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Characterization of Triacylglycerol Enantiomers Using Chiral HPLC/APCI-MS and Synthesis of Enantiomeric Triacylglycerols

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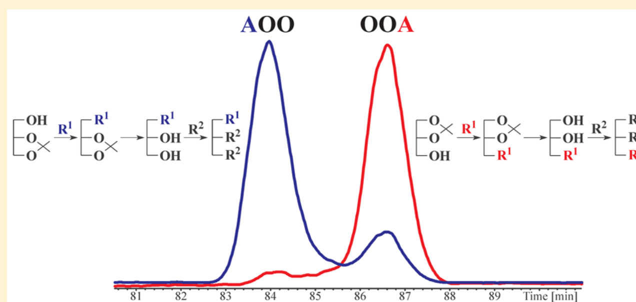
Ⓢ *Supporting Information*

ABSTRACT: In this work, the first systematic characterization of triacylglycerol (TG) enantiomers in real samples using chiral high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization mass spectrometry (APCI-MS) is performed. Our chiral HPLC/APCI-MS method is based on the use of two cellulose-tris-(3,5-dimethylphenylcarbamate) columns connected in series using a gradient of hexane-2-propanol mobile phase. All TG enantiomers containing 1−8 DBs and different fatty acyl chain lengths are separated using our chiral HPLC method except for TGs having a combination of saturated and di- or triunsaturated fatty acyls in *sn*-1 and *sn*-3 positions. In our work, the randomization reaction of monoacyl TG standards is used for the preparation of all TG enantiomers and regioisomers in a mixture, while the stereospecific esterification of 1,2- or 2,3-isopropylidene-*sn*-glycerols by selected fatty acids is used for the synthesis of TG enantiomers. The composition of TG enantiomers and regioisomers in hazelnut oil and human plasma samples is determined. Unsaturated fatty acids are preferentially esterified in *sn*-2 position in hazelnut oil, while no significant preference of saturated or unsaturated fatty acyls is observed in case of human plasma sample. Fatty acids with the higher number of DBs are preferred in *sn*-1 position of TG enantiomers in hazelnut oil unlike to moderate *sn*-3 preference in human plasma. The characterization of cholesteryl esters from TG fraction of human plasma sample using our chiral HPLC/APCI-MS method is presented as well.



Triacylglycerols (TGs) are important components of human diet used as a source of energy, fatty acids (including essential ones), fat soluble vitamins, and other nonpolar compounds. The variety of TG species is enormous due to a high number of fatty acids present in natural samples. They differ in fatty acyl chain lengths, number, positions, and cis-/trans- configuration of double bonds (DBs) and their position on the glycerol skeleton (regioisomers, enantiomers), all having great differences in their biological and nutritional properties. Nonaqueous reversed-phase (NARP) and silver-ion HPLC/MS represent the most widespread analytical techniques used for the characterization of natural TG mixtures. NARP-HPLC enables the separation of tens to hundreds TGs in natural samples[1−12] according to their equivalent carbon number (ECN)[3−6,9] even with the separation of cis-/trans-isomers,[13−15] DB positional isomers,[16−19] and linear/branched isomers.[15,20] Silver-ion chromatography provides the separation of TGs mainly according to their degree of unsaturation,[21−28] DB positional isomers[25,26] and cis-/trans-isomers.[13,21,23] Moreover, regioisomers can also be resolved under carefully optimized chromatographic conditions.[13,21,26] Good orthogonality of NARP and silver-ion chromatographic modes is used in two-dimensional HPLC in online[29−31] or off-line[13,32] setup for the characterization of complex TG mixtures. Gas chromatography with liquid stationary phase[33] can be also applied for the characterization of complex mixtures of cis-/trans-isomers as their fatty acid methyl esters. The prevailing fatty acid in *sn*-2 position can be determined using atmospheric pressure chemical ionization (APCI)[6,14,28,34] due to the lower relative abundance of fragment ion $[M+H-R^2COOH]^+$ formed by the neutral loss of fatty acid from this position.

