Chemical Research in Toxicology®

Article

Trans Lipid Library: synthesis of docosahexaenoic acid (DHA) monotrans isomers and regioisomer identification in DHA-containing supplements

GEORGIA MENOUNOU, GIORGIA GIACOMETTI, ROBERTA SCANFERLATO, Paolo Dambruoso, anna sansone, Itziar Tueros, JAVIER AMEZAGA, Chryssostomos Chatgilialoglu, and Carla Ferreri

Chem. Res. Toxicol., **Just Accepted Manuscript** • DOI: 10.1021/acs.chemrestox.8b00021 • Publication Date (Web): 27 Feb 2018

Just Accepted

ACS Publications

Chemical Research in Toxicology is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Trans Lipid Library: synthesis of docosahexaenoic acid (DHA) monotrans isomers and regioisomer identification in DHA-containing supplements

Georgia Menounou[1‡], Giorgia Giacometti[1‡], Roberta Scanferlato[1], Paolo Dambruoso[1], Anna Sansone[1], Itziar Tueros[2], Javier Amézaga[2], Chryssostomos Chatgilialoglu[1], Carla Ferreri[1,]*

[1]*ISOF, Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129 Bologna (Italy)*

[2]*AZTI, Food and Health, Parque Tecnológico de Bizkaia, Astondo Bidea 609, 48160 Derio (Spain)*

‡ contributed equally to the work

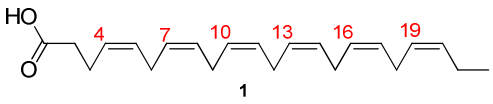
KEYWORDS. Omega-3 fatty acids, trans fatty acid isomers, isomer identification, deodorization, nutraceuticals.

ABSTRACT: Docosahexaenoic acid (DHA) is a semi-essential polyunsaturated fatty acid (PUFA) for eukaryotic cells, found in natural sources such as fish and algal oils and widely used as ingredient for omega-3 containing foods or supplements. DHA effects are connected to its natural structure with six cis double bonds, but geometrical monotrans isomers can be formed during distillation or deodorization processes, as an unwanted event that alters molecular characteristics and annihilates health benefits. The characterization of the six monotrans DHA regioisomers is an open issue to address for analytical, biological and nutraceutical applications. Here we report the preparation, separation and first identification of each isomer by a dual approach consisting of: i) the direct thiyl radical-catalyzed isomerization of cis-DHA methyl ester, and ii) the two-steps synthesis from cis-DHA methyl ester via mono-epoxides as intermediates, which are separated and

identified by bidimensional nuclear magnetic resonance spectroscopy, followed by elimination for the unequivocal assignment of the double bond position. This mono-trans DHA isomer library with NMR and GC analytical characterization was also used to examine the products of thiyl radical catalysed isomerization of a fish oil sample and to evaluate the trans isomer content in omega-3 containing supplements commercially available in Italy and Spain.

INTRODUCTION

Cis polyunsaturated fatty acids (PUFA) are essential components of human fats, particularly important for biological functions, such as for example to form membrane phospholipids and signalling molecules. In vivo, the omega-3 fatty acid docosa-4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-hexaenoic acid (cis-DHA) (Figure 1) is produced through several steps of elongation and desaturation from alpha-linolenic acid (octa-9*Z*,12*Z*,15*Z*-decatrienoic acid), the essential precursor taken exclusively from the diet [1]. This nine-steps biosynthesis is variably efficient in humans, therefore the semi-essential nature of DHA is now worldwide recognized by Health Agencies. Due to its importance for a correct human growth, adequate daily intakes of 100-200 mg have been established by the main international food safety and health agencies from dietary sources, such as algae or fish, especially in children and pregnant women [2]. This led to an increased marketing of DHA-rich formulas, either as functional foods and supplements. On the other hand, the multiple roles of DHA for molecular pathways and signalling have attracted interest in the last decade, all biological activities being strictly dependent from the structural requisite of the cis geometry [3]. When high temperatures and low pressures are used in deodorization processes, to eliminate the unpleasant fishy smell of oils, the natural cis structure of the polyunsaturated fatty acids is affected and the formation of geometrical trans isomers was demonstrated [4,5]. The health consequences of trans PUFA are matters of several studies that evidenced harmful roles for cardiovascular health and pregnancy [6,7], whereas it is worth noting that omega-3 supplementation in persons with cardiovascular diseases and pregnant women is highly recommended.



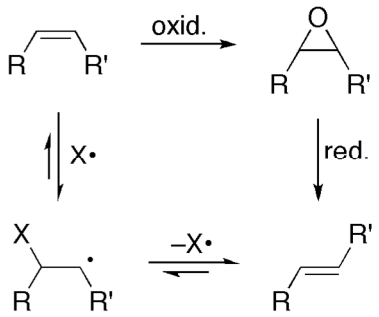
1

Figure 1. Structure of DHA (4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid)

In the frame of our research on the free radical modifications of biomolecules, we studied geometrical trans fatty acids (TFA) obtained from an isomerization process catalysed by sulfur-centered radicals, which occurs via an addition-elimination mechanism without involving the shift of the double bond, with interesting insights for polyunsaturated fatty acids [8,9]. The number of geometrical isomers for the unsaturated fatty acid is calculated as to 2^n, where the n is the number of double bonds, therefore in case of long chain PUFA, such as DHA, a high number of isomers can be calculated (2^6=128). However, due to the step-by-step mechanism of this reaction, monotrans isomers are the first and major products and they are also the most relevant products of radical stress in biological systems, where the low radical concentration produced in vivo is able to involve one double bond, as examined by using the biomimetic model of membranes in the form of unilamellar liposomes [9]. Indeed, monotrans isomers of arachidonic acid in the 5 and 8 positions were found the most relevant products created during oxidative metabolism with production of thiyl radicals, distinguishable from TFA obtained from the dietary intake and metabolic transformation of chemically manipulated oils containing trans isomers of linoleic acid [10-13]. Some attention has been given to the biological effects of specific monotrans isomers, showing differences from the natural cis analogues [14-17], however extension of such studies is limited by the availability of TFA molecular library and by the few commercially available TFAs.

Synthetic routes to PUFA monotrans isomers are useful to build up the trans lipid library and address analytical protocols and biological studies. We first characterized the monotrans isomers of arachidonic acid (4 isomers)[8] and eicosapentaenoic acid (EPA, 5 isomers)[18] using gas chromatography (GC) and carbon-13 nuclear magnetic resonance (^{13}C NMR) in combination for the assignment of each isomer structure. In the case of EPA,

we tested the efficiency of a dual synthetic approach to obtain the five mono-trans isomers in comparison with the isomers coming from the free radical-catalysed isomerization, as shown in Scheme 1. The strategy of separation and assignment of the mono-epoxide structures, prior to the elimination, successfully worked for the first unambiguous determination of the trans alkene position, integrating previous data in the literature [19-21].



Scheme 1. Dual synthetic approach to obtain the geometrical trans isomer: from cis-alkene via epoxide formation and ring opening, or via radical-based isomerization.

We considered the dual approach shown in Scheme 1 to be useful also for the synthesis of the mono-trans DHA isomers. Only the 4E regioisomer is commercially available as methyl ester and can be used as GC reference, whereas in literature monotrans DHA isomers were analysed by several authors after deodorization/isomerization reactions of DHA-rich oils, assaying various conditions of GC analysis but lacking a specific regioisomer assignment [4,5,16,20,21]. As far as the synthesis of the DHA mono-epoxides is concerned, the classical epoxidation reaction can lead to these compounds [22], which are named epoxydocosapentaenoic acid (EDP). 4,5-EDP and 19,20-EDP were known [19,23-25] also due to their biological relevance as metabolites of cytochrome P450 [26,27]. The strategy of the separation of the intermediate mono-epoxide regioisomers was successful to unambiguously individuate the epoxidation of the double bond position, using mono- and bidimensional nuclear magnetic resonance (NMR) experiments. Consequently, after ring opening, dibromide formation and elimination from each mono-epoxide regioisomer the double bond was unequivocally

established, thus allowing for the assignment of the corresponding peaks in the GC analysis. This double approach clarified that the direct isomerization by free radical-catalysed reaction gives the six isomers as exclusive products within few minutes. The usefulness of such identification was then assayed for analysis of fish oil and its isomerization products, also in view of applying the analytical route to the purity of fish oil preparations, which is a hot topic of food research [5, 28, 29]. We studied trans-containing triglycerides, and we showed that NMR represents a powerful analytical methodology to apply directly on the oil without transformation to fatty acid methyl esters [30]. The monotrans DHA library and GC characterizations were finally applied to the analysis of omega-3 containing supplements in capsules, commercially available in Italy and Spain, evidencing trans isomer contamination in the oil ingredient.

Experimental Section

Materials and Methods

Chloroform, methanol, *n*-hexane, 2-propanol and acetonitrile were purchased from Merck (HPLC purity). Absolute ethanol was purchased from BDH Prolabo VWR (AnalaR NORMAPUR) and 2-mercaptoethanol was purchased from Aldrich. Silver nitrate, 30% aq NH_4OH and anhydrous sodium sulfate (Na_2SO_4) were purchased from Carlo Erba. All fatty acid methyl esters (FAME) used as reference standard for GC analyses were purchased from Aldrich, Fluka or Sigma and used without further purification. 4E, 7Z10Z,13(Z) 16(Z) 19(Z) DHA-methylester was purchase from Lipidox (Lidingö, Sweden). Deuterated benzene and deuterated $CDCl_3$ were purchased from Eurisotop (France). Silica gel thin-layer chromatography (analytical and preparative) was performed on Merck silica gel 60 plates (0.25 and 2 mm thickness, respectively).

