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ORIGINAL PAPER

^{13}C-NMR Regioisomeric Analysis of EPA and DHA in Fish Oil Derived Triacylglycerol Concentrates

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Abstract The regio-isomeric distribution of the omega-3 polyunsaturated fatty acids (PUFA) *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) in the triacylglycerols (TAG) of anchovy/sardine fish oil was determined by ^{13}C nuclear magnetic resonance (NMR) analysis under quantitative conditions. From the measurements of *sn*-1,3 and *sn*-2 carbonyl peak areas it was established that EPA was mainly located in the *sn*-1,3 positions, whereas DHA primarily occupied the *sn*-2 position. Reconstituted TAG prepared by *Candida antarctica* lipase-B (CALB) glycerolysis of the ethyl ester (EE) or the free fatty acid (FFA) forms of anchovy/sardine fish oil, displayed a different pattern: EPA was equally distributed, while DHA was preferentially attached to the *sn*-1,3 positions. TAG concentrates of varying EPA and DHA molar fractions were prepared by CALB-catalyzed glycerolysis of the corresponding EE and FFA. ^{13}C-NMR analysis of the purified products revealed a lack of CALB regioselectivity for EPA and a slight *sn*-1,3 regioselectivity for DHA. Since this pattern was observed in all cases of this study, it was concluded that the lipase regioselectivity during TAG synthesis is independent of both the acyl donor type (carboxylic acid or ester) and the fatty acid content of the oil substrate.

Keywords ^{13}C NMR · Regioselectivity · Regio-isomeric distribution · Triacylglycerol · Anchovy · Sardine · Fish oil concentrate · EPA · DHA

Introduction

The health benefits of consuming omega-3 polyunsaturated fatty acids (PUFA), principally *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), are well documented [1, 2]. As a result, omega-3 PUFA have become a mainstream nutritional supplement for people actively working on their health, particularly those trying to prevent cardiovascular disease [1], boost their mental capacity, or slow down their cognitive decline [2].

Concentrated forms of omega-3 PUFA are sold globally as health supplements; most are sold as fatty acid ethyl esters (EE). Some of studies suggest EE are more susceptible to oxidation [3, 4] and are metabolized at slower rate than their triacylglycerol (TAG) counterparts [5, 6]. Although, strong data supporting these claims are not available, many customers require TG forms for food or dietary supplement use, mainly due to regulatory differences.

From a technological standpoint, the conversion of fish oil to EE followed by short path distillation or rectification is an effective strategy for the concentration of omega-3 PUFAs. Transformation of the concentrated EE back into TAG requires an additional glycerolysis step. Lipases are efficient catalysts to transform between such fatty acid types. *Candida antarctica* lipase-B (CALB; EC 3.1.1.3)

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shows low selectivity among fatty acid types, unsaturation, or chain length when catalyzing glycerolysis under anhydrous, solvent-free conditions at elevated temperatures. This is important for the preparation of TAG having the same fatty acid composition as the starting ethyl esters or free fatty acids [7].

The regio-isomeric distribution of omega-3 PUFA in TAG prepared by enzymatic glycerolysis of EE or FFA concentrates is an important structural parameter to accurately characterize [8–13]. The regio-isomeric distribution is influenced by the enzyme's fatty acid glycerol regioselectivity and substrate fatty acid composition, and may differ from the starting fish oils.

Indirect analysis of regio-isomeric distribution in TAG using chromatographic techniques often gives erroneous measurements. Chemical or enzymatic hydrolyses [14–17] are complicated by acyl migration, incomplete reactions, or selectivity issues [18, 19]. Because of its 1,3-regioselectivity, pancreatic lipase is often used for analysis of oils. However, pancreatic lipase cannot be used with fish oils because it also has fatty acid selectivity, leading to hydrolytic products still containing PUFA in the *sn*-1,3 positions [20, 21]. The official IUPAC method for the enzymatic analysis reports that it is not applicable to marine oils [22].

Direct analysis using spectroscopic methods, in particular high resolution ^{13}C nuclear magnetic resonance (NMR), has emerged as a versatile technique for the analysis of fish lipids. Research by Gunstone [23, 24] and Aursand [25] first established the relationship between omega-3 PUFA structure and chemical shifts.

This relationship allowed characterization by ^{13}C NMR of the omega-3 PUFA regio-isomeric distribution in oils from a number of fish species [23–29]. Other applications include discrimination of fish oils and commercial encapsulated marine oil supplements according to PUFA composition, to degree of refinement and adulteration [30], as well as to geographical and wild/farmed origin [31]. Authentication of fish oils is possible based on differences in DHA *sn*-2 content estimated by ^{13}C-NMR analyses [32]. Researchers have also used a combination of ^{13}C-NMR, ^{1}H-NMR and 2D-NMR techniques to detect and identify a wide range of compounds in commercially available marine oil supplements [33].

