosapentaenoyl-glycerol (OOE). In this fraction no species contained less than five unsaturations. Docosapentaenoic acid (22:5, n-3) was found in some species. The major TAGs in this fraction were palmitoyl-eicosapentaenoyl-docosahexaenoyl-glycerol (PED) and dieicosapentaenoyl-palmitoyl-glycerol (EEP), with nearly one half of the total TAGs. Fraction 2 contained species from didocosahexaenoyl-palmitoleoyl-glycerol (PoDD) to dipalmitoyl-docosahexaenoyl-glycerol (PPD). In this fraction one species was found containing only one double bond: dimyristoyl-palmitoleoyl-glycerol (MMPo). Docosapentaenoic acid (22:5, n-3) was found in the major species, with 20.42% of the total TAGs. Fraction 3 contained species from didocosahexaenoyl-palmitoyl-glycerol (DDP) to dioleoyl-palmitoyl-glycerol (POO). In this fraction some of the TAGs contained

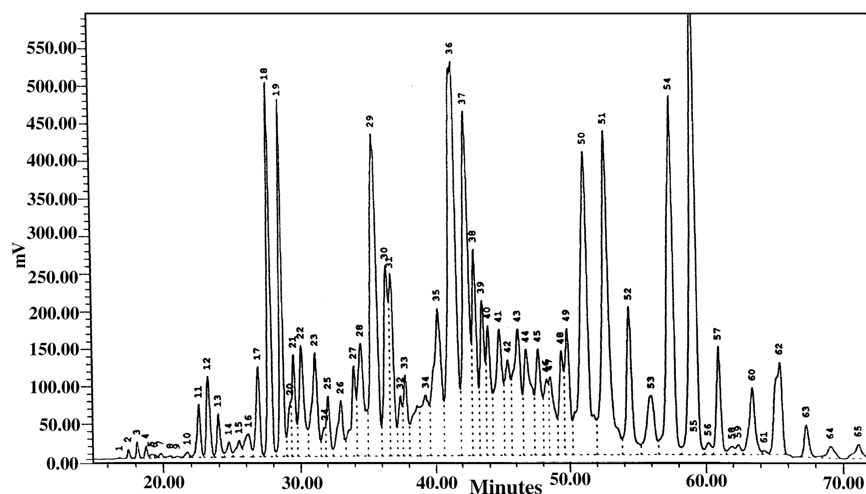

Figure 1. RP-HPLC chromatogram of sardine oil triacylglycerols. Numbers correspond to the peaks of Table 2. Details of elution conditions are described in Experimental.

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few double bonds and even a trisaturated TAG (trimyristin) was found. The major TAGs in this fraction were dioleoyl and dipalmitoyl species combined with eicosapentaenoic and docosahexaenoic acids (OOD, OOE, PPD, and PPE). The last fraction contained TAGs from palmitoyl-oleoyl-docosahexaenoyl-glycerol (POD)/palmitoyl-oleoyl-eicosapentaenoyl-glycerol (POE) to distearoyl-palmitoyl-glycerol (SSP). The main TAGs were composed of saturated (myristic and stearic) or monounsaturated (oleic or palmitoleic) acids.

When these results were applied to the whole chromatogram obtained through the analysis of fish oil by RP-HPLC, Figure 1 was obtained. Sixty-five molecular species of TAGs are reported, from which 59 are identified. Only one TAGs was finally assigned to the majority of chromatographic peaks and on the contrary of what occurred in the TAG fractions, in this case, no peak contained more than two TAGs assigned. Table 2 shows the distribution of TAGs within the chromatographic profile, which resulted to be very regular, as the major TAG is trimyristin, with 8.22% of the total. Important amounts of dioleoyl-acyl-glycerol (OPO, OOE), dipalmitoyl-acyl-glycerol (PPO, PPPo), dipalmitoleoyl-acyl.glycerol (PoPoO), and dieicosapentaenoyl-acyl-glycerol (EEP) species are found. Many TAGs with the same ECN value are separated. However resolution of eicosapentaenoic or docosahexaenoic acid contining TAGs was difficult and some species containing these PUFA remained unresolved (DDpM/EDpM, MMD/MME, and PoOD/PoOE).