GC analyses. Fatty acid methyl esters were analyzed by gas chromatograph (Agilent 6850, Milan) equipped with a 60m × 0.25mm × 0.25μm (50%-cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA). The instrument has a flame ionization detector (FID) that requires air (450 mL / min) and hydrogen (40 mL / min)

and is maintained at a temperature of 250 ° C. Two identical equipment conditions were used for the analyses, applying the same injection temperature (230°C) but different oven conditions and carrier gas. Method A: from an initial temperature of 165°C held for 3 min, followed by an increase of 1°C/min up to 195°C, held for 40 min. A final ramp, with a temperature increase of 10°C/min up to a maximum temperature of 240°C, was maintained for 10 min for column purge. A constant pressure mode (29 psi) was chosen with helium as carrier gas. Methyl esters were identified by comparison with the retention times of commercially available standards or trans fatty acid references, obtained as described elsewhere. Method B: temperature started from 195°C, held for 26min, followed by an increase of 10°C/min up to 205°C, held for 13min, followed by a second increase of 30°C/min up to 240°C, held for 10min. A constant pressure mode (29 psi) was chosen with hydrogen as carrier gas. **GC/MS spectra** were recorded on a Clarus 500 GC apparatus equipped with a Clarus 560S mass spectrometer (GC/MS transfer line temperature 230°C) and a 60m x 0.25mm x 0.25µm (50%-cyanopropylphenyl)-dimethylpolysiloxane column (DB225 ms, Agilent, USA), using injector temperature of 230°C, a split ratio of 50:1 and helium as carrier gas at a constant flow of 1.2 mL/min, with the following oven conditions: temperature started from 195°C, held for 52min, followed by an increase of 3°C/min up to 205°C, held for 10min, followed by a second increase of 3°C/min up to 225°C, held for 15min and a final increase of 5°C/min up to 230°C, held for 10min.

Photolysis. Photolysis was carried out in a quartz photochemical reactor (Sigma Aldrich) equipped with a 5.5 W low-pressure mercury lamp. The temperature was maintained at (22 ± 2)°C by means of a thermostat bath.

Nuclear magnetic resonance (NMR). All NMR spectra were collected with a fully automated Agilent NMR system, consisting of a 54-mm bore, 500 MHz (11.7 T) Premium Shielded superconducting magnet, a DD2 Performa IV NMR console and the Agilent OneNMR probe. All samples were dissolved in C_6D_6 and transferred in 3 mm thin wall NMR tubes (Wilmad 335-PP-8). After having calibrated each sample pw90, the

following 7 NMR experiments were collected on each sample (2D experiments were collected with 50% NUS and F2 sw (^{1}H): 10 ppm, from-0.5 to 9.5 ppm):

- ^{1}H NMR spectra: pw 90 was calibrated for each sample, sw: -0.5 to 9.5 ppm
- one gHSQC: F1 sw (^{13}C) 135 ppm, from 5.0 to 140 ppm; nt=32; ni=512; res: 33 Hz (0.26 ppm)

  F2 sw (^{1}H): 10 ppm, from -0,5 to 9,5 ppm, NUS: 50%
- two bsgHSQCAD: *first* F1 sw (^{13}C): 60 ppm, from 5.0 to 65 ppm; nt=32; ni=512; res: 15 Hz (0.12 ppm)

  F2 sw (^{1}H): 10 ppm, from -0.5 to 9.5 ppm, NUS: 50%

  second: F1 sw (^{13}C): 20 ppm, from 120.0 to 140 ppm; nt=32; ni=512; res: 5 Hz (0.04 ppm)

  F2 sw (^{1}H): 10 ppm, from -0.5 to 9.5 ppm, NUS: 50%
- one gHMBCAD: F1 sw (^{13}C): 175 ppm, from 5.0 to 180 ppm; nt=64; ni=512; res: 43 Hz (0.34 ppm)

  F2 sw (^{1}H): 10 ppm, from -0.5 to 9.5 ppm, NUS: 50%
- two bsgHMBC: *first* F1 sw (^{13}C): 60 ppm, from 5.0 to 65 ppm; nt=64; ni=512; res: 15 Hz (0.12 ppm)

  F2 sw (^{1}H): 10 ppm, from -0.5 to 9.5 ppm, NUS: 50%

  second F1 sw (^{13}C): 60 ppm, from 120.0 to 180 ppm; nt=64; ni=512; res: 15 Hz (0.12 ppm)

  F2 sw (^{1}H): 10 ppm, from -0.5 to 9.5 ppm, NUS: 50%.

Epoxidation of Methyl All-(Z)-4,7,10,13,16,19-Docosahexaenoate (DHA–Me, 1). Synthesis of epoxydocosapentaenoic acid methyl ester (EDP-Me) regioisomers. A 5.84 mM solution of *meta*-chloroperoxybenzoic acid in dichloromethane (2.5 mL corresponding to 0.0146 mmol; 2.51 mg) was added dropwise to a solution of DHA methyl ester **1** (50 mg; 0.146 mmol) in dichloromethane (2.5 mL). After the addition was completed, the mixture was stirred for 10 min at room temperature under argon atmosphere,

following the formation of mono-epoxides with traces of di-epoxides by TLC (see **Figure S1** in Supporting Information). Eluent: 7/3 *n*-hexane /diethyl ether; DHA-Me R_f = 0.9, first mixture (**5**, **6** and traces of **4**) R_f = 0.7, second mixture (**3** and **7**) R_f = 0.6, pure isomer (**2**) R_f = 0.5, diepoxides $R_f \leq 0.4$). Work-up was carried out by addition of 5 mL of ice-cold $NaHCO_3$ (25% w/v) stirred for 2 min. Then, the reaction mixture was transferred in a separating funnel, the aqueous layer was discarded and the organic layer was washed two times with $NaHCO_3$, deionized H_2O and dried over Na_2SO_4. The crude was purified with flash chromatography (eluent: 9/1 *n*-hexane/diethyl ether) to give a first fraction containing an inseparable mixture (colourless oil; 8 mg; 0.0223 mmol; 15.3% yield) containing 13,14-EDP-Me (**5**), 16,17-EDP-Me (**6**) with traces of 10,11-EDP **4** and of residual starting material (**1**). Structures **5** and **6** in **Figure 2**, were found in a 29:71 ratio, respectively, as calculated by the integration of protons of C-22 on ^{1}H NMR spectrum (trace II in **Figure 5**). EDP-Me regioisomers **5** and **6** were further purified by preparative TLC using 100 mL toluene/500 µL isopropanol as the eluent: (R_f = 0.5, pure isomer **6**, and R_f = 0.4, pure isomer **5**) to give pure **6** (3 mg; 0.0084 mmol; 37.5% yield) with only traces of **4**.

The second fraction contained an inseparable mixture of 7,8-EDP-Me (**3**) and 19,20-EDP-Me (**7**) (colourless oil; 7mg; 0.0195 mmol; 13.4% yield) (**Figure 2**) found in a 43:57 ratio, respectively, as calculated by the integration of protons of C-22 on ^{1}H NMR spectrum (trace III in **Figure 5**). The subsequent fraction contained pure 4,5-EDP-Me (**2**) regioisomer (**Figure 2)** (colourless oil; 5 mg; 0.0134 mmol; 9.2% yield) identified as shown in **Figure 5** (trace IV) for the C-22 triplet at ^{1}H NMR.

The starting material DHA-Me (**1**) recovered was 30 mg (0.0877 mmol; 60% recovered yield).

The structural assignment of each regioisomer in the fractions was performed by dissolving the fractions in C_6D_6 as NMR solvent in which they are stable, and carrying out mono- and bi-dimensional experiments. **Table S1 and Figure S2 in Supporting Information** report the detailed ^{1}H and ^{13}C NMR resonances attributed to the

regioisomers as well as to cis-DHA methyl ester. For the ^{1}H NMR of the mono-epoxides see **Figures S6** and **S7** in the **Supporting Information**. 4,5-EDP (**2**) and 19,20-EDP (**7**) were previously reported in $CDCl_3$ (cfr., **Table S2**) [22,23]. In **Supporting Information** the 2D NMR experiments for the assignment of the C and H resonances in the EDP regioisomers are shown (**Figures S4** and **S5**).

Here below, a summary of the main NMR spectroscopy for the EDP regiosiomers assignment is provided.

*Methyl (Z)-13,14-Epoxy-all-(Z)-4,7,10,16,19-docosahexaenoate (**5**)*. ^{1}H NMR (C_6D_6, 500 MHz): δ_H 5.25 - 5.55 (m, 10H), 3.31 (s, 3H), 2.72 - 2.83 (m, 8H), 2.25 - 2.41 (m, 4H), 2.12 (s, 4H), 1.93 - 2.00 (m, 2H), 0.88 (t, *J* = 7.30 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ_C 172.35 (C1), 131.90 (C20), 130.53 (C17), 130.19 (C10), 129.02 (C5), 128.32 (C8), 128.11 (C4), 127.87 (C7), 126.82 (C19), 124.76 (C11), 124.55 (C16), 55.66 (C14), 55.65 (C13), 50.65 (C1`), 33.63 (C2), 26.35 (C15), 26.34 (C12), 25.78 (C9), 25.68 (C18), 25.57 (C6), 22.75 (C3), 20.53 (C21), 14.03 (C22).

*Methyl (Z)-16,17-Epoxy-all-(Z)-4,7,10,13,19-docosahexaenoate (**6**)*. ^{1}H NMR (C_6D_6, 500 MHz): δ 5.25 - 5.55 (m, 10H), 3.31 (s, 3H), 2.72 - 2.83 (m, 8H), 2.25 - 2.41 (m, 4H), 2.12 (s, 4H), 1.87 - 1.94 (m, 2H), 0.86 (t, *J* = 7.30 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ 172.35 (C1), 133.81 (C20), 130.19 (C13), 129.02 (C5), 128.40 (C10), 128.32 (C8), 128.11 (C4), 127.87 (C7), 127.70 (C11), 124.76 (C14), 123.66 (C19), 55.79 (C17), 55.65 (C16), 50.65 (C1`), 33.63 (C2), 26.34 (C15), 26.22 (C18), 25.78 (C12), 25.66 (C9), 25.57 (C6), 22.75 (C3), 20.53 (C21), 14.00 (C22).

Methyl (Z)-7,8-Epoxy-all-(Z)-4,10,13,16,19-docosahexaenoate (**3**). ^{1}H NMR (C_6D_6, 500 MHz): δ 5.35 - 5.51 (m, 10H), 3.30 (s, 3H), 2.69 - 2.85 (m, 8H), 2.19 - 2.32 (m, 4H), 2.08 - 2.14 (m, J = 7.30 Hz, 4H), 1.93 - 2.04 (m, 2H), 0.89 (t, J = 7.50 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ 172.30 (C1), 131.82 (C20), 130.17 (C11), 130.08 (C4), 128.35 – 127.80 (C13, C14, C16 and C17), 127.06 (C19), 125.77 (C5), 124.78 (C10), 55.65 (C8),

55.63 (C7), 50.66 (C1`), 33.51 (C2), 26.35 (C9), 26.23 (C6), 25.78 (C12), 25.75 – 25.60 (C15), 25.58 (C18), 22.83 (C3), 20.55 (C21), 14.07 (C22).

*Methyl (Z)-19,20-Epoxy-all-(Z)-4,7,10,13,16-docosahexaenoate (**7**)*. ^{1}H NMR (C_6D_6, 500 MHz): δ 5.25 - 5.52 (m, 10H), 3.31 (s, 3H), 2.73 - 2.86 (m, 9H), 2.60 (dt, *J* = 4.20, 6.40 Hz, 1H), 2.25 - 2.37 (m, 3H), 2.04 - 2.16 (m, *J* = 7.30 Hz, 3H), 1.40 (ddq, *J* = 7.30, 7.30, 14.10 Hz, 1H), 1.28 (ddq, *J* = 6.10, 7.30, 13.50 Hz, 1H), 0.84 (t, *J* = 7.70 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ 172.32 (C1), 130.03 (C16), 129.06 (C5), 128.35 – 127.80 (C7, C8, C10, C11, C13 and C14), 128.08 (C4), 124.96 (C17), 57.38 (C20), 55.75 (C19), 50.65 (C1`), 33.64 (C2), 26.31 (C18), 25.79 (C15), 25.75 – 25.60 (C9 and C12), 25.58 (C6), 22.76 (C3), 21.10 (C21), 10.45 (C22).

*Methyl (Z)-4,5-Epoxy-all-(Z)-7,10,13,16,19-docosahexaenoate (**2**)*. ^{1}H NMR (C_6D_6, 500 MHz): δ 5.23 - 5.55 (m, 10H), 3.29 (s, 3H), 2.65 - 2.88 (m, 10H), 2.13 - 2.27 (m, 3H), 1.95 - 2.09 (m, 3H), 1.68 - 1.76 (m, 1H), 1.58 - 1.68 (m, 1H), 0.89 (t, *J* = 7.63 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ 172.35 (C1), 131.89 (C20), 130.27 (C8), 128.63/128.38/128.11/128.96 (C13, C14, C16 and C17), 128.42 (C11), 127.95 (C10), 127.19 (C19), 124.78 (C7), 56.07 (C5), 55.32 (C4), 50.85 (C1`), 30.76 (C2), 26.38 (C6), 25.86 (C9), 25.75 (C12 and C15), 25.66 (C18), 23.42 (C3), 20.66 (C21), 14.17 (C22).

Methyl All-(Z)-4,7,10,13,16,19-Docosahexaenoate (**1**). ^{1}H NMR (C_6D_6, 500 MHz): δ 5.24 - 5.47 (m, 12H), 3.31 (s, 3H), 2.73 - 2.88 (m, 10H), 2.29 (dddd, *J* = 0.80, 1.40, 7.34, 14.70 Hz, 1H), 2.29 (dddd, *J* = 0.80, 1.40, 7.30, 14.70 Hz, 1H), 2.11 (t, *J* = 7.40 Hz, 2H), 1.95 - 2.03 (m, 2H), 0.89 (t, *J* = 7.61 Hz, 3H). ^{13}C NMR (C_6D_6, 126 MHz): δ 174.32 (C1), 131.78 (C20), 129.08 (5), 128.50 (C17), 128.22 (C14), 128.15 (C8, C10 and C11), 128.11 (C7, C13 and C19), 128.06 (C4), 127.91 (C16), 50.65 (C1`), 33.65 (C2), 25.67 (C15), 25.66 (C9), 25.65 (C18), 25.57 (C6), 25.56 (C12), 22.75 (C3), 20.54 (C21), 14.08 (C22).

Transformation of EDP-Me regioisomers to Monotrans DHA–Me Isomers. The reaction is described for 16,17-EDP-Me and 13,14-EDP-Me (first fraction of the EDP chromatographic separation), and the same steps were followed for the other isolated EDP-Me fractions.

Step 1. (Bromination of Epoxide). To a freshly prepared solution of triphenylphosphine dibromide (7 mg; 0.0167 mmol) in dry dichloromethane (50 μL) a solution containing a mixture of EDP-regioisomers, **6** and **5**, (3 mg; 0.0084 mmol containing traces of 10,11-EDP **4**) in dry dichloromethane (100 μL) and pyridine (1 μL) was added, stirring at 0°C under argon atmosphere. The mixture was left stirring overnight at 5°C, and then was quenched with a 1 M aqueous solution of hydrochloric acid (0.5 mL) and extracted three times with chloroform/ethanol (4/1). The reaction was monitored by TLC for the ring opening as dibromide (7/3 *n*-hexane/diethyl ether, starting material $R_f = 0.7$ and dibromide product $R_f = 0.4$). The organic layers were collected, dried over Na_2SO_4, and evaporated under vacuum. The crude containing methyl 16,17-dibromo-(4Z,7Z,10Z,13Z,19Z)-docosapentaenoate and 13,14-dibromo-(4Z,7Z,10Z,16Z,19Z)-docosapentaenoate (with traces of the 10,11-dibromoderivative) was used for the next step. The same reaction was also performed for the inseparable mixture of the EDP-Me regioisomers **3** and **7** and for the EDP-ME regioisomer **4**. In all cases the reaction crude was used for the next step.

Step 2. (Elimination). To an ice-cooled slurry of activated zinc (4.7 mg; 0.072 mmol), acetic acid (2 μL), and *N*,*N*-dimethylformamide (150 μL) a solution of methyl 16,17-dibromo-(4Z,7Z,10Z,13Z,19Z)-docosapentaenoate and traces of 13,14-dibromo-(4Z,7Z,10Z,16Z,19Z)-docosapentaenoate (3 mg; 0.0060 mmol) in *N*,*N*-dimethylformamide (100 μL) was added and the mixture was left stirring at 0°C for 14 hrs. The reaction was monitored by TLC (eluent: 7/3 *n*-hexane/diethyl ether) evidencing the formation of alkenes ($R_f = 0.9$) in the presence of traces of starting material ($R_f = 0.4$). At this point, the reaction was stopped by addition of a 1 M aqueous hydrochloric acid solution (1 mL), and the precipitate was filtered off. The filtrate was extracted three times with a mixture of chloroform/methanol (3/1). The organic layers were collected and dried over

Na_2SO_4. The solvent was removed under vacuum to afford a colourless oil (1.5 mg; 0.0044 mmol; 73% yield). The reaction crude was analysed by Ag-TLC (eluent: 1/9 *n*-hexane/diethyl ether with 0.4% MeOH) evidencing the presence of two spots, referred to monotrans DHA-Me and cis DHA-Me (see **Figure S8 (b)** in **Supporting Information**). The other two crude reaction mixtures of dibromides were treated for elimination as described above. The debromination reaction occurred also in the second fraction transformed into the dibromide intermediates affording methyl (4Z,7Z,10Z,13Z,16Z,19E)-docosahexaenoate and methyl (4Z,7E,10Z,13Z,16Z,19Z)-docosahexaenoate (colourless oil; 3.5 mg; 0.0010 mmol; 86% yield), as well as for the last EDP fraction affording methyl (4E,7Z,10Z,13Z,16Z,19Z)-docosahexaenoate (colourless oil;
The elimination products were then analysed by GC taking advantage of the parallel NMR assignments of regioisomers, thus allowing for the attribution of the trans double bond position. **Figures S11-S13** in **Supporting Information** are the GC traces of the elimination of the three EDP fractions, in comparison with the trans isomer mixture obtained by photolysis and with literature data [4,5,16,20,21]. The assignment of the monotrans isomer peaks in the GC analysis was obtained on the basis of the starting mono-epoxide assignment (**Figure 4**).

Scheme 2 in the main text summarizes the formation of mono-epoxides and the chromatographic separation of fractions that underwent the bromination-elimination step affording the separation of the monotrans regioisomers.

Synthesis of Monotrans DHA-Me Isomers by Photolysis. A 15 mM solution of DHA-Me ester (20 mg, 0.058 mmol) in 2-propanol (3.87 mL) was transferred into a quartz photochemical reactor (Sigma Aldrich) equipped with a 5.5 W low-pressure mercury lamp. The reaction mixture was degassed with argon for 20 min, then an aliquot of a previously degassed stock solution of 2-mercaptoethanol in 2-propanol was added (0.03 mmol) and the UV lamp was turned on for 5 minutes at the temperature of 22 ± 2 °C, kept by means of a thermostat bath. The reaction was monitored by Ag-TLC, spraying the plate with cerium ammonium sulfate/ammonium

molybdate reagent (CAM), to evidence the formation of the monotrans fraction together with ditrans, tritrans products, in order to stop the reaction when monotrans isomers were prevalently formed in the presence of remaining cis-DHA (see **Figure S8 (a)** in **Supporting Information**). The solvent was removed under vacuum, and addition of chloroform with further evaporation under vacuum helped the removal of 2-mercaptoethanol. The crude of the reaction was subsequently dissolved in 1 mL of *n*-hexane and loaded onto a preparative Ag-TLC plate. A rapid and efficient separation was obtained using 1:9 *n*-hexane/diethyl ether with 0.4% MeOH as mobile phase. After elution, the plate portion corresponding to the monotrans isomer fraction (R_f = 0.58), detected by spraying a small portion of the plate with CAM, was scraped off. Silica was washed with chloroform (3 x 5 mL). The solvent was evaporated to give a solid material, which is the Ag-fatty acid complex insoluble in *n*-hexane. This material was dissolved in a 5% aqueous solution of NH_4OH, vigorously stirred (600 rpm) for 10 min and extracted with aliquots of *n*-hexane (3 x 5 mL). Finally the organic phase was dried over Na_2SO_4, filtered and evaporated. The unreacted all-cis DHA-Me was recovered and subjected to a second isomerization cycle. This procedure leaded to the collection of a colourless oil that corresponded to the monotrans DHA methyl ester isomer mixture (5.2 mg, 0.0152 mmol, overall yield from two isomerization cycles 26%) (see **Figure 2**). GC analysis was carried out under the conditions described in Materials and Methods (see Supporting Information, see trace II, **Figure 4** or trace IV in **Figure 6** of the main text). ^{13}C NMR (**Figures S9** and **S10** in Supporting Information) of the mixture of monotrans DHA-Me isomers obtained from the isomerization and from the purification were also performed.

^{13}C NMR (C_6D_6) δ 10.94, 13.72, 14.12, 20.44, 20.56, 22.76, 25.26, 25.57, 25.66, 27.12, 28.01, 28.17, 28.57, 29.24, 30.39, 33.67, 33.71, 35.64, 45.29, 50.64, 50.73, 61.17, 126.93, 127.12, 127.48, 127.67, 127.86, 128.14, 128.48, 128.78, 128.81, 129.28, 129.37, 131.66, 131.68, 131.71, 131.75, 131.97, 131.99, 132.00, 132.23, 132.27, 132.49,132.52, 132.55, 172.35, 172.37.

After purification the ^{13}C NMR of the pure monotrans fraction was carried out (see **Figure S10** in **Supporting Information**): ^{13}C NMR (C_6D_6) δ 14.10, 20.55, 22.75, 30.40, 33.65, 50.65, 127.47, 127.67, 127.86, 131.68, 131.72, 131.77, 132.02, 132.28, 172.33.

Isomerization of fish oil by photolysis. A sample of commercially available fish oil, used as omega-3 containing ingredient for food and nutraceutical preparation, was isomerized following the procedure above described for the DHA-Me isomerization. After 30 minutes, 30 % of total trans content was obtained as determined after transesterification to the corresponding FAME and GC analysis. In **Supporting Information** the ^{13}C NMR of the isomerized oil are reported (**Figures S21** and **S22**). By GC analysis of the FAME obtained from the triglyceride transesterification the cis and trans fatty acid contents were also determined. In **Figure 6** of the main text the GC separation of the DHA isomers is shown, in comparison with the monotrans DHA methyl ester isomer library. In **Figure S23-S26** in **Supporting Information** two different GC methods were used with hydrogen or helium as carrier gas in order to evidence the best fatty acid separation.

Analysis of monotrans DHA isomers from commercially available supplements. Nineteen DHA-containing supplements in soft-gel capsules were examined for their cis and trans fatty acid contents, being six products commercially available in Italy and thirteen in Spain. The oily content was taken from the capsules and the lipids were extracted according to the Folch method [31]. Briefly, approx. 3 mg of each sample were dissolved in 2:1 chloroform/methanol (3 mL) and partitioned with brine (1 mL); the extracted organic phases (3 x 3mL) containing lipids were joined and dried with anhydrous Na_2SO_4. After filtration, the solvent was eliminated using a rotary evaporator and the oily residue was left under vacuum for 10 minutes to remove any possible trace of water. The lipid extract was checked by TLC (mobile phase: *n*-hexane/diethyl ether 7: 3) and then treated with freshly prepared 0.5 M KOH/MeOH solution for 30 min at room temperature under stirring. Reaction was quenched using brine and fatty acid methyl esters (FAME) were extracted using *n*-hexane (3x 3mL); the organic phase was collected and dried with anhydrous Na_2SO_4. After filtration and solvent

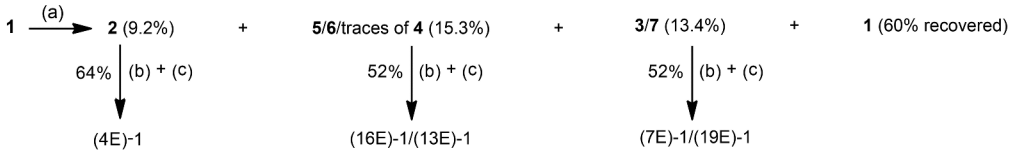
evaporation, the FAME mixture was dissolved in 10 μL n-hexane and 1 μL was injected for GC analysis. **Tables S2 and S3** in **Supporting Information** report the FAME content: products **1-6** are omega 3 supplements present in the Italian market and products **7-19** are present in the Spanish market.

RESULTS AND DISCUSSION

The dual synthetic approach for DHA transformation and monotrans DHA isomer identification. According to Scheme 1 we approached the dual synthetic strategy first by carrying out the direct radical-catalysed isomerization of DHA methyl ester, as previously described by us for EPA [17], thus obtaining a mixture of the six monotrans DHA isomers (**Figure 2**). Ag-TLC was used to purify the monotrans DHA isomers from the starting material (**Figure 3**, lane C). The recognition of each monotrans isomer was not realizable at this stage, however satisfactory separation of these isomers could be obtained by gas chromatography, the GC trace showing five out of six separable peaks, meaning that only two isomers are superimposed. It is also worth noting that one monotrans DHA isomer elutes similarly to cis-DHA (**Figure 4**, GC trace II). GC analysis, under the conditions described in Materials and Methods (see Supporting Information: trace II, **Figure 4** or trace IV in **Figure 6** of the main text) gave a pattern of peaks similar to those described in literature [4,5,20,22]. At this point the assignment of each monotrans isomer could not be performed. Only by elution of the commercially available 4*E*-DHA-Me, the third eluting peak could be assigned to this isomer (**Figure 4**, trace I).

By the second synthetic route DHA methyl ester was transformed in two-steps to give the corresponding monotrans alkenes (**Scheme 1**), via monoepoxide formation, ring-opening of the epoxide, using the stereoselective formation of the dibromide derivatives, followed by elimination reaction, that provide the trans geometry of each starting double bond. In this strategy, the key step is the separation and characterization of the

mono-epoxide products, in order to assign the double bond position for the subsequent elimination step. *Meta*-chloroperoxybenzoic acid (*m*-CPBA) epoxidation followed by purification by flash chromatography (with 9/1 *n*-hexane/diethyl ether as the eluent; see TLC separation with 7/3 *n*-hexane/diethyl ether in **Figure S1**, **Supporting Information**) provided three fractions of monoepoxide regioisomers (**Scheme 2**). The structures of the six regioisomers are shown in **Figure 2**.



Scheme 2. Two-steps transformation of all-cis DHA-Me into monotrans isomers (yields of the products are in parenthesis): (a) *m*-CPBA, DCM, RT, 10 min; (b) dry DCM, pyridine, Ph_3PBr_2, 5°C, overnight followed by (c) DMF, activated Zn, AcOH, 0°C, 10 h.

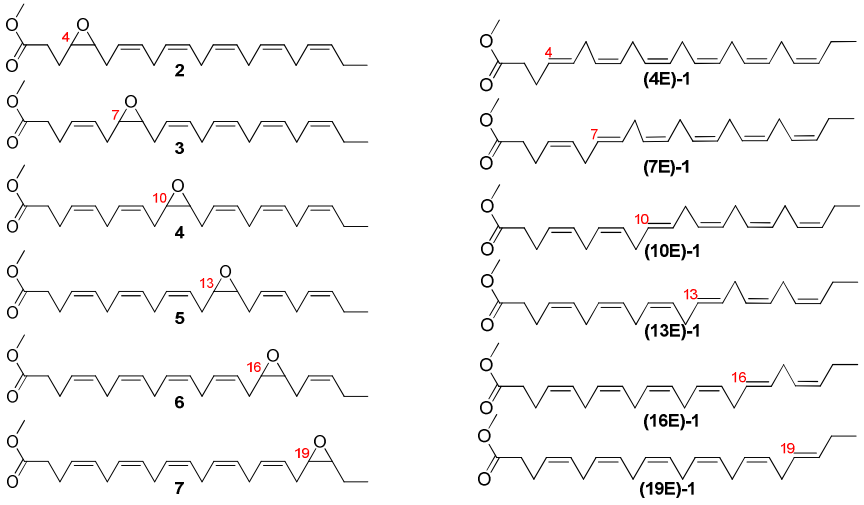


Figure 2. Structures of the six EDP regioisomers (left) and the corresponding monotrans DHA methyl ester isomers (right).

Under our experimental conditions, described in the Experimental Section, the 4-monoepoxide (compound **2)** was isolated in pure form as third fraction of the chromatography. The other regioisomers were obtained in the first and second chromatographic fractions, each fraction as a mixture of two compounds. EDPs are biologically relevant compounds obtained from DHA by cytochrome P450 enzyme, exhibiting a variety of biological effects in inflammation, pain, angiogenesis and cancer [26]. Actually cytochrome P450 is able to provide the stereoselective epoxidation of DHA, preferably at the last double bond obtaining 19,20-EDP [25]. However, also 16,17-EDP, 13,14-EDP, 10,11-EDP and 7,8-EDP have been detected and separated by HPLC analysis, whereas 4,5-EDP was reported to be unstable [27]. To the best of our knowledge, the full EDP isomer analysis by NMR spectroscopy has not been reported so far. We were interested to carry out mono- and bi-dimensional NMR experiments in order to assign the epoxide position in each compound of the reaction mixture. It is worth noting that the use of deuterated benzene (C_6D_6) as NMR solvent is required, in order to avoid decomposition during the experiment in the slightly acidic environment of deuterated chloroform. We performed also the spectrum of DHA-Me in deuterated benzene for examining the epoxidation results using the same experimental conditions (see **Figure S2** in **Supporting Information**). **Table S1** in **Supporting Information** collects the ^{13}C NMR data of the EDP isomers and the 10,11-EDP regioisomer is not presented since it was formed only in traces. The reasons for the scarce formation of this stereoisomer in the epoxidation reaction were not investigated. In **Supporting Information Figure S2** shows the structures of the five EDP isomers with their proton and carbon atom resonances as assigned in this study using deuterated benzene as solvent. The NMR spectra of all the five EDP isomers are reported in the Experimental Procedures. In order to assign the monoepoxide resonances we examined ^{1}H and ^{13}C NMR resonances comparing with DHA-Me for each fraction separated by column chromatography as described in the next section dedicated to NMR. The subsequent transformation of mono-epoxides, forming in situ the corresponding dibromides followed by elimination to the corresponding alkenes, was performed in one-step for each fraction. The latter reaction

sequence was adapted for DHA methyl ester from a procedure described for arachidonic acid methyl ester [10,14] and successfully applied to eicosapentanoic acid methyl ester (EPA) [18].

During the *in situ* transformation of epoxides to dibromide derivatives and the subsequent elimination to trans alkenes, the concerted elimination is not the only occurring mechanism for which the double bond can be formed. Therefore, the presence of cis-DHA was detected in all the resulting elimination crude mixtures. Having recognized in each starting fraction the mono-epoxide structures as major and minor isomers, as described in the NMR section and summarized in **Scheme** 2, the assignment of the corresponding monotrans DHA isomers could be satisfactorily carried out examining the peaks present in the GC traces of the DHA-Me isomers after the two-steps synthesis, as shown in **Figure 4** (traces II, IV, V).

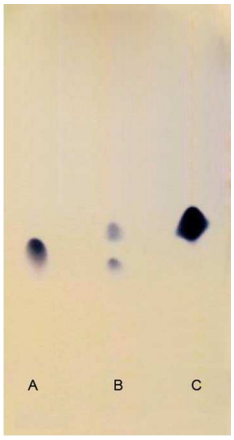


Figure 3. Ag-TLC (eluent: 1/9 *n*-hexane/diethyl ether with 0.4% MeOH) of the transformation of one mono-epoxide fraction (second fraction) via bromination and elimination reactions. A: standard all-cis DHA-Me. B: elimination reaction showing the presence of all-cis-DHA-Me and monotrans isomers (19E and 7E) of DHA-Me. C: mixture of monotrans DHA-Me obtained by thiyl-radical catalysed isomerization after purification as reported in Experimental Procedures.

The isomer overlapping with DHA-Me is the 16*E* monotrans DHA-Me, and the 7*E* and 10*E* monotrans DHA-Me are also superimposed (lanes (III) and (IV) corresponding to the conversion of these two mono-epoxide fractions according to **Scheme 2**). It is worth recalling that the 10*E* isomer was obtained from the traces of the 10,11-EDP, formed with the lowest yield among all EDP isomers, as ascertained by the study of the NMR spectra reported in the following section. Finally, the following GC order of elution could be assigned: 19E < 16E = all-cis < 4E < 13E < 7E = 10E.

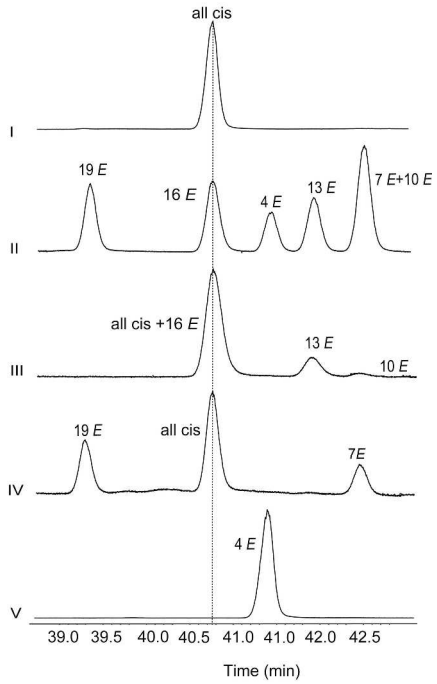


Figure 4. GC chromatograms of the 39.0-42.5 min region referred to: (I) all *cis*-DHA methyl ester; (II) mixture of monotrans DHA methyl ester isomers obtained by photolysis in the presence of thiyl radicals; (III) 16*E*-isomer superimposed with all cis-DHA methyl ester, 13*E* and traces of 10*E*; (IV) 19E, all cis-DHA methyl ester and 7*E*; and (V) 4*E* (commercially available product).

This is the first time the assignment of mono-trans geometrical isomers of the omega-3 fatty acid DHA is made, by a combination of Ag-TLC, NMR spectroscopy, gas chromatography, and this result contributes to the

library of non-commercially available trans isomers developed by us for the long-chain polyunsaturated fatty acids (LC-PUFA) omega-6 and omega-3.

NMR study of the EDP isomers and the monotrans DHA isomers. As previously mentioned, the ^{1}H and ^{13}C NMR spectra of the EDP and monotrans DHA-Me isomers obtained in this work (see **Figure 2**) were carried out in C_6D_6, primarily used to avoid decomposition of the mono-epoxide compounds. The ^{1}H NMR spectra of the EDP regioisomers was very diagnostic for the terminal methyl group (C-22), that appears as triplet in the 0.80-0.91 ppm region and is influenced by the relative position of the epoxide functionality. As shown in **Figure 5**, in the starting material DHA-Me the triplet is centred at 0.885 ppm (trace I), whereas the pure 4,5-EDP isomer (isolated by chromatography) shows the triplet centred at 0.888 ppm (trace IV). Two other fractions obtained from the chromatographic separation contained other four isomers (see **Scheme 2**): i) a mixture with the 16,17-EDP isomer with the triplet centred at 0.853 ppm and the 13,14-EDP isomer with the triplet centred at 0.880 ppm, with traces of the 10,11-EDP isomer (**Figure 5**, trace II); ii) a mixture with the 19,20-EDP isomer with the triplet at 0.838 ppm (major isomer) and the 7,8-EDP isomer with the triplet at 0.892 ppm. The assignment of the triplet was made by correlation experiments, crossing the ^{1}H and ^{13}C resonances of the methyl group and epoxide carbon atoms, respectively (see **Table S2** in Supporting Information).

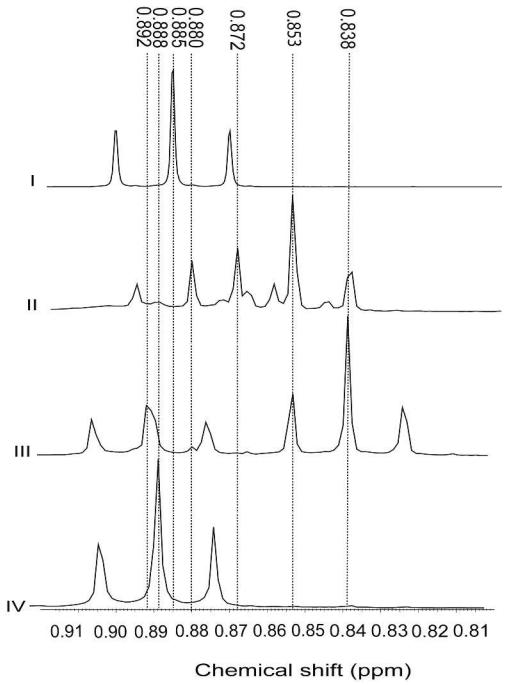


Figure 5. ^{1}H NMR region (0.81-0.91 ppm) related to the terminal methyl group (C-22) of EDP regioisomers (spectra run in C_6D_6). The triplet is centred at different ppm in relationship with the position of the epoxide along the fatty acid chain; (I) DHA-Me all-cis; (II) mixture of 13,14-EDP **5**, 16,17-EDP **6** (traces of 10,11-EDP **4**); (III) mixture of 7,8-EDP **3** and 19,20-EDP **7;** (IV) 4,5-EDP **2**.

Examining the ^{13}C NMR spectrum of the EDP regioisomers and using the assigned resonances of starting DHA-Me, the carbon atom resonances of the epoxide function were determined. In fact, for the 16,17-monoepoxide two peaks at 55.79 and 55.65 ppm can be assigned to the carbon atoms of the epoxide function, since at the same time in the spectrum are absent the chemical shifts of the ethylenic carbon atoms assigned at the C16-C17 double bond of DHA-Me (128.50 and 127.91 ppm, respectively). The 13,14-EDP has two peaks at 55.66 and 55.65 ppm assigned to the epoxide function, since the C13-C14 double bond resonances of DHA-Me at 128.22 and 128.11 ppm are not present. The 19,20-EDP has the epoxide carbon atoms at 57.38 and 55.75 ppm, and correspondently the chemical shifts at 131.78 and 127.11 ppm (C19-C20 of DHA-Me) are absent. In the 19,20-EDP regiosiomer the chemical shift of the C-22 is noticeably moved at 10.45 ppm (instead of the

range 14.08 – 14.00 ppm for all the other EDP regioisomers), indicating an up-field shift due to a different electronic distribution influencing the end of the carbon atom chain (i.e., the ω position). The 7,8-EDP showed the chemical shifts of the epoxy carbon atoms at 55.65 and 55.63 ppm, corresponding to the absence of the resonances at 128.15 and 128.11 ppm (C7-C8 in the DHA-Me). Finally, for the EDP in the position 4,5 the epoxy function was individuated by the chemical shifts at 56.07 and 55.32 ppm corresponding to the absence of the peaks at 129.08 and 128.06 ppm (C4-C5 of DHA-Me).

Bidimensional experiments can also be performed to acquire more data on these assignments. A representative description of the assignment of resonances to 7,8- and 19,20-EDP isomers obtained by HSQC/HMBC experiments is shown in **Supporting Information** (**Figures S4** and **S5**). By this additional information we could increase data on the epoxide assignments, however the above described ^{1}H and ^{13}C NMR characteristics satisfactorily individuated the EDP regioisomers.

As far as the DHA monotrans isomers are concerned, they were obtained from the EDP two steps transformation as shown in **Scheme 2**, as well as mixture of the six isomers by radical-catalyzed isomerization of the all-*cis* DHA-Me. This latter mixture was examined by NMR and **Figure 6** shows a trace of the enlargement of the ^{13}C NMR region corresponding to the C-20 resonance (traces I and II, panel b), which was found to be diagnostic for the geometrical isomers. The C-20 atom is the most deshielded among the olefinic peaks, and its distance from the position of the trans double bond in the fatty acid chain influences its chemical shift. Five different chemical shifts for this carbon atom could be individuated in the monotrans isomer mixture isolated from the isomerization (trace II in Figure 6, panel b), one of them representing two overlapped resonances, and they are clearly different from the C-20 of DHA-Me appearing at 131.78 ppm (*cfr.*, trace I in **Figure 6**, panel b). The importance of the last vinylic carbon atom of the PUFA fatty acid chain was previously described for arachidonic and eicosapentaenoic acids [10,18]. The present data support the use of NMR spectroscopy for the analysis of natural and marine oils, as already shown by other groups [32-34] and recently

reviewed [35]. The herein described chemical synthesis of monotrans DHA isomers, as well as our previously published work on trans lipid library [8-11], certainly increase availability of reference compounds and will help to gather more information by the powerful NMR methodology.

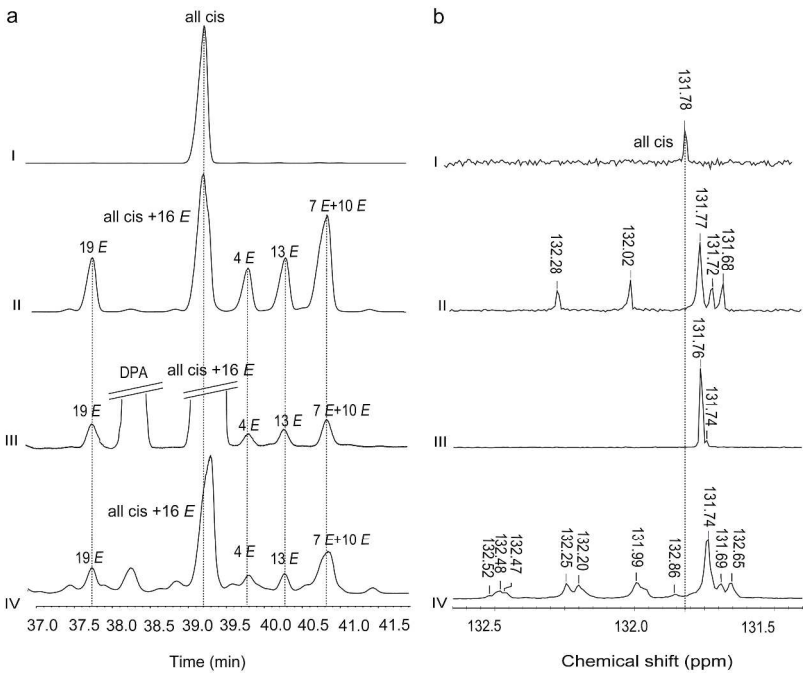


Figure 6. Panel **a)** Partial GC traces of: (I) all-*cis* DHA-Me; (II) the six DHA-Me monotrans isomers and all-*cis* DHA-Me; (III) FAME obtained from the transesterification of DHA-containing fish oil. The presence of monotrans DHA isomers as contaminants is visible when the trace is expanded (× 10); (IV) DHA-Me isomers obtained after isomerization and transesterification of fish oil (this mixture contains mono-, di- and tri-trans isomers). Panel **b)** Enlargement of the ^{13}C NMR region (131.5-132.5 pm) corresponding to the C-20 ethylenic carbon atom of: (I) all-*cis* DHA-Me; (II) the six DHA-Me monotrans isomers and all-*cis* DHA-Me; (III) DHA-containing triglycerides from fish oil; (IV) DHA-containing triglycerides from fish oil after isomerization (this mixture contains mono-, di- and tri-trans isomers).

Isomerization of DHA-containing fish oil and determination of the monotrans DHA isomer content in commercially available supplements. Natural oils are important sources of cis PUFA, and trans isomers of

EPA and DHA are contaminants after deodorization or distillation procedures that are used in order to eliminate the fishy odour. PUFA isomerization in oils has been described by heating procedures [4,21] or *p*-toluene sulfinic acid treatment [28], whereas we reported the isomerization of some oils by thiyl radical catalysed process [30], but not of fish oils. Here we performed the isomerization of a sample of DHA-containing fish oil, commercially available as raw material for food or supplements. The reaction was carried out as described in the Experimental section and GC analysis was performed after transesterification of the triglycerides into the corresponding FAMEs. **Figure 6** (panel a) shows the GC regions corresponding to FAMEs from fish oil in the starting material (trace III) and it is worth noting that in this sample mono-trans DHA isomers are already present as contaminants, as it can be seen by comparison with the FAME of the oil after free radical-catalysed isomerization (trace IV), and related cis and monotrans DHA-Me isomers (traces I and II). In the same **Figure 6** (panel b) the enlarged region of the ^{13}C NMR spectra corresponding to the C-20 (ethylenic carbon atom) resonance is shown for the DHA-containing triglycerides of fish oil (trace III) in comparison with the all-*cis* DHA-Me (trace I). It is gratifying to see that the C-20 chemical shift is similar for FAME and triglycerides that contains the DHA moieties. Moreover, after fish oil isomerization, trans-DHA-containing triglycerides show strong similarity of the C-20 resonances for the monotrans DHA-Me isomers that were previously described (*cfr.*, trace IV with trace II). In **Supporting Information** the full ^{13}C NMR spectra of the fish oil triglycerides before and after the isomerization are shown (**Figures S21** and **S22**).

It is worth mentioning that the GC resolution of monotrans isomers achieved under our analytical conditions, using a specific oven program with a 60 m × 0.25 mm × 0.25 μm (50%-cyanopropyl)-methylpolysiloxane column, were obtained after testing several gas chromatographic conditions, in particular concerning the carrier gas. Using natural sources of fatty acids as marine oils, it was envisaged that other long-chain fatty acids can elute in the region of the DHA-Me and its monotrans isomers. This was the case of C22-PUFAs, such as 22:5 ω-3 (DPA) and 22:6 ω-6, as well as long chain saturated and monounsaturated fatty acids, such as C24:0 and

C24:1. In **Supporting Information** two GC experimental conditions are described in Materials and Methods, and **Figures S23-26** show the peak resolution connected with these two methods. We were aware that a very careful study was carried out with a 200 m SLB IL111 ionic liquid column verifying the accuracy of the proposed method using Standard Reference Material (SRM) 3275 "Omega-3 and Omega-6 Fatty Acids in Fish Oil" from the National Institute of Standards and Technology (NIST) [29]. We could compare the reported results with our method using hydrogen as carrier gas (trace III in **Figure 6** and **Figure S26** in **Supporting Information**) and it was gratifying to see that interferences with other fatty acids can be avoided also under our conditions. As already shown for EPA isomers [18], the use of hydrogen as carrier gas together with specific oven temperature program is satisfactory, in terms of no interferences by C22-PUFAs and long chain saturated and monounsaturated fatty acids.

The combination of GC and ^{13}C NMR analyses, the latter using the resonance of the C-20, can be of diagnostic value for individuating monotrans DHA isomers, with the advantage that ^{13}C NMR can be performed directly on the triglycerides without any transformation. In this case, it is also worth noting that by studying the region between 130.40 and 132.60 ppm, also in different NMR solvents, the overview of omega-6 and omega-3 monotrans isomer content can be obtained, as shown by previous and actual work by us. This overall picture can be relevant for further developments in metabolomics research.

With these data in our hands we performed the analysis of the triglyceride fractions contained in DHA-containing supplements available on the markets in Italy and Spain. As matter of facts, analytical protocols to run quality control of supplements are increasingly addressed for the safety of producers and consumers, and researchers raised problems of oxidized or trans contaminants by evaluating commercial products of different countries [28, 29]. **Table 1** shows our results in terms of fatty acids, such as omega-3 DPA, DHA, the DHA monotrans isomers and the monounsaturated 24:1, detected in some representative supplements available in Italian and Spanish markets, presenting low and high-content of monotrans DHA isomers. The values of these

fatty acids are shown as relative percentages (% rel) in the whole fatty acid composition determined on the natural oil ingredient of the capsules. The full fatty acid analysis referred to 19 products of Italian and Spanish markets are available in **Supporting Information** (**Tables S2 and S3** and **Figures S23-26** of representative GC chromatograms).

Table 1. Representative fatty acid contents in terms of DPA, DHA, 24:1 and monotrans DHA isomers eluting in the GC time window 37-41.5 min (see **Figure 6** and **Figures S23-26**) obtained from natural oils of commercially available supplements in Italy and Spain. The values are expressed as percentages relative to 100% of the fatty acid peak areas identified in the GC analysis. In **Tables S2 and S3** (**Supporting Information**) the complete analysis for 19 products is reported.

	1	**2**	**4**	**5**	**8**	**13**	**14**	**15**	**19**
22:6 ω3 trans-Δ19	**0.12±0.01**	**0.06±0.00**	**0.13±0.08**	**0.12±0.04**	**0.09±0.01**	**0.27±0.03**	**0.34±0.03**	**0.31±0.02**	**0.66±0.24**
22:5 ω3 (DPA)	3.67±0.07	4.31±0.02	3.91±0.18	0.15±0.02	2.10±0.03	9.94±0.32	1.17±0.02	13.32±0.04	2.09±0.33
22:6 ω3 (DHA)	18.51±0.10	20.64±0.37	20.98±0.07	57.35±0.15	14.60±0.15	83.68±0.17	86.19±0.11	78.78±0.11	76.75±0.41
22:6 ω3 trans-Δ4	**0.08±0.00**	**0.01±0.00**	**0.07±0.00**	**0.17±0.00**	**0.08±0.00**	**0.09±0.03**	**0.16±0.02**	**0.18±0.03**	**0.30±0.01**
22:6 ω3 trans-Δ13	**0.19±0.02**	**0.02±0.00**	**0.14±0.03**	**0.33±0.01**	**0.09±0.01**	**0.10±0.04**	**0.46±0.03**	**0.35±0.02**	**0.74±0.02**
24:1	0.34±0.03	0.10±0.00	0.66±0.01	nd	0.24±0.01	0.14±0.03	nd	nd	0.34±0.14
22:6 ω3 trans-Δ7+Δ10	**0.26±0.04**	**0.02±0.00**	**0.29±0.02**	**0.50±0.02**	**0.07±0.00**	**0.20±0.03**	**0.68±0.01**	**0.49±0.03**	**1.00±0.14**
TOTAL monotrans DHA	**0.75±0.04**	**0.11±0.00**	**0.63±0.08**	**1.12±0.04**	**0.33±0.01**	**0.66±0.04**	**1.64±0.03**	**1.33±0.03**	**2.70±0.25**

In Table 1 is shown that the percentage of monotrans DHA isomers was found between 0.11% and 2.70%, with the highest trans contents contained in the DHA-richest formulas (*cfr.*, product 19 and product 2 in Table 1). This would suggest that the procedures in order to enrich DHA content in the oil be responsible of raising the isomer presence in the resulting material. Our studies on the trans lipid library support previously reported analyses of PUFA-containing oils and supplements, discovering contaminations either as oxidation and isomerization products [4,5,20,21,28,29], addressing the important issue of the precise monotrans isomer

identification in case of EPA and DHA. This is strongly needed especially because health benefits are highly discussed for omega-3 supplements, whereas contaminants can certainly annihilate positive effects in a dose-proportional manner, as shown by us in a murine model [15].

In conclusion, here we showed the extension of the trans lipid library to include an important long chain PUFA such as DHA, with the individuation of its structural changes due to chemical treatment as well as free radical reactivity, converting the cis geometry into trans. It is expected that this new knowledge will successfully integrate other analytical methodologies for the quality control of trans fats in health care and food products needed by regulatory bodies all over the world [36].

Conflict of Interest. The authors declare no conflict of interests.

Supporting Information. The following files are available free of charge: a) EDP-regioisomer identification with full assignment of ^{1}H and ^{13}C NMR resonances and examples of the 2D NMR experiments to corroborate assignments; b) experimental details of the monotrans DHA isomer preparation, separation and identification; c) further analytical information on triglyceride isomerization; d) fatty acid contents of commercially available supplements and representative gas chromatographic analyses in different conditions.

AUTHOR INFORMATION

Corresponding Author

*Carla Ferreri; email: carla.ferreri#@isof.cnr.it

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Funding Sources

The funding of the MSCA-ITN-2014-ETN - Marie Skłodowska-Curie ITN project CLICKGENE (#642023) supported research activity as part of PhD training program of GM and GG. RS received a grant from the Di Bella Foundation. This work was partially funded by the Basque Country Government (Department of the Environment, Regional Planning**,** Agriculture and Fisheries). J.A. is recipient of a PhD grant from AZTI.

ACKNOWLEDGMENT

C.F. is grateful to Dr. Matxalen Uriarte (AZTI) for her strong commitment to introduce the subjects of lipidomic analyses and personalized health in the Basque Country.

ABBREVIATIONS

ACOH, acetic acid; Ag-TLC, silver-thin layer chromatography, CAM, cerium ammonium sulfate/ammonium molibdate reagent; C_6D_6, deuterated benzene; $CDCl_3$, deuterated chloroform; DCM, dichloromethane; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EDP, epoxydocosapentaenoic acid; EPA, eicosapentaenoic acid FAME, fatty acid methyl esters; *m*-CPBA, meta-chloroperoxybenzoic acid; PUFA, polyunsaturated fatty acid; TFA, trans fatty acid.

REFERENCES

(1) Siddiqui, R. A., Harvey, K., and Stillwell, W. (2008) Anticancer properties of oxidation products of docosahexaenoic acid. *Chem. Phys. Lipids 153*, 47–56.

(2) Scientific Opinion of the Panel on Dietetic products, Nutrition and Allergies on a request from European Commission related to labelling reference intake values for n-3 and n-6 polyunsaturated fatty acids. (2009) *The EFSA Journal 1176*, 1–11.

(3) Sanders, TA. (2009) DHA status of vegetarians. *Prostaglandins Leukot. Essent. Fatty Acids 81*, 137–141.

(4) Fournier, V., Destaillats, F., Hug, B., Golay, P.A., Joffre, F., Juanéda, P., Sémon, E., Dionisi, F., Lambelet, P., Sébédio, J.L., and Berdeaux, O. (2007) Quantification of eicosapentaenoic and docosahexaenoic acid geometrical isomers formed during fish oil deodorization by gas-liquid chromatography. *J. Chromatogr. A 1154*, 353–359.

(5) Sciotto, C., and Mjøs, S.A. (2012) Trans isomers of EPA and DHA in omega-3 products on the European market. *Lipids 47*, 659–667.

(6) Liu, X.-R., Deng, Z.-Y. Deng, Hu, J.-N-, and Fan, Y.-W. (2013) Erythrocyte membrane trans-fatty acid index is positively associated with a 10 year CHD risk probability. *Br. J. Nutr. 109*, 1695–1703.

(7) Anderson, A. K., McDougald, D. M., and Steiner-Asiedu, M. (2010) Dietary trans fatty acid intake and maternal and infant adiposity. *Eur. J. Clin. Nutr. 64*, 1308–1315.

(8) Chatgilialoglu, C., Ferreri, C., Melchiorre, M., Sansone, A., and Torreggiani, A. (2014) Lipid geometrical isomerism: from chemistry to biology and diagnostics. *Chem Rev. 114*, 255–284.