The stereospecific analysis of individual TGs represents a challenging task in the lipidomics due to different stereo-availability of fatty acyls in the stereospecific environment of human body. The stereospecific analysis using silver-ion HPLC or APCI mass spectra enables the determination of fatty acids in *sn*-2 position (TG regioisomers), but without the resolution of *sn*-1 and *sn*-3 positions for TG enantiomers. Nowadays, the analysis of TG enantiomers is usually performed using chemical derivatization methods or chiral HPLC after their partial hydrolysis to diacylglycerols (DGs) by *sn*-1/3 stereoselective pancreatic lipase[35,36] or more often by Grignard reagent[37−39] without the specificity to any fatty acid and less fatty acyl migration. The chemical derivatization method uses the conversion of formed DGs to different derivatives (e.g., phospholipids[35]) followed by another *sn*-2 specific hydrolysis (e.g., using phospholipase A). Drawbacks of stereospecific

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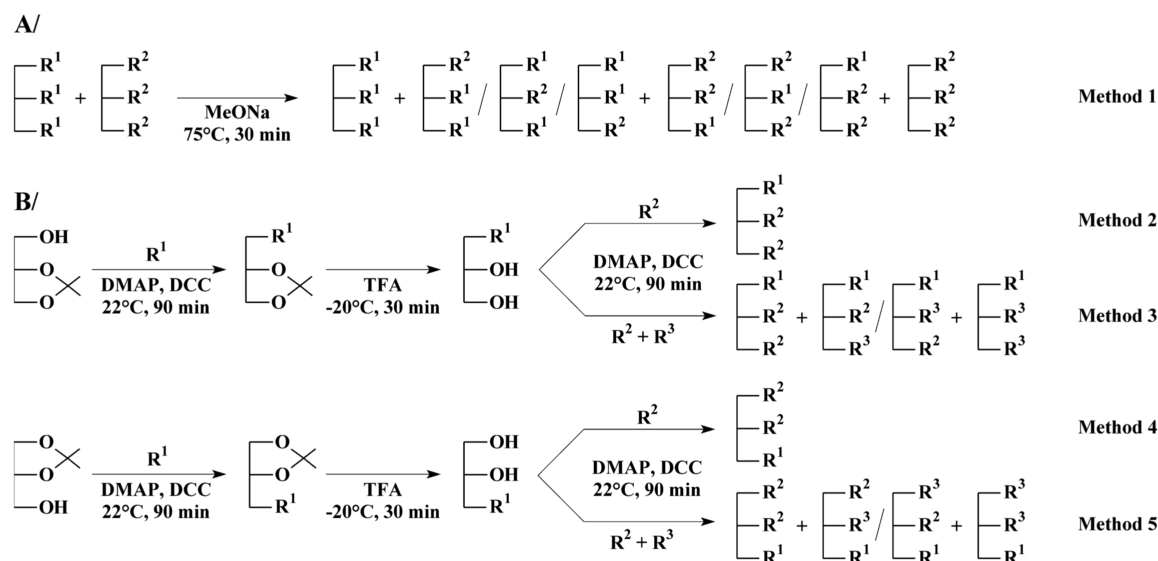

Figure 1. Scheme of synthetic procedures used for the preparation of triacylglycerol isomers: A/synthesis of the mixture of triacylglycerol regioisomers and enantiomers using the randomization reaction of monoacyl triacylglycerol standards, B/synthesis of enantiomers using the stereospecific esterification of 1,2- and 2,3-isopropylidene-*sn*-glycerols. MeONa, sodium methoxide; DMAP, 4-dimethylaminopyridine; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; R^1, R^2, R^3, different fatty acids esterified on the glycerol skeleton.

analysis using the chemical derivatization are laborious and time-consuming derivatization steps with a potential risk of fatty acyl migration strongly depending on reaction conditions.

The chiral HPLC uses the derivatization of DGs with chiral agents to form diastereoisomers (e.g., diastereoisomeric naphthylethylurethanes[37,39,40] or phenylethylcarbamates[36]), which can be easily separated using the conventional silicagel column in nonchiral normal-phase HPLC systems. Nonchiral agents can be used to form chiral derivatives (e.g., 3,5-dinitrophenylurethanes[38,41−43]), which are easily resolved using the chiral column. Intact DGs[44] or monoacylglycerols (MGs)[45−47] can also be directly separated by chiral HPLC without any derivatization. No systematic study of retention behavior of intact TG enantiomers or the analysis of real samples by chiral HPLC have been published so far. Polysaccharide-based chiral HPLC column with cellulose-tris-3,5-dimethylphenylcarbamate coated on the silicagel as a stationary phase and hexane-2-propanol mobile phase have been used for the separation of two pairs of intact TG enantiomers,[48] but only with fatty acyls with a great difference in the fatty acyl chain length and saturation degree (combination of two C8:0 with C20:5 or C22:6 fatty acyls). Such combinations do not occur in nature, where C16 and C18 fatty acyls with 0 to 3 DBs are strongly prevailing. The same column with methanol as the mobile phase has been used with so-called recycle chromatography for the partial separation of three TG enantiomeric pairs with retention times in the range of 150−190 min.[49] A great challenge in the stereospecific analysis of TGs is the lack of commercial standards of TG enantiomers. These standards are usually synthesized in the laboratory, which involves laborious synthesis in several steps using glycerol with blocked hydoxyl groups in specific positions, that is, 1,2- and 2,3-isopropylidene-*sn*-glycerols.[50,51]