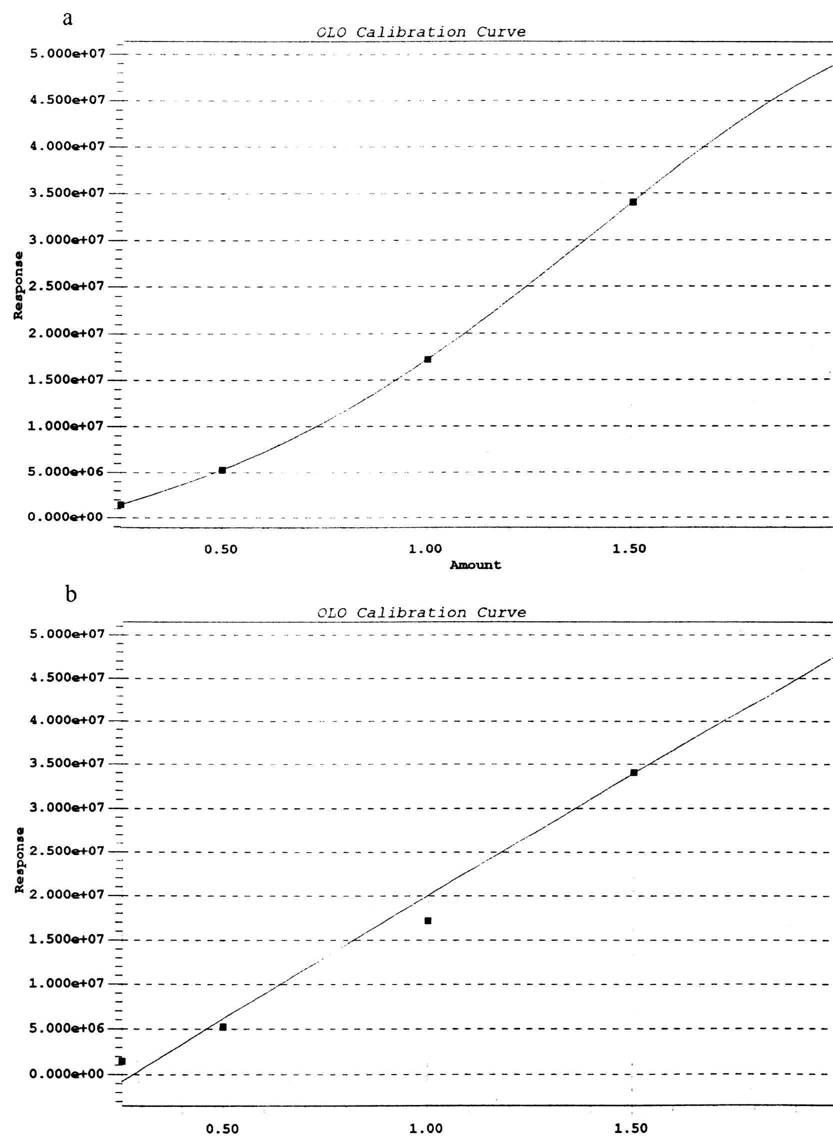

Figure 2. Calibration curves for 1,3-dileoyl-2-linoleoyl-glycerol (OLO) standard analyzed by HPLC and light-scattering detection. The detector response (mV) is plotted against concentration of the standard (µg/µL, 10 µL injected). Fourth (a) and linear (b) regression was applied.

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Detector Response

Sigmoidal response curves of the light scattering detector (ELSD) were observed for all the standards determined. As examples, Figure 2 shows calibration curves of 1,3-dioleoyl-2-linoleoyl-glycerol (OPO). All standards showed correlation factors (r^2) equal to 1 when cubic or fourth regression was applied. However, linear regression could also be valid (r^2=0.95-0.99) in the range of concentrations studied, between 0.25 and 2.5 mg/mL. The resulting equations calculated for the calibration curves are depicted in Table 3.

DISCUSSION

In this study TAGs from sardine oil were analyzed using RP-HPLC. To the best of our knowledge, this is the first paper reporting a complete separation and identification of molecular species of TAGs in fish oil. Until the present, the chromatographic techniques used, including silver-ion TLC, silver-ion HPLC, or even RP-HPLC, were just able to separate fractions of TAGs, which were subsequently the subject of fatty acid analysis. However, no TAG assignments could be made to the resulting chromatograms. Thus, only predictions or supposals were reported.[7,8,22]

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Due to the difficulties present in the TAG analysis of fish oils, most authors were not able to achieve the molecular species separation before. Christie et al.[7,8,23] performed a number of analyses to determine the TAG composition of fish oils by silver-ion HPLC, obtaining up to 26 fractions in herring oil.[8] Other attempts by silver-ion HPLC obtained even less fractions.[9] RP-HPLC was used by Pagnucco et al.[23] achieving the separation of up to 19 fractions. Bergqvist et al.[22] attempted to resolve 35 single peaks from fish oil. Unfortunately they only were able to identify 3 of them.

In the method reported in the present study, a rather simple binary gradient of acetone in acetonitrile achieved excellent resolution of chromatographic peaks. This is a common mixture employed for TAG analysis and has already been applied to a variety of fats by several authors.[13,16,25] The light-scattering detector (ELSD) showed excellent behaviour, taking into account its main drawback, the non-linear response. A sigmoidal response of the ELSD has already been observed before.[26] For calibration purposes investigators searched for linear relationships and, as they found exponential responses of the detector, logarithms had to be calculated. Herslof et al.[27] represented an amount/response graphic for saturated standards, finding exponential trends. In the range of amounts tried (0-30 µg) correlation coefficients (r^2) from 0.984 to 0.995 were found. Applying an exponential curve fitting, r^2 was improved to 0.998.

Table 3

Resulting Equations for the Calibration Curves Calculated for Standards, Applying Fourth or Linear Regression

Standard	Equation	r	r^2
$C_{13}C_{13}C_{13}$	$y=4.59\cdot10^3x^4-1.34\cdot10^5x^3+1.43\cdot10^6x^2-3.23\cdot10^6x+2.59\cdot10^6$	1.00	1.00
	$y=3.50\cdot10^6x-7.13\cdot10^6$	0.99	0.97
LLL	$y=5.15\cdot10^3x^4-1.58\cdot10^5x^3+1.74\cdot10^6x^2-3.96\cdot10^6x+3.24\cdot10^6$	1.00	1.00
	$y=4.17\cdot10^6x-8.05\cdot10^6$	0.99	0.98
LOL	$y=8.43\cdot10^3x^4-2.72\cdot10^5x^3+3.10\cdot10^6x^2-9.51\cdot10^6x+9.05\cdot10^6$	1.00	1.00
	$y=4.71\cdot10^6x-7.39\cdot10^6$	0.99	0.99
MMM	$y=1.41\cdot10^5x^4-1.95\cdot10^6x^3+9.54\cdot10^6x^2-1.43\cdot10^6x+6.58\cdot10^6$	1.00	1.00
	$y=5.75\cdot10^6x-7.39\cdot10^6$	0.97	0.95
OLO	$y=7.64\cdot10^3x^4-2.25\cdot10^5x^3+2.32\cdot10^6x^2-5.42\cdot10^6x+4.41\cdot10^6$	1.00	1.00
	$y=4.74\cdot10^6x-9.23\cdot10^6$	0.9	0.98
OOO	$y=7.65\cdot10^3x^4-2.07\cdot10^5x^3+1.86\cdot10^6x^2-4.21\cdot10^6x+6.08\cdot10^6$	1.00	1.00
	$y=1.91\cdot10^6x-5.56\cdot10^6$	1.00	0.99
OPO	$y=7.82\cdot10^3x^4-2.10\cdot10^5x^3+1.99\cdot10^6x^2-4.65\cdot10^6x+3.75\cdot10^6$	1.00	1.00
	$y=4.16\cdot10^6x-9.21\cdot10^6$	0.97	0.95
PPP	$y=1.17\cdot10^3x^3+5.58\cdot10^5x^2-1.00\cdot10^6x+9.16\cdot10^6$	1.00	1.00
	$y=4.96\cdot10^6x-1.07\cdot10^7$	0.9	0.98
OSO	$y=6.70\cdot10^3x^4-1.96\cdot10^5x^3+2.04\cdot10^6x^2-4.64\cdot10^6x+3.80\cdot10^6$	1.00	1.00
	$y=4.68\cdot10^6x-9.33\cdot100^6$	0.99	0.98

Fatty acid: C_{13}=decatrienoic acid, 13:0; M=myristic acid, 14:0; P=palmitic acid, 16:0; S=stearic acid, 18:0; O=oleic acid, 18:1; L=linoleic acid, 18:2. Triacylglycerols: PPP=sn-glycerol-triaalmitate; OLO=snpglycerol-1,3-linoleate-2-oleate, etc. r=correlation factor

Recently Ruiz-Sala et al.[28] calculated correlation coefficients for seven TAG standards detected by ELSD, including trilinolein (LLL). The linearity of the plots log area/log amount was poorer, with r^2 below 0.975. With the use of cubic or fourth regression curves there is no need to work with logarithms and r^2 can be extremely close to 1 (0.9999-1).

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In spite of the improvements in the HPLC analysis, the problem of the identification of the chromatographic peaks was still present. This has been one of the main problems authors have encountered when analyzing TAGs by HPLC. As purchase of standards of all species of TAGs is almost impossible some other methods for identification have been proposed. Parameters such as Partition number (PN) and Equivalent Carbon Number (ECN), as well as mathematical models and equations derived from them, have been used with considerable results.[29,30] However, when working with complex mixtures of TAGs the identification process becomes enormously complicated and thus, improved methods must be applied. The problem is that the larger the number of fatty acids is in a fat, the larger the number of possible TAGs there is. Therefore, a method to reduce the number of possible TAGs was needed. The inclusion of silver-ion TLC in the methodology was revealed as a helpful tool for this aim.

Each fraction of TAGs obtained from silver-ion TLC contained less number of fatty acids, and so, the number of possible TAGs was also less. In this way, once all the possible TAGs were calculated, they were assigned to the peaks according to their ECN. Indeed, from the standards, and applying the equations described in Section 2, the ECN of the peaks was also calculated. Almost all peaks could be identified by this method.

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Differences in the fatty acid and TAG composition of fish oils are due to species variations and, among same species, due to several other reasons. For example, season variations, have been observed as causes of differences in the fat content of sardine, as food availability, water temperature, and the sexual state of the animal are affected.[31] In spite of this, the fatty acid content of sardine oil was as expected, with palmitic acid (16:0) as the main fatty acid and important amounts of oleic, eicosapentaenoic and docosahexaenoic acids. It is worth mentioning the relatively high content of myristic acid, being trimyristin (MMM) the main TAG. Sardine has been shown to contain even more than 10% of this fatty acid.[9,24]

The only authors who attempted to make a suggestion of the TAG composition of sardine oil were Pagnucco et al.[24] From the main TAGs they hypothesized to be present in sardine, almost all of them are in agreement with our results. Only MPoE, SOE, SOD, PoSO, and MOE were suggested to be contained in sardine oil but were not identified by our method. On the other hand, these authors did not achieve detection of TAGs below PN 38 whereas we were able to identify species of PN 30.

We conclude that RP-HPLC is capable of completely separating the TAG molecular species of fish oil. However, RP-HPLC needs help in the identification step. Silver-ion TLC and GC were revealed as powerful techniques as tools for identification purposes. Although silver-ion TLC is a

complicated technique to carry out, the separation of only four bands by silver-ion TLC was enough to permit the identification. As we did so with fish oil, we believe this method would be very helpful for the analysis of any other complex mixture of TAGs.

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