In this publication, we examined the regio-isomeric distribution of EPA and DHA in reconstituted TAG prepared by enzymatic glycerolysis of the EE and FFA forms of anchovy/sardine fish oil. We also examined the region-isomeric distribution of EPA and DHA in TAG concentrates obtained upon CALB-catalyzed glycerolysis of EE and FFA concentrates of varying EPA and DHA composition. This study establishes the effect of both the acyl donor type (carboxylic acid or ester) and the EPA/DHA composition of the oil on the enzyme glycerol regioselectivity for these acids during TAG synthesis. To our knowledge, such data have not been previously reported.

Experimental Procedures

Fish oil ethyl esters 2009EE, 4329EE, 6003EE and 0453EE (first two digits refer to the content of EPA and the last two digits refer to the content of DHA, both in gas chromatography (GC) peak area percentages) were manufactured by Ocean Nutrition Canada Limited (Mulgrave, NS, Canada). The corresponding free fatty acid forms 2009FFA, 4329FFA, 6003FFA and 0453FFA, were prepared according to a standard internal protocol. Novozym435, immobilized CALB (10,000 PLU/g) on a Lewatit VPOC 1600 matrix were purchased from Novozymes (Franklinton, NC, USA).

Thin layer chromatography (TLC) was conducted on silica gel-coated aluminium- backed TLC plates (0.25 mm thickness) containing F-254 UV indicator (Silicycle Inc., Quebec City, PQ, Canada) and developed in mixtures of 90/10/1 (v/v) hexanes/ethyl acetate/acetic acid or 70/30/1 (v/v) hexanes/ethyl ether/acetic acid. Spots were visualized by staining with an ethanolic solution of phosphomolybdic acid (20% wt.).

Flash chromatography was conducted on silica gel (particle size 40–63 μm; Silicycle Inc., Quebec City, PQ, Canada) using a 24/40 cm glass column (ChemGlass Life Sciences, Vineland, NJ, USA) equipped with a 250 ml solvent reservoir. TAG prepared by enzymatic glycerolysis of FFA were isolated in one step by eluting with a 90/10 (v/v) hexanes/ethyl acetate mixture. TAG prepared by glycerolysis of EE were purified using the same solvent mixture following an initial elution of the residual EE with a 98/2 (v/v) hexanes/ethyl acetate mixture.

Solvents were purchased from Caledon Laboratories Ltd. (Georgetown, ON, Canada). Deuterated chloroform (99.8%) for NMR analyses was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents, with the exception of GC standard mixtures and homo-TAG standards for ^{13}C-NMR analysis were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standard mixtures of fatty acid methyl esters (FAME) and ethyl esters (FAEE) for GC analyses, and homo-triacyl glycerol standards for ^{13}C-NMR analysis were purchased from Nu-Check Prep, Inc. (Elysian, MN, USA). The latter were: tripalmitin (C16:0), tripalmitolein (C16:1ω-7, Δ9), triolein (C18:1ω-9, Δ9), trieicosatrienoin (C20:3ω-6, Δ8), trigammalinolein (C18:3ω-6, Δ6), trieicosapentaenoin (C20:5ω-3, Δ5) and tridocosahexaenoin (C22:6ω-3, Δ4), with Δ referring to the position of the first double bond relative to the carbonyl center (C-1). Tri-docosapentaenoin (C22:5ω-3, Δ7) was synthesized by a procedure developed in-house.

Glycerolysis of Ethyl Esters and Free Fatty Acids to Produce Reconstituted Triacylglycerols

Fish oil EE and FFA were mixed in separate vessels (250-ml round bottom flasks) with glycerol and Novozym435 (4% wt. based on the starting oil weight) and the mixture stirred under vacuum (0.1 torr) at elevated temperature. The molar ratios of reactants specific to each reaction are given in Table 1. Glycerolysis of FFA was conducted at 70 °C for a 24 h period. Glycerolysis of EE was conducted at 85 °C and extended for 48 h. Progress of reactions was monitored by TLC and by lipid class analysis (see below). The immobilized enzyme was filtered off and the TAG then purified by flash chromatography.

Fatty Acid Composition Analysis

The fatty acid profile of pure reconstituted TAG was determined by gas chromatography analysis of the corresponding FAME according to the procedure described [34], and expressed as molar percentage. TAG were transesterified into methyl esters in the presence of BCl_3/CH_3OH and analyzed on an Agilent 6890 gas chromatograph equipped with a computer workstation (Chemstation B.04.01 SP1-647 version software), SP 2560 glass capillary column (100 m length), split injection (50:1) and flame ionization detection. The GC oven was programmed from an initial 160 °C (held 3 min) to 200 °C at 1.25 °C/min rate, maintained for 5 min at this temperature and increased to 230 °C at 2.5 °C/min. The individual FAME were identified by comparison with the retention times of standard mixtures. The fatty acid profile of fish oil EE was determined by direct GC analysis of the FAEE and expressed as molar fractions.

Table 1 Molar ratios of reactants required for completion of glycerolysis reactions, yield and purity of the reconstituted triacylglycerols

Comp.[a]	Molar excess of oil (%)[b]		Y/P (%)[c]	
	EE → TAG	FFA → TAG	EE → TAG	FFA → TAG
2009	0	5	84.3/99.9	90.3/99.1
4329	35	25	68.4/99.1	82.7/98.4
6003	35	5	76.3/99.2	91.1/99.2
0453	85	40	88.2/99.4	87.0/98.9

Purity (P) is based on area % of TAG *sn*-2 glycerol relative to the total area of *sn*-2 glycerol peaks, calculated from the glycerol region of the ^{13}C-NMR spectrum of purified triacylglycerols

[a] Composition: the first two digits refer to the content of EPA and the last two digits refer to the content of DHA, both in area percentages

[b] Refers to the % excess relative to equimolar amounts (no excess) that would be 3:1 oil/glycerol

[c] The yield (Y) is based on the area % of the TAG peak calculated from lipid class analysis of the final reaction mixture

Lipid Class Analysis

Analysis of the composition of non-polar lipids was employed to monitor the formation of TAG during the course of enzymatic glycerolysis. The different classes of compounds were separated using a size exclusion chromatography (SEC-HPLC) based methodology employing a series of three TSKgel-G1000Hxl columns (Tosoh Biosciences LLC, Montgomeryville, PA, USA) with internal diameter 7.8 mm, 30 cm length and 5 μm particle size. TAG, diacylglycerols (DAGs), monoacylglycerols (MAGs), EE and FFA were separated using an isocratic elution mode (0.6 ml/min flow rate) in tetrahydrofuran at 30 °C. The elution was monitored by refractive index detection. Identification was performed by comparison with the retention times of in-house developed standards.

^{13}C-NMR Spectroscopy

Quantitative broad band ^{13}C-NMR spectra of mixtures of fish oils and TAG concentrates were recorded under continuous ^{1}H decoupling at 20 °C in a Bruker AV-III 700 MHz spectrometer, equipped with a 5 mm TCI cryoprobe using the standard TopSpin V 2.1 (Bruker Biopin Canada, Milton, ON, Canada) software. The spectrometer was located at the Biomolecular Magnetic Resonance Facility at the National Research Council of Canada, Institute for Marine Biosciences (Halifax, NS, Canada). The data were acquired at a ^{13}C frequency of 176.09 MHz using the following acquisition parameters: 128 k complex data points, spectral width of 41,666 Hz (237 ppm), pulse width 90°, acquisition time 1.573 s, and collection of 256 scans. A repetition time of 16.6 s was employed, of which 1.6 s corresponded to acquisition time and the remaining 15 s to relaxation delay. Prior to Fourier transformation, all free induction decays (FID) were zero filled to 128 k real data points and apodized using exponential multiplication (0.3–0.7 Hz line broadening) for sensitivity enhancement. Carbonyl peak integrals were measured accurately using the standard deconvolution algorithm from TopSpin V 2.1 software, assuming a pure Lorentzian lineshape. The spectra of fish oils and pure reconstituted TAG were collected in 200 mg pure samples dissolved in 0.7 ml of 99.8% $CDCl_3$. The spectra of homo-TAG standard mixtures were collected on mixtures containing between 15 and 20 mg of each compound dissolved in 0.7 ml 99.8% $CDCl_3$ using the same experimental conditions, except that the relaxation delay that was adjusted to 0.5 s.

A series of ^{13}C-NMR spectra of natural anchovy/sardine fish oil 2009TAG were collected on increasing relaxation

delay (at constant acquisition time) to obtain the dependency of carbonyl peak area ratios with the repetition time. The spectra were acquired under continuous ^{1}H decoupling at 20 °C at a ^{13}C frequency of 125 MHz a in a Bruker Avance-500 MHz spectrometer on 100 mg sample dissolved in 0.7 ml 99.8% $CDCl_3$. Experimental parameters were: spectral width 29,762 Hz, 128 k complex data points, pulse width 90° and 512 scans. The acquisition time was kept constant at 2.2 s and the relaxation delay adjusted to 0.1, 1, 2, 5, 10 and 20 s. The spectra were Fourier transformed and processed as above.

In all spectra, ^{13}C chemical shifts were expressed in parts per million (ppm) relative to $CDCl_3$ at 77.16 ppm [35].

Results and Discussion

Preparation of Triacylglycerol Concentrates

The FFA forms of fish oil or fish oil concentrates were reconstituted into TAG by glycerolysis in the presence of immobilized CALB. Reconstituted TAG were also synthesized by enzymatic glycerolysis of EE. These processes were slower than the glycerolysis of FFA, requiring higher temperatures (85 °C) and longer time for completion (48 h).

The molar ratios of EE and FFA to glycerol were adjusted in each case to obtain 90% conversion, or higher, of the desired TAG product. In most cases, especially in the glycerolysis of ethyl esters, these levels of TAG conversion could not be achieved. Instead, the ratios were adjusted to minimize the residual DAGs in the final reaction mixture to levels below 10%, at which point the reactions were considered to be complete. For each reaction, the conditions that led to completion are summarized in Table 1.

As shown in Table 1, glycerolysis of the low DHA oils 2009FFA and 6003FFA required only 5% FFA excess for completion; concentrates with higher DHA content (4329FFA and 0453FFA) required 25 and 40% FFA excess, respectively, after 24 h. Glycerolysis of 2009EE came to successful completion after 48 h on stoichiometric amounts of EE and glycerol. Glycerolysis of 4329EE and 6003EE required 35% ethyl esters excess for completion; glycerolysis of the high DHA oil 0453EE required almost double the amount of ethyl esters for completion. The purity of the TAG preparations recovered by flash chromatography of crude reaction mixtures was in all cases 90% and higher (Table 1). High purity levels of TAG minimize the likelihood of erroneous estimation of peak areas from NMR measurements, resulting from overlapping of carbonyl peaks of fatty acid chains esterified as TAG and DAG.

Regio-isomeric Analyses of EPA and DHA

The proportion of EPA and DHA chains attached to the region-isomeric *sn*-1,3 and *sn*-2 positions of reconstituted TAG was calculated from the relative areas of previously assigned carbonyl peaks in broadband decoupled ^{13}C-NMR spectra acquired under quantitative conditions [23–28].

Prior to the analysis, the relationship between fatty acid molecular structure and ^{13}C carbonyl chemical shifts was established from spectral analysis of mixtures of homo-TAG, containing saturated fatty acids and unsaturated fatty acids with specific degree and positions of unsaturation. In agreement with published data [23–28], carbonyl carbons of *sn*-1,3 and *sn*-2 acyl chains were separated by a systematic shift of about 0.4 ppm forming clusters of *sn*-1,3 an *sn*-2 peaks (Fig. 1a, Fig. S-1). In addition, carbonyl chemical shifts of unsaturated chains showed a marked dependency on the proximity to the first double bond [36], shifting to lower frequencies as the first double bonds moved closer to the carbonyl center (lower Δ in Fig. S-1).

In Fig. 1b, the carbonyl region of the broad band decoupled ^{13}C-NMR spectrum of 2009TAG natural anchovy/sardine fish oil is displayed; the top part (Fig. 1a) displays the spectrum of a mixture of eight homo-TAG standards for comparison. Chemical shifts were assigned in the spectrum of the fish oil by singling out pairs of resonances that are split by 0.4 ppm, starting from the highest frequency peak at 173.28 ppm, assigned to saturated chains attached to *sn*-1,3 positions. Each pair of *sn*-1,3/*sn*-2 chains was then assigned to individual fatty acids on the basis of the progressive decreasing shift that is induced on the carbonyl chemical shifts as unsaturation occurs closer to the carbonyl center [23–28, 36]. The assignments are listed in Table 2.

The longitudinal relaxation rates (T_1) of *sn*-1,3 and *sn*-2 carbonyl peaks of EPA and DHA were estimated by the standard inversion recovery sequence on a sample of natural anchovy/sardine 2009TAG fish oil, to establish the proper repetition time to measure peak areas quantitatively from one spectrum simultaneously. These measurements revealed that the equilibrium spin population difference of EPA chains was relaxed faster than for DHA. For EPA, *sn*-2 carbonyl chains displayed longer T_1 (2.835 s) than the *sn*-1,3 carbonyls (2.437 s) by a factor of almost 16%. The relaxation rates of *sn*-1,3 and *sn*-2 DHA carbonyls differed only by 2% (3.231 and 3.303 s, respectively). These values suggested that the proper repetition time for simultaneous quantitative analysis of EPA and DHA carbonyl peaks should be set to 16.5 s, corresponding to five times the longest T_1 (3.303 s) [37].