(9) Chatgilialoglu, C., Ferreri, C., Guerra, M., Samadi, A., Bowry, V. (2017) The Reaction of Thiyl Radical with Methyl Linoleate: Completing the Picture. *J. Am. Chem. Soc. 139*, 4704–4714.

(10) Ferreri, C., Sassatelli, F., Samadi, A., Landi, L., and Chatgilialoglu, C. (2004) Regioselective cis-trans isomerization of arachidonic double bonds by thiyl radicals: the influence of phospholipid supramolecular organization. *J. Am. Chem. Soc. 126*, 1063−1072.

(11) Ferreri, C., Faraone Mennella, M. R., Formisano, C., Landi, L., and Chatgilialoglu, C. (2002) Arachidonate geometrical isomers generated by thiyl radicals: the relationship with trans lipids detected in biological samples. *Free Radic. Biol. Med. 33*, 1516−1526.

(12) Zambonin, L., Ferreri, C., Cabrini, L., Prata, C., Chatgilialoglu, C., and Landi, L. (2006) Occurrence of trans fatty acids in rats fed a trans-free diet: a free radical-mediated formation? *Free Radic. Biol. Med. 40*, 1549–1556.

(13) Ferreri, C., Kratzsch, S., Brede, O., Marciniak, B., and Chatgilialoglu C. (2006) Trans lipid formation induced by thiols in human monocytic leukemia cells. *Free Radic. Biol. Med. 38*, 1180–1187.

(14) Souabni, H., Thoma, V., Bizouarn, T., Chatgilialoglu, C., Siafaka-Kapadai, A., Baciou, L., Ferreri, C., Houée-Lévin, C., and Ostuni, M.A. (2012) *Trans* arachidonic acid isomers inhibits NADPH-oxidase activity by direct interaction with enzyme components. *BBA Biomembranes 1818*, 2314–2324.

(15) Aoun, M., Feillet-Coudray, C., Fouret, G., Chabi, B., Crouzier, D., Ferreri, C., Chatgilialoglu, C., Cabello, C., Cristol, J. P., Carbonneau, M.A., and Coudray, C. (2012) Rat liver mitochondrial membrane characteristics and mitochondrial functions are more profoundly altered by dietary lipid quantity than by dietary lipid quality: effect of different nutritional lipid patterns. *Br. J. Nutr. 107*, 647–659.

(16) Grandgirard, A., Piconneaux, A., Sébédio, J. L., O'Keefe, S. F., Semon, E., and LeQuere, J.L. (1989) Occurrence of geometrical isomers of eicosapentaenoic and docosahexaenoic acids in liver lipids of rats fed heated linseed oil. *Lipids 24*, 799−804.

(17) Vendel Nielsen, L., Krogager, T.P., Young, C., Ferreri, C., Chatgilialoglu, C., Nørregaard Jensen, O., and Enghild, J.J. (2013) Effects of elaidic acid on HepG2 lipid metabolism, investigated by an integrated approach of lipidomics, transcriptomics and proteomics. *Plose One 8*, e74283.

(18) Ferreri, C., Grabovskiy, S., Aoun, M., Melchiorre, M., Kabal'nova, N., Feillet-Coudray, C., Fouret, G., Coudray, C., and Chatgilialoglu, C. (2012) Mono-trans eicosapentaenoic acid isomer library: a dual synthetic strategy and follow-up of rats fed deodorized fish oil diets. *Chem. Res. Toxicol*, *25*, 687–694.

(19) Vatéle, J.M., Doan, H.D., Chardigny, J.M., and Grandgirard, A. (1994) Synthesis of methyl (5Z, 8Z, 11Z, 14Z, 17E)-eicosapentaenoate and methyl (4Z, 7Z, 10Z, 13Z, 16Z, 19E) docosahexaenoate. *Chem. Phys. Lipids 74*, 185−193.

(20) Mjøs, S. A. (2005) Properties of trans isomers of eicosapentaenoic acid and docosahexaenoic acid methyl esters on cyanopropyl stationary phases. *J. Chromatogr., A 110*, 185–192

(21) Mjøś, S.A., and Solvang, M.(2006) Geometrical isomerization of eicosapentaenoic acid and docosahexaenoic acid at high temperatures. *Eur. J. Lipid Sci. Technol*. *108*, 589−597.

(22) VanRollins, M., Frade, P. D., and Carretero, O. A. (1989) Synthesis of epoxide and vicinal diol regioisomers from docosahexaenoate methyl esters. *J. Lipid Res*. *30*, 275–286.

(23) Gjerde, M., Jakobsen, A.V., and Trond, V.H. (2012) Concise synthesis of three ω-3 polyunsaturated fatty acids. *Tet. Lett. 54*, 5837−5839.

(24) Kato, T., Ishimatu, T., Aikawa, A., Taniguchi, K., Kurahashi, T., and Nakai, T. (2000) Preparation of the enantiomers of 19-epoxy docosahexanoic acids and their 4-hydroxy derivatives. *Tetrahedron: Asymm*. *11*, 851−860.

(25) Lucas, D., Goulitquer, S., Marienhagen, J., Fer, M., Dreano, Y., Schwaneberg, U., Amet, Y., and Corcos, L. (2010) Stereoselective epoxidation of the last double bond of polyunsaturated fatty acids by human cytochromes P450. *J. Lipid Res*. *51*, 1125−1133.

(26) Zhang, G., Kodani, S., and Hammock, B.D. (2014) Stabilized epoxygenated fatty acids regulate inflammation, pain, angiogenesis and cancer. *Progr. Lipid Res. 53*,108–123.

(27) Fer, M., Dréano, Y., Lucas, D., Corcos, L., Salaün, J.P., Berthou, F., and Amet Y. (2008) Metabolism of eicosapentaenoic and docosahexaenoic acids by recombinant human cytochromes P450. *Arch. Biochem. Biophys. 471* 116–125.

(28) Albert, B.B., Derraik, J. G., Cameron-Smith, D., Hofman, P. L., Tumanov, S., Villas-Boas, S. G., Garg, M. L., and Cutfield, W.S. (2015) Fish oil supplements in New Zealand are highly oxidised and do not meet label content of n-3 PUFA. *Sci. Rep. 5*, 7928. DOI: 10.1038/srep07928.

(29) Tyburczy Srigley, C., and Rader, J. I. (2014) Content and composition of fatty acids in marine oil omega-3 supplements. *J. Agr. Food Chem*. *62*, 7268–7278.

(30) Samadi, A., Andreu, I., Ferreri, C., Dellonte, S., and Chatgilialoglu, C. (2004) Thiyl radical catalysed isomerization of oils: an entry to the trans lipid library. *J. Am. Oil Chem. Soc*. *81*, 753–758.

(31) Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem*. *226*, 497–509.

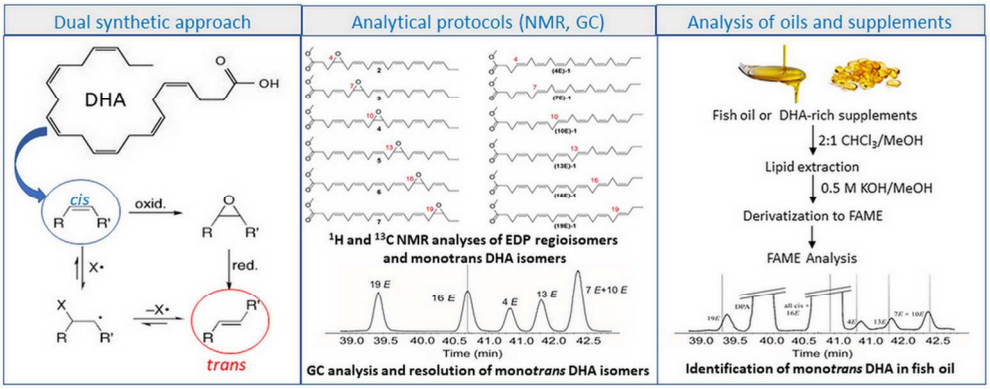
(32) Dais, P., Misiak, M., Hatzakis, E. (2015) Analysis of marine dietary supplements using NMR spectroscopy. *Anal. Methods 7*, 5226–5238.

(33) Siddiqui, N., Sim, J., Silwood, C. J. L., Toms, H., Iles, R. A., Grootveld, M. (2003) Multicomponent analysis of encapsulated marine oil supplements using high-resolution ^{1}H and ^{13}C NMR techniques. *J. Lipid Res. 44*, 2406–2427.

(34) Knothe, G., Kenar, J. A. (2004) Determination of the fatty acid profile by ^{1}H-NMR spectroscopy. *Eur. J. Lipid Sci. Technol. 106*, 88–96.

(35) Alexandri, E., Ahmed, R., Siddiqui, H., Choudhary, M. I., Tsiafoulis, C. G., Gerothanassis, I. P. (2017) High resolution NMR spectroscopy as a structural and analytical tool for unsaturated lipids in solution. *Molecules 22*, 1663; doi:10.3390/molecules22101663.

(36) Song, J., Park, J., Jung, J., Lee, C., Gim, S. Y., Ka, H., Kim, M.-J., Kim, C., and Lee, J. (2015) Analysis of trans fat in edible oils with cooking process. *Toxicol. Res. 31*, 307–312.



TOC graphics

127x50mm (300 x 300 DPI)