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L. Cossignani · M. S. Simonetti · A. Neri
P. Damiani

Structural analysis of triacylglycerol fraction and some its sub-fractions from an eicosapentaenoic acid/docosahexaenoic acid dietary supplement

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Abstract Ag^+- and RP-HPLC have been used to study the triacylglycerol fraction of a dietary supplement containing fish oil. The fatty acid (FA) distribution in the glycerol backbone of the triacylglycerol fraction was determined by stereospecific analysis. Eicosapentaenoic acid, the most abundant poly unsaturated FA, was predominantly located in the *sn*-3 position, while docosahexaenoic and docosapentaenoic acids were generally located in the *sn*-2 position. The nutritional significance of these findings is briefly discussed.

Key words Triacylglycerols · Silver-ion and reverse-phase high-performance liquid chromatography · Structural analysis · Eicosapentaenoic acid · Docosahexaenoic acid

Introduction

The effects of the introduction of poly unsaturated fatty acids *n*-3 (PUFA *n*-3) into the human diet have been investigated [1] for several years. PUFA *n*-3 are modulators of the metabolism of arachidonic acid to eicosanoids [2] and they are important for the normal growth of the retina and brain [3]. Many studies on hyperlipidemic patients have shown that the consumption of PUFA *n*-3 protects then against cardiovascular diseases and hypertension in several ways [1, 4]. Dietary saturated fatty acids (FA) (especially palmitic acid) and cholesterol accelerate atherogenesis, whereas mono unsaturated FA and PUFA *n*-3 reduce the levels of plasmatic lipoproteins (i.e. very low-density lipoproteins and low-density lipoproteins) and cholesterol [4–6]. Many studies have demonstrated that dietary changes can be related to a rapid change in the FA composition of plasma lipids [6, 7]. Among foods, the lipid fraction of fish is rich in PUFA *n*-3 as eicosapentaenoic acid (EPA, C20:5 *n*-3) and docosahexaenoic acid (DHA, C22:6 *n*-3); for these reasons dietary supplements based on fish oil are commonly used.

The lipid fraction of fish oil is also characterized by various FA which differ in terms of length of C chain, and number and position of double bonds. This leads to the presence of a great number of molecular species of triacylglycerols (TAG).

The FA composition of TAG (% mol of total lipid fraction) of fish oil has been determined by Ag^+-HPLC [8]. Some authors [9] have combined Ag^+-HPLC, to obtain TAG separation according to the degree of unsaturation, with RP-HPLC in which the retention parameters of TAG molecular species, depending on chainlength and/or on total number of double bonds, have been evaluated in terms of partition number [10], equivalent C number [11–14] and other analogous algorithms such as selectivity [15].

Since dietary lipids are absorbed mainly as free FA or as *sn*-2 monoacylglycerols resulting from the action of lipases, the position occupied by FA in the glycerolic backbone is important for physiological and nutritional reasons; consequently, stereospecific analysis is also an important tool with which to establish the nutritional quality of TAG [16, 17].

In this work the stereospecific analysis of a fish oil dietary supplement was carried out to determine the distribution of FA (especially PUFA *n*-3) in the TAG glycerolic backbone. Ag^+-HPLC analysis of TAG was achieved and the most abundant sub-fractions obtained were submitted to RP-HPLC to obtain the required information.

Materials and methods

Materials. The dietary supplement was refined fish oil concentrate.

Sample preparation. The TAG fraction was isolated from the dietary integrator by TLC (silica gel plates, 0.25 mm, 20 cm × 20 cm)

L. Cossignani · M. S. Simonetti · A. Neri · P. Damiani (✉)
Istituto di Chimica Bromatologica, Università degli Studi,
Via S. Costanzo, P. O. Box 346, I-06100 Perugia, Italy
e-mail: dapi@unipg.it

using petroleum ether/diethyl ether/formic acid (70/30/1, v/v/v) as eluent, with subsequent extraction of the relevant band with hexane/diethyl ether (50/50, v/v). After centrifugation, solution recovery and solvent evaporation, the recovered TAG were dissolved in hexane (approximately 0.5 ml) and stored at low temperature (approximately 4 °C), with addition of buthylhydroxytoluene as antioxidant.

The TAG were dissolved in 1,2-dichloroethane (0.5/0.6 ml) before injection for Ag^+-HPLC analysis.