The main goal of our work is the development of chiral HPLC/APCI-MS method for the analysis of TG enantiomers using polysaccharide based chiral HPLC columns. Two different approaches for the synthesis of TG standards are developed based on the randomization reaction and stereospecific esterification of glycerol applied for the synthesis of wide range of TG regioisomers and enantiomers used for the characterization of their retention behavior in the chiral HPLC. The developed method is applied for the stereospecific analysis of TGs in biological samples and preferences of individual fatty acyls in specific positions are discussed as well.

■ EXPERIMENTAL SECTION

Materials. Hexane, dichloromethane, chloroform (all HPLC grade), 2-propanol (HPLC/MS grade), methanol (HPLC gradient grade), sodium methoxide, sodium hydroxide, 1,2-isopropylidene-*sn*-glycerol, and 2,3-isopropylidene-*sn*-glycerol, 4-dimethylaminopyridine (DMAP), dicyclohexylcarbodiimide (DCC), and trifluoroacetic acid (TFA) (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The model mixture of TG standards GLC#435 (all saturated monoacyl TGs from C7:0 to C22:0), tripalmitin (PPP, C16:0), tristearin (SSS, C18:0), triarachidin (AAA, C20:0), trielaidin (EEE, Δ*9t*-C18:1), tripetroselinin (PePePe, Δ*6*-C18:1), tri-*cis*-vaccenin (cVacVacVa, Δ*11*-C18:1), triolein (OOO, Δ*9*-C18:1), trilinolein (LLL, Δ*9,12*-C18:2), trilinolenin (LnLnLn, Δ*9,12,15*-C18:3), trigamma-linolenin (γLnγLnγLn, Δ*6,9,12*-C18:3), and palmitic (P, C16:0), stearic (S, C18:0), arachidic (A, C20:0), oleic (O, Δ*9*-C18:1), linoleic (L, Δ*9,12*-C18:2), and linolenic (Ln, Δ*9,12,15*-C18:3) acids were purchased from Nu-ChekPrep (Elysian, MN, USA).

Sample Preparation. The fraction of TGs from plasma sample was collected after the HILIC separation of total lipid extract prepared using chloroform−methanol−water extraction procedure[52] (see Supporting Information). TGs from hazelnuts were extracted using hexane[7] (see Supporting Information). The samples were dissolved in hexane at the appropriate concentration before the HPLC analysis.

Synthesis of TG Isomers Using the Randomization Reaction. Mixtures of TG enantiomers and regioisomers with equal molar concentrations were prepared from monoacyl TG standards (i.e., AAA, OOO, LLL, etc.) using the randomization procedure[26] (Figure 1A, Method 1). Twenty mg of each TG standard and 100 mg of sodium methoxide were weighed into a

Table 1. Composition of Triacylglycerols in Synthesized Randomization Mixtures According to Method 1 Described in Figure 1

randomization mixture	composition of triacylglycerols[a]
AAA/OOO/LnLnLn	AAA; AAO/AOA/OAA; AOO/OAO/OOA; OOO; ALnA/AALn/LnAA; ALnO/OLnA/LnAO/LnOA/AOLn/OALn; OLnO/LnOO/OOLn; LnLnA/ALnLn/LnALn; LnLnO/OLnLn/LnOLn; LnLnLn
OOO/LLL/LnLnLn	OOO; OLO/LOO/OOL; OLnO/LnOO/OOLn; LLO/OLL/LOL; LLnO/LnLO/OLnL; LnOL/OLLn/LOLn; LLL; LnLnO/OLnLn/LnOLn; LnLL/LLnL/LLLn; LnLnL/LLnLn/LnLLn; LnLnLn
PPP/LLL/OOO	PPP; PPO/POP/OPP; POO/OPO/OOP; OOO; PLP/LPP/PPL; PLO/OLP/POL/LOP/LPO/OPL; OLO/LOO/OOL; LLP/PLL/LPL; LLO/OLL/LOL; LLL
SSS/LLL	SSS; SSL/SLS/LSS; SLL/LLS/LSL; LLL
PPP/LLL	PPP; PLP/LPP/PPL; LLP/PLL/LPL; LLL