In a separate set of experiments, the area ratios of carbonyl peaks of EPA and DHA chains was measured as function of the repetition time (at constant acquisition time)

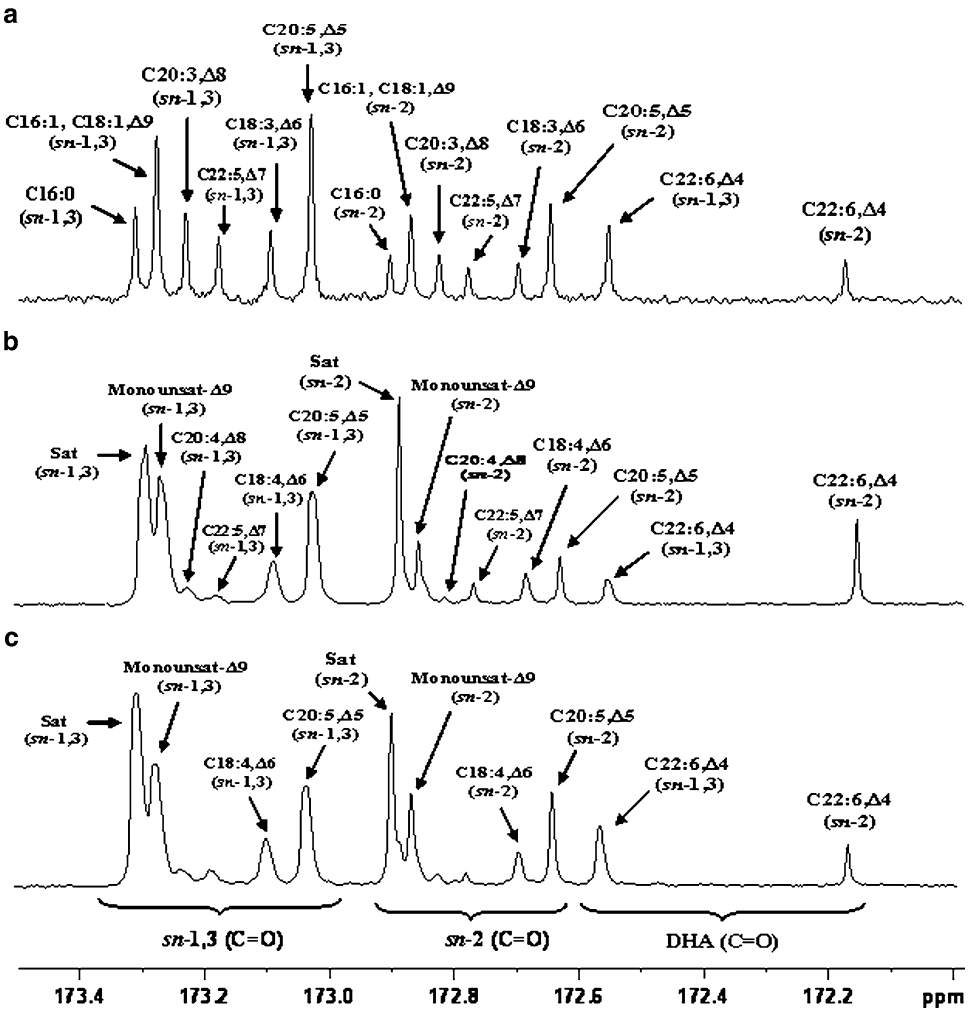

Fig. 1 Carbonyl region of the broad band decoupled ^{13}C-NMR spectrum of: **a** mixture of eight homo-TAG standards; **b** natural anchovy/sardine fish oil 2009TAG; **c** reconstituted 2009TAG obtained upon glycerolysis of 2009FFA. The assignment of *sn*-1,3 and *sn*-2 regioisomeric peaks to individual fatty acids is annotated

using continuous ^{1}H decoupling. These conditions were used based on the finding of Wollenberg [38] and Vlahov [39], that NOE enhancement factors for *sn*-1,3 and *sn*-2 carbonyls in TAG do not differ appreciably. According to Fig. 2, peak areas of DHA carbonyls could be estimated semi-quantitatively at repetition times shorter than 16.5 s, given that the *sn*-1,3/*sn*-2 areas ratio remained invariable on increasing repetition times. Meanwhile, comparison of *sn*-1,3 and *sn*-2 carbonyl peaks of EPA should be made at repetition times longer than 12 s. At lower repetition times, the *sn*-1,3/*sn*-2 peak area ratio for EPA decreased, most likely as a result of incomplete recovery of the excess spin population of *sn*-1,3 chains. From these measurements of relaxation rates, quantitative analysis of EPA carbonyl peaks requires 14.2 s (5 × 2.835 s) repetition time; the areas could be estimated semi-quantitatively at repetition times between 12 and 14.2 s.

The regio-isomeric distribution of the omega-3 fatty acids EPA, DHA and stearidonic acid (STA; C18:4, Δ6) in the TAG of anchovy/sardine fish oil was calculated from the area under the *sn*-1,3 and *sn*-2 peaks of these acids after spectral deconvolution of the carbonyl region of the ^{13}C-NMR spectrum collected using 16.6 s relaxation delay (Table 3). The regio-isomeric distribution was expressed as the percentage of *sn*-1,3 (or *sn*-2) chains over total chains (*sn*-1,3 plus *sn*-2). A random (equal) distribution is defined as a *sn*-1,3 position specificity of 67 mol% and a *sn*-2 position specificity of 33 mol%. The omega-3 fatty acids, C20:4; Δ8 and C22:5; Δ7, were also identified but their regio-isomeric distribution was not reported here.

Our ^{13}C-NMR experiments (Table 3) showed that the omega-3 fatty acids EPA, DHA and STA were assembled differently in the glycerol backbone of anchovy/sardine fish oil TAG. Stearidonic acid chains appeared to be equally distributed, judging from the 66.9 and 33.1 mol% of *sn*-1,3 and *sn*-2 chains, respectively. However, EPA preferentially resided in the *sn*-1,3 positions (80.9%), whereas DHA was preferentially attached to the *sn*-2 position (61.7% mol).

Figure 1c shows the ^{13}C-NMR spectrum of the purified TAG prepared by enzymatic re-esterification with glycerol of FFA from natural 2009TAG anchovy/sardine fish oil.

Table 2 Assignment of carbonyl resonances to individual fatty acids or groups of fatty acids in the ^{13}C-NMR spectra of natural anchovy/sardine fish oil 2009TAG and reconstituted TAG obtained upon glycerolysis of 2009EE and 2009FFA

Fatty acid assignment	Chemical shift (ppm)
Saturated (*sn*-1,3)	173.31
Monounsaturated, Δ9 (*sn*-1,3)	173.28
C20:4, Δ8 (*sn*-1,3)	173.24
C22:5, Δ7 (*sn*-1,3)	173.19
C18:4, Δ6 (*sn*-1,3)	173.10
C20:5, Δ5 (*sn*-1,3)[a]	173.04
Saturated (*sn*-2)	172.90
Monounsaturated, Δ9 (*sn*-2)	172.87
C20:4, Δ8 (*sn*-2)	172.83
C22:5, Δ7 (*sn*-2)	172.78
C18:4, Δ6 (*sn*-2)	172.69
C20:5, Δ5 (*sn*-2)[a]	172.64
C22:6, Δ4 (*sn*-1,3)[a]	172.56
C22:6, Δ4 (*sn*-2)[a]	172.17

[a] These assignments also apply to reconstituted TAG concentrates from 4329, 6003 and 0453EE and FFA

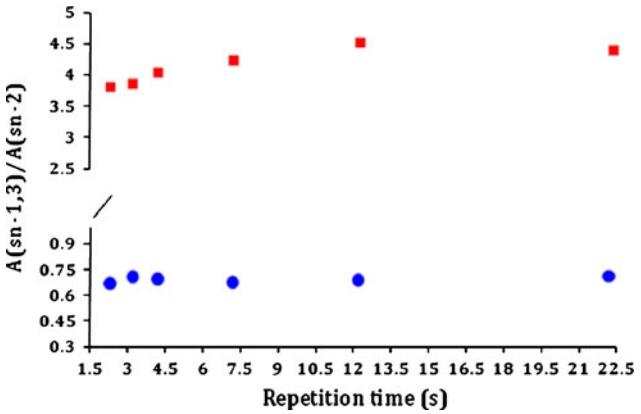

Fig. 2 Variation of carbonyl *sn*-1,3/*sn*-2 peak areas ratios of EPA and DHA in natural anchovy/sardine 2009TAG as a function of the repetition time in a ^{1}H continuous decoupling sequence

Analysis of *sn*-1,3 and *sn*-2 peak areas revealed that on enzymatic re-esterification, EPA and STA residues were equally distributed among *sn*-1,3 and *sn*-2 positions, whereas DHA resided in a slight excess in the *sn*-1,3 positions (70%). Similar results were found for the TAG prepared by direct enzymatic glycerolysis of 2009EE (Table 3).

Enzymatic synthesis of TAG from 2009EE and 4329EE and the corresponding FFA (2009FFA and 4329FFA) resulted in final products with almost identical fatty acid profiles to those of the starting materials, as measured by GC analysis (Tables S-1, S-2). Triacylglycerols prepared by enzymatic esterification of 6003FFA with glycerol displayed a similar fatty acid profile to that of the starting material, whereas the TAG formed on enzymatic glycerolysis of the corresponding ethyl esters (6003EE) contained lower amounts of EPA (48% from original 60%) and higher amounts of C18:1ω-9 (oleic acid; 20% from original 11%) (Table S-3). Glycerolysis of 0453EE and the corresponding 0453FFA concentrate resulted in lower levels of DHA in the TAG than those in the starting materials (37 and 40%, respectively, compared to 53%). The levels of saturates were higher (11%, including 6% of C16:0) than in the starting materials (3%) (Table S-4). This may indicate higher reactivity of shorter and more flexible saturated chains compared to DHA.

The carbonyl region of the ^{13}C-NMR spectra of the purified TAG concentrates prepared by enzymatic esterification of 4329FFA, 6003FFA and 0453 FFA are shown in Fig. 3. Analysis of peak area ratios indicated that in all reconstituted TAG, EPA was equally distributed among *sn*-1,3 (66–68%) and *sn*-2 (32–34%) positions, whereas DHA resided in excess in the *sn*-1,3 positions (% area 70% and higher; Table 3). The NMR spectral analysis of reconstituted TAG prepared by enzymatic glycerolysis of EE followed the similar pattern in terms of EPA and DHA regio-isomeric distribution (Table 3).

The regio-isomeric distribution of EPA and DHA in Peruvian anchovy/sardine fish oils evaluated in this study is in close agreement with a previous report by Gunstone [28], on South African anchovy fish oil consisting of 24.6% EPA and 9.8% DHA. In his report no data were mentioned for STA. To our knowledge, the regio-isomeric distribution of STA chains has only been reported by Aursand and coworkers [29] in lipids extracted from white muscle of Atlantic salmon and cod liver oil. In the former, STA was preferentially attached to the *sn*-2 position (73.4%) whereas in the latter, it was equally distributed among *sn*-1,3 (66.8%) and *sn*-2 (33.2%) positions. The random distribution pattern we found in Peruvian anchovy/sardine fish oil was similar to that found in cod liver oil.

The enzymatic synthesis of TAG from non-concentrated 2009EE and the corresponding 2009FFA led to different regio-isomeric distributions of EPA and DHA in the resulting products compared to the starting natural 2009TAG fish oil: 14% of EPA chains in re-constituted TAG were relocated from the *sn*-1,3 to the *sn*-2 positions, whereas 33% of DHA chains were relocated from the *sn*-2 to the *sn*-1,3 positions. The relationship between these structural differences and the bioavailability, stability and sensory profiles of these oils has not yet been fully examined. For instance, if a correlation between the positional distribution of EPA and DHA (and/or perhaps even other fatty acid residues) and oxidative stability of the natural oils is found, only the potentially most stable crude

Table 3 Regio-isomeric distributions (molar %) of EPA and DHA in natural anchovy/sardine fish oil and in reconstituted TAG prepared by enzymatic glycerolysis of EE and FFA of various EPA/DHA concentrations

	EPA[a]		DHA[a]		SA[a]	
	sn-1,3 (%)	*sn*-2 (%)	*sn*-1,3 (%)	*sn*-2 (%)	*sn*-1,3 (%)	*sn*-2 (%)
2009TAG natural anchovy/sardine fish oil	80.9	19.1	38.3	61.7	66.9	33.1
2009FFA → TAG	66.3	33.7	70.0	30.0	66.2	33.8
2009EE → TAG	66.7	33.3	69.8	30.2	66.1	33.9
4329FFA → TAG	67.3	32.7	70.6	29.4	–	–
4329EE → TAG	66.3	33.7	71.6	28.4	–	–
6003FFA → TAG	68.2	31.8	73.3	26.7	–	–
6003EE → TAG	67.8	32.2	75.9	24.1	–	–
0453FFA → TAG	66.5	33.5	69.9	30.1	–	–
0453EE → TAG	67.2	32.8	69.7	30.3	–	–

[a] From three spectral fittings the percentage of relative standard deviation (RSTD) was less than 1% in each case

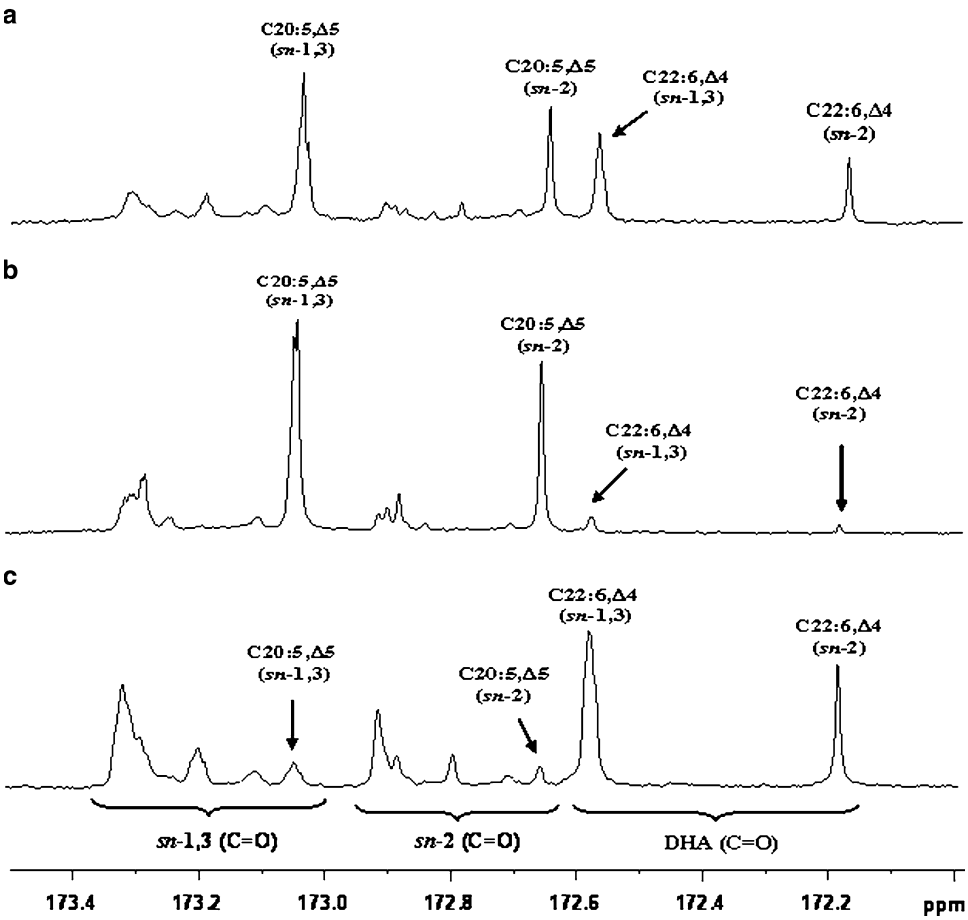

Fig. 3 Carbonyl region of the ^{13}C-NMR spectrum of reconstituted TAG obtained upon glycerolysis of: **a** 4329FFA; **b** 6003FFA; **c** 0453FFA. The region-isomeric distributions of EPA (C20:5, Δ5) and DHA (C22:6, Δ4) among *sn*-1,3 positions and the *sn*-2 position are annotated

oils would be selected for producing food and pharmaceutical grade omega-3 oils and concentrates that need to have superior sensory profiles. Initial steps towards the stability and sensory investigation of these oils have been made. Ultimately, our goal is to evaluate differences between bioavailability of natural and restructured omega-3 oils and concentrates.

Fatty acid profiles of reconstituted TAG similar to those of the starting materials (2009 and 4329 substrates) result from random acylation stemming from the lack of CALB

fatty acid selectivity. During reconstitution of high concentrated DHA omega-3 oils (0453 EPA/DHA composition), the enzyme discriminated strongly against DHA. Consequently, glycerolysis of 0453EE and FFA substrates not only required significant excess of acyl donor groups for completion (85 and 40%, respectively), but reconstituted TAG of the same fatty acid profile could not be prepared; instead they contained lower levels of C22:6 (37% and 42 area%). Discrimination against EPA was observed on reconstitution of the high EPA concentrate 6003EE, but not on enzymatic esterification of the corresponding FFA oil 6003FFA.

In a spite of the fatty acid discriminatory effect on TAG re-synthesized from high EPA and DHA concentrates, the enzyme displayed lack of glycerol *sn*-1,3/*sn*-2 regioselectivity for EPA chains, and some *sn*-1,3 regioselectivity for DHA chains on TAG assembly. This pattern was observed in all cases in this study, indicating that it occurs independently of the substrate composition and the nature of the acyl donor group. Thus, in TAG re-synthesized by direct glycerolysis of EE and by esterification of FFA with glycerol, the maximum attainable proportion of EPA omega-3 chains attached to the *sn*-2 positions is about 33% of total chains, whereas for DHA omega-3 chains it is between 25 and 30%.

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