Apparatus and conditions. The LC apparatus consisted of a pump (model 9012; Varian, Walnut Creek, Calif.), a sample injection valve (model 7125; Rheodyne, Cotati, Calif.) with a 20-μl loop, a Chromospher 5 Lipid column (250 mm × 4.6 mm, 5 μm; Chromopack, Middelburg, The Netherlands); for the Ag^+-HPLC, a DDL 21 light-scattering detector (LSD; Cunow, Cergy St. Christophe, France) and a Hewlett-Packard HP 3394 integrator (Palo Alto, Calif.) were used.

The analysis was carried out as already described [9], using the following eluents and solvent gradient: solvent A, 1,2-dichloroethane/dichloromethane (1/1, v/v); solvent B, acetone; solvent C, acetone/acetonitrile (2/1, v/v). The best gradient conditions were: from 100% A to 50% A, 50% B over 10 min; to 70% B, 30% C over 30 min; to 50% B, 50% C over 60 min and then to 100% C over a further 20 min. The flow rate was 1.0 ml/min and the LSD was set-up with an evaporator temperature of 30 °C, N-inlet pressure of 60 psi and photomultiplier voltage of 600 V. The eluate from the Ag^+-HPLC column was split using a back-pressure regulator as splitting system (Alltech, Deerfield, Ill.) so as to recover enough sample for subsequent HRGC and RP-HPLC analyses.

The HRGC analysis was carried out on the methyl esters of FA (FAME) obtained by transmethylation [18] of each sub-fraction, to which a suitable amount of internal standard (nonadecanoic acid methyl ester had been added). The Ag^+-HPLC separation and the 15 sub-fractions obtained from the TAG are given in Fig. 1.

For the RP-HPLC analysis of the main TAG sub-fractions obtained by Ag^+-HPLC, the same pump, splitting system and detector were used. The column was an endcapped Superspher 100 RP-18 (5 μm; Merck, Darmstadt, Germany) and the analysis was carried out using the following eluents and gradient: solvent A, acetonitrile/isoctane (90/10, v/v); solvent B, acetonitrile/ethanol/isoctane (40/35/20, v/v/v). The best gradient conditions were: from 100% A to 100% B over 120 min, and the LSD was set-up with an evaporator temperature of 40 °C, N-inlet pressure of 60 psi and photomultiplier voltage of 600 V. The splitting system was set to recover enough amount from each peak for subsequent HRGC analysis.

All the solvents used were HPLC grade.

The HRGC quantitative analysis of constituent FA was carried out on FAME obtained by transmethylation of each peak collected from the different TAG subfractions.

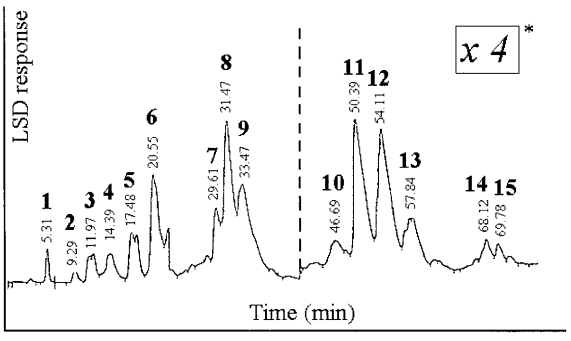

Fig. 1 Ag^+-HPLC profile of the dietary supplement – separation of triacylglycerol subfractions. *On the right of the dashed line* attenuation is 4 times that on the left

The HRGC of FAME was always carried out using a 9001 GC (Chrompack), supplied with a split/splittless injection system, a CP-Wax 58 CB (Chrompack) capillary column (25 m × 0.25 mm i.d., 0.2 μm), and a flame-ionization detector. This apparatus was interfaced with a PC, with Mosaic software for the integration and plots. The column temperature program was: 2 min at 120 °C, then to 240 °C at 3 °C/min, held at the final temperature for 3 min. The carrier gas was He, flow rate 1.8 ml/min.

Stereospecific analysis of TAG. *sn*-1,3/*sn*-1,2(2,3)-Diacylglycerols (DAG) were prepared by partial chemical deacylation using ethyl magnesium bromide in anhydrous ethyl ether. *sn*-1,3-DAG were separated from *sn*-1,2(2,3)-DAG by TLC. This was followed by synthesis of *sn*-1,2(2,3) phosphatidylcholines PC, then enzymatic incubation with phospholipase A_2 and isolation of *sn*-1-lysophosphatidylcholines (*sn*-1-LP) and free FA (FFA) by TLC [18–20]. HRGC analysis of constituent FA of TAG and *sn*-1-LP as FAME as well as of FFA methylated by diazomethane was carried out.

Results and discussion

Due to the large number of TAG molecular species of the lipid fraction of fish oil, their separation posed a problem. The complexity of FA in the TAG fraction of the dietary supplement examined is shown in Table 1 (column I). This data is compared with the combined data describing the FA composition (column II), obtained from the sum of each FA (% mol) in the TAG sub-fractions separated by Ag^+-HPLC (determined by quantifying the FAME by using the internal standard

Table 1 Comparison between the fatty acid composition (%) determined by direct analysis of the triacylglycerol fraction (*I*) and by combined data of the fatty acid composition (%) of the 15 triacylglycerol sub-fractions (*II*). *PUFA n-3* Polyunsaturated fatty acids *n*-3

Fatty acid	I Fatty acid content of triacylglycerols (%)	II Fatty acid content of triacylglycerols (combined data, %)
C14:0	8.3	9.7
C14:1 (*n*-5)	0.7	0.4
C16:0	17.7	19.8
C16:1 (*n*-9 + *n*-7)	8.8	9.4
C18:0	3.6	3.9
C18:1 (*n*-9 + *n*-7)	14.6	19.1
C18:2 (*n*-6)	1.5	2.8
C20:0	0.4	0.3
C18:3 (*n*-6)	0.2	0.1
C18:3 (*n*-3)	0.7	0.9
C20:1 (*n*-9)	1.7	1.3
C18:4 (*n*-3)	2.7	1.8
C20:3 (*n*-6)	0.2	0.1
C22:1 (*n*-9)	0.8	0.8
C20:3 (*n*-3)	0.0	0.0
C20:4 (*n*-6)	0.3	0.2
C20:4 (*n*-3)	0.8	0.6
C20:5 (*n*-3)	19.6	17.1
C21:5 (*n*-3)	0.5	0.4
C22:4 (*n*-6)	0.7	0.2
C22:5 (*n*-6)	0.5	0.2
C22:5 (*n*-3)	2.9	2.1
C22:6 (*n*-3)	12.9	9.3
PUFA *n*-3	40.3	32.3

Table 2 Fatty acid composition (%) of triacylglycerol sub-fractions separated by Ag^+-HPLC

Fatty acid	Sub-fractions														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C14:0	14.2	9.3	5.2	4.5	10.7	9.1	15.5	10.2	8.4	7.4	6.6	3.3	3.4	0.7	1.2
C14:1 (*n*-5)	0.2	2.5	0.8	0.6	1.0	0.8	0.2	0.2	0.2	0.2	0.3	0.1	0.4	0.0	0.0
C16:0	26.8	18.9	17.2	15.8	22.6	22.6	29.4	22.4	16.5	17.2	15.9	11.0	10.0	3.5	5.9
C16:1 (*n*-9+*n*-7)	12.1	24.0	6.8	15.8	13.2	10.7	5.7	10.8	14.2	5.0	3.2	5.6	6.8	0.5	0.2
C18:0	5.0	3.2	9.1	4.0	4.1	5.3	5.2	4.1	2.9	3.4	2.8	2.9	3.0	1.2	1.7
C18:1 (*n*-9+*n*-7)	35.5	36.2	36.6	44.5	21.8	21.7	9.9	16.0	18.9	9.1	17.6	14.3	12.6	5.9	5.2
C18:2 (*n*-6)	2.9	1.3	6.1	7.8	10.2	14.1	0.9	0.8	2.4	1.2	1.8	1.1	3.3	2.1	1.2
C20:0	0.4	0.0	2.2	0.6	0.4	0.0	0.2	0.2	0.2	0.2	0.0	0.1	0.2	0.0	0.0
C18:3 (*n*-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.0	0.0	0.0	0.1	0.0	0.0
C18:3 (*n*-3)	0.4	0.0	3.2	0.8	10.5	3.5	0.2	0.2	0.4	0.5	0.5	0.0	0.4	0.0	0.0
C20:1 (*n*-9)	1.8	3.1	4.2	2.6	2.5	1.5	1.6	1.5	2.4	0.4	0.0	0.5	0.4	0.0	0.0
C18:4 (*n*-3)	0.0	0.6	3.8	1.3	1.0	0.0	1.9	0.6	0.0	4.6	3.6	1.4	1.5	5.0	2.5
C20:3 (*n*-6)	0.0	0.0	0.0	0.1	0.0	0.8	0.0	0.0	0.1	0.2	0.0	0.4	0.0	0.0	0.0
C22:1 (*n*-9)	0.8	0.8	4.7	1.0	0.4	1.0	0.9	0.8	1.2	0.4	0.0	0.5	0.2	0.0	0.0
C20:3 (*n*-3)	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C20:4 (*n*-6)	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.1	0.0	1.1	0.7	0.0	0.4	0.9	0.0
C20:4 (*n*-3)	0.0	0.0	0.0	0.0	1.0	0.3	0.7	0.2	0.2	1.4	0.9	2.2	0.6	1.4	0.6
C20:5 (*n*-3)	0.0	0.0	0.0	0.0	0.3	1.8	25.1	18.0	8.5	33.2	34.3	27.5	17.7	55.5	43.4
C21:5 (*n*-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.8	0.4	1.1	0.0	1.0	0.6	0.0	0.0
C22:4 (*n*-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	1.1	0.0	0.0	1.8	1.3
C22:5 (*n*-6)	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.1	0.7	0.5	0.0	0.2	0.0	0.0
C22:5 (*n*-3)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8	2.0	4.5	4.3	5.7	2.3	7.7	3.2
C22:6 (*n*-3)	0.0	0.0	0.0	0.0	0.4	6.9	0.9	10.0	20.6	8.3	6.0	22.5	36.0	13.6	33.5
Sub-fraction (%)	5	3	7	5	3	3	10	17	10	6	13	9	6	2	1

Table 3 Content (%) of iso unsaturated fatty acid groups (where *S, M, D, T, Q, P* and *E* represent the groups of fatty acids with 1, 2, 3, 4, 5 and 6 double bonds, respectively) and calculated unsaturation degree (*CUD*) of triacylglycerol sub-fractions separated by Ag^+-HPLC

Iso unsaturated fatty acid groups	Sub-fractions														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S	48.7	33.2	35.4	26.3	40.0	39.8	54.0	40.4	31.2	31.5	28.4	19.8	19.1	6.3	10.4
M	48.2	65.0	51.6	63.3	37.9	34.6	17.8	29.5	37.8	15.6	21.6	22.3	21.9	6.9	5.8
D	2.7	1.3	6.0	7.6	9.7	13.7	0.9	0.8	2.4	1.2	1.8	1.1	3.4	2.3	1.3
T	0.4	0.0	3.2	1.3	10.0	4.2	0.3	0.3	0.7	0.7	0.5	0.4	0.6	0.0	0.0
Q	0.0	0.5	3.8	1.5	1.8	0.3	2.4	1.0	0.2	7.0	6.1	3.6	2.5	9.4	4.6
P	0.0	0.0	0.0	0.0	0.3	1.6	23.7	19.5	10.1	36.7	36.4	32.6	20.1	62.5	46.7
E	0.0	0.0	0.0	0.0	0.3	5.8	0.8	8.5	17.7	7.2	5.2	20.2	32.4	12.5	31.2
CUD[a]	1.6	2.1	2.7	2.7	2.9	3.6	4.6	5.5	6.1	8.3	7.9	9.7	10.1	13.1	13.4

[a] $CUD = (M + 2 \times D + 3 \times T + 4 \times Q + 5 \times P + 6 \times E)/33.33$

in all the TAG sub-fractions). The comparison showed a satisfactory agreement between the data, inspite of the critical Ag^+-HPLC separation due to matrix complexity. The results showed relatively high levels of PUFA *n*-3 (approximately 40% of total FA), in particular those of EPA and DHA (ratio EPA/DHA around 1.5). The most abundant and/or best separated sub-fractions are presented by peaks 7, 8, 9, 10, 11, 12, 13 and 14, with a maximum retention time of 70 min, containing higher amounts of both EPA and DHA in the TAG backbone (Fig. 1, Table 2).

The percentages of iso unsaturated FA in the TAG sub-fractions obtained by Ag^+-HPLC are reported in Table 3, together with the calculated unsaturation degree (CUD) for each TAG sub-fraction. This parameter (values as given in Table 3) is an index of the efficiency of the chromatographic separation. The obtained values for CUD increased as they should, but were often not whole numbers, and were sometimes very similar for neighbouring sub-fractions. These results are due to the fact that many molecular species in the TAG fraction, even if with different degrees of unsaturation, produced several chromatographic overlaps. For this reason, RP-HPLC analysis of TAG sub-fractions is very important when defining their component molecular species. The RP-HPLC chromatograms of the TAG sub-fractions showed good separation of peaks (Fig. 2). From the FA percentage composition of the main RP-HPLC peaks, it was possible to identify and to quantify with satisfactory precision the TAG

Table 4 Tentative attribution of triacylglycerol molecular species to the main peaks obtained by RP-HPLC of each triacylglycerol sub-fraction (peak nos. *7, 9, 13, 14* in Fig. 2), by their fatty acid composition (%). *M* miristic acid (C14:0), *P* palmitic acid (C16:0), P_o palmitoleic acid (C16:1), *S* stearic acid (C18:0), *O* oleic acid (C18:1), *EPA* eicosapentaenoic acid (C20:5 *n*-3), *DPA* docosapentaenoic acid (C22:5 *n*-6), *DHA* docosahexaenoic acid (C22:6 *n*-3), *L* linoleic acid (C18:2 *n*-6), *T* linolenic acid (C18:3 *n*-6), *Q* octadicatetraenoic acid (C18:4 *n*-3), *Eq* docosatetraenoic acid (C20:4 *n*-3)

Fatty acid	Sub-fraction 7		
	Peak 2	Peak 5	Peak 8
M	47.7	24.3	6.2
P	6.1	31.9	52.7
Po	0.0	1.7	0.0
S	0.0	1.0	6.3
O	5.4	3.5	3.9
EPA	34.3	23.1	29.5
DPA	0.0	2.6	0.0
DHA	6.6	3.3	1.4
Attribution	M:M:EPA	M:P:EPA	P:P:EPA
Double bonds	5	5	5

Fatty acid	Sub-fraction 9			
	Peak 2	Peak 5	Peak 7	Peak 11
M	6.1	3.2	5.3	2.2
P	7.9	9.5	12.5	22.2
P	36.6	20.0	10.2	6.4
S	1.9	2.5	1.7	3.9
O	9.0	26.5	11.6	21.2
L	4.1	2.9	0.6	0.2
T	1.6	1.2	0.8	0.8
Q	1.5	1.1	1.8	2.9
EPA	25.7	24.1	0.7	0.5
DPA	0.3	1.4	1.4	0.3
DHA	4.0	6.3	48.2	28.4
Attribution	Po:Po:EPA	Po:O:EPA	P:Po:DHA	P:O:DHA
Double bonds	7	7	7	7

Fatty acid	Sub-fraction 13				
	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
M	1.1	12.5	0.4	0.0	2.0
P	2.9	9.9	3.0	32.3	7.2
Po	25.3	5.1	0.0	0.0	3.0
S	0.9	2.7	0.8	0.0	24.9
O	4.0	6.2	33.1	1.2	8.2
EPA	32.7	2.4	27.1	0.0	0.0
DPA	1.0	1.3	2.7	3.6	1.6
DHA	32.2	53.0	32.9	62.9	53.2
Attribution	Po:EPA:DHA	M:DHA:DHA	O:EPA:DHA	P:DHA:DHA	S:DHA:DHA
Double bonds	12	12	12	12	12

Fatty acid	Sub-fraction 14	
	Peak 1	Peak 2
M	0.9	1.6
P	2.5	6.2
Po	0.7	1.4
S	1.1	1.5
O	2.8	7.7
Q	7.5	2.1
Eq	1.0	6.4
EPA	64.8	38.9
DPA	0.9	19.4
DHA	15.2	12.5
Attribution	EPA:EPA:DHA	EPA:DPA:DHA
Double bonds	16	16

molecular species that gave each RP-HPLC peak in each sub-fraction. As examples, the tentative identification of some molecular species of four sub-fractions (peak numbers 7, 9, 13 and 14) is reported in Table 4, even if these molecular species were not always abundant or well separated. To verify the structures attributed to each peak, the molecular species were also identified by "1-random, 2-random, 3-random distribu-

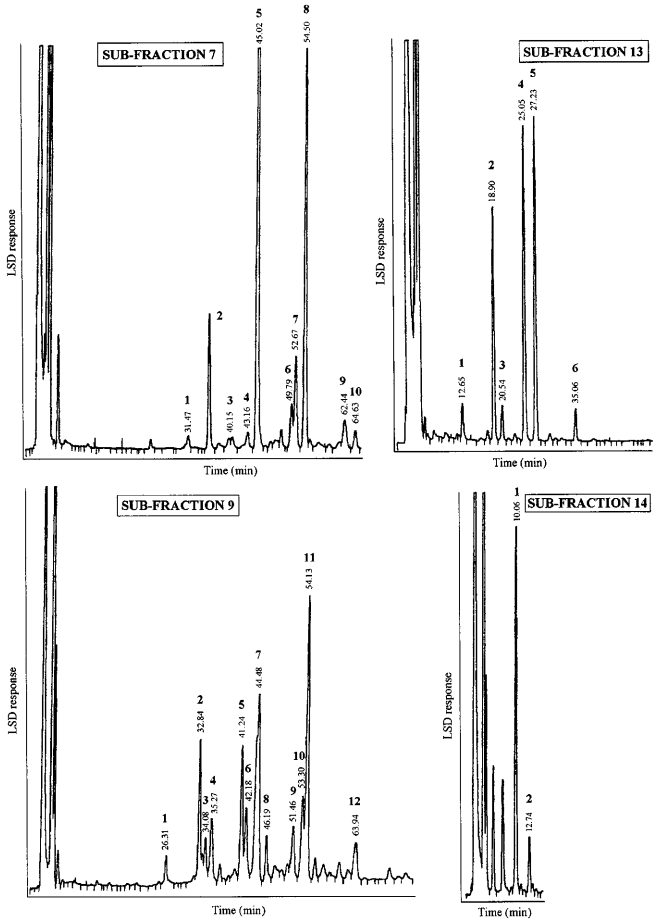

Fig. 2 RP-HPLC profiles of triacylglycerol molecular species from the four sub-fractions examined. *LSD* Light scattering detector

Table 5 Stereospecific analysis of the triacylglycerol fraction of dietary supplement

Fatty acid	*sn*-1	*sn*-2	*sn*-3
C14:0	6.6	12.0	6.2
C14:1 (*n*-5)	0.8	0.8	0.4
C16:0	22.4	20.8	10.0
C16:1 (*n*-9+*n*-7)	11.0	9.0	6.6
C18:0	7.4	2.9	0.6
C18:1 (*n*-9+*n*-7)	24.3	7.1	12.4
C18:2 (*n*-6)	2.0	1.3	1.1
C20:0	0.3	0.4	0.4
C18:3 (*n*-6)	0.3	0.3	0.1
C18:3 (*n*-3)	1.1	0.5	1.1
C20:1 (*n*-9)	1.5	0.9	1.9
C18:4 (*n*-3)	2.8	2.4	2.8
C20:3 (*n*-6)	0.2	0.2	0.2
C22:1 (*n*-9)	0.0	1.1	0.0
C20:4 (*n*-6)	0.6	0.0	0.3
C20:4 (*n*-3)	1.3	0.5	0.7
C20:5 (*n*-3)	14.3	12.3	32.3
C21:5 (*n*-3)	0.6	0.4	0.6
C22:4 (*n*-6)	0.5	0.6	1.1
C22:5 (*n*-6)	0.0	0.6	1.0
C22:5 (*n*-3)	0.8	5.3	2.6
C22:6 (*n*-3)	1.5	21.0	16.2
PUFA *n*-3 (%)	22.4	42.4	56.3

tion" theory, using the results of the stereospecific analysis of the TAG fraction reported in Table 5. From these data, the distribution of FA amongst the three positions of the glycerolic backbone was obtained. The saturated FA generally were located in *sn*-1 and *sn*-3 positions, as expected, whereas the percentages of PUFA *n*-3 showed that *sn*-3 and *sn*-2 were the preferential positions of these compounds. EPA, the most abundant PUFA *n*-3, was situated much more frequently in the *sn*-3 position, while DHA and DPA docosapentaenoic acid (C22:5 *n*-3) were generally found in the *sn*-2 position.

All these observations, obtained by stereospecific analysis of TAG, confirm the importance of the intramolecular TAG structure on the bioavailability, lymphatic transfer and incorporation of these PUFA *n*-3 and their preferential and rapid supply to plasma TAG and phospholipids [17, 21].

The next phase of this research will involve the stereospecific analysis of each TAG subfraction in order to determine the positions of PUFA *n*-3. The outcome of this research could possibly be used to improve technological processes for the enrichment and/or fractionation of these, or similar, products.

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