[a]Sorted according to retention times in our chiral HPLC/APCI-MS method.

dry boiling flask with the addition of 2 mL of hexane dried with molecular sieves. The mixture was heated at 75 °C for 30 min in water bath under the reflux.Then, the mixture was extracted with water and methanol to remove sodium methoxide. The hexane phase containing synthesized TGs was evaporated using the gentle stream of nitrogen and redissolved before the HPLC/MS analysis in hexane at the appropriate concentration.

Stereospecific Synthesis of TG Enantiomers. TG enantiomers of $R^1R^2R^2$ (Method 2)/$R^2R^2R^1$ (Method 4) type and enantiomers with mixed fatty acyls $R^1R^2R^3$ (Method 3)/$R^3R^2R^1$ (Method 5) (Figure 1B) were synthesized from 1,2- and 2,3-isopropylidene-*sn*-glycerols. Ten milligrams of 1,2- or 2,3-isopropylidene-*sn*-glycerol, 20 mg of fatty acid, 10 mg of DMAP and 15 mg of DCC in 1 mL of dichlormethane were stirred in a vial for 1.5 h at ambient temperature. Then, residual hydroxyl groups in glycerol were deprotected by the reaction with 0.25 mL of ice cold TFA for 30 min at −20 °C and then TFA was neutralized with 2 mL of 2 mol/L ice cold sodium hydroxide. Formed MGs were extracted from the reaction mixture using 3 mL of chloroform - methanol (4:1, v/v) mixture and the chloroform layer was evaporated using the gentle stream of nitrogen. Prepared 1-MG (Methods 2 and 3) or 3-MG (Methods 4 and 5) were stirred with the total amount of 40 mg of fatty acids (one fatty acid in the case of Methods 2 and 4 or more fatty acids for Methods 3 and 5), 20 mg of DMAP and 30 mg of DCC in 1 mL of dichlormethane for 1.5 h at ambient temperature. Synthesized TG enantiomers were extracted from the reaction mixture using hexane, evaporated using the gentle stream of nitrogen and redissolved before the HPLC/MS analysis in hexane at the appropriate concentration.

Chiral HPLC/APCI-MS. HPLC experiments were performed on a liquid chromatograph Agilent 1200 Series (Agilent Technology, Waldbronn, Germany). The final HPLC method for analyses of TGs used the following conditions: two chiral chromatographic columns Lux Cellulose-1 with cellulose-tris-(3,5-dimethylphenylcarbamate) coated silicagel as the stationary phase (250 mm × 4.6 mm, 3 μm, Phenomenex, Torrance, CA, U.S.A.) connected in series, the flow rate 1 mL/min, the injection volume for standard mixtures and analyzed samples 1 μL, column temperature 35 °C and the mobile phase gradient: 0 min −90% A + 10% B, 180 min 60% A + 40% B, where A is hexane and B is hexane-2-propanol (99:1, v/v) mixture. The column was conditioned 60 min before each analysis to achieve good reproducibility. The ion trap analyzer Esquire 3000 (Bruker Daltonics, Bremen, Germany) with positive-ion APCI was used in the mass range m/z 50−1200 with the following setting of tuning parameters: pressure of the nebulizing gas 50 psi, drying gas flow rate 3 L/min, temperatures of the drying gas and APCI heater 350 and 400 °C, respectively. Reconstructed ion current chromatograms of protonated molecules and fragment ions were used to support the identification and integration of coeluting peaks.

Definition of Abbreviations. Identified TG species were annotated using initials of fatty acid trivial names sorted according to their stereochemical positions (*sn*-1, *sn*-2, and *sn*-3), for example, 1-octadec-9-enoyl-2-octadeca-9,12-dienoyl-3-hexadecanoyl-*sn*-glycerol was annotated as OLP. Abbreviations of fatty acids: M, myristic (C14:0); P, palmitic (C16:0); Po, palmitoleic (Δ9-C16:1); Ma, margaric (C17:0); S, stearic (C18:0); Pe, petroselinic (Δ*6*-C18:1); O, oleic (Δ*9*-C18:1); E, elaidic (Δ*9t*-C18:1); cVa, *cis*-vaccenic (Δ*11*-C18:1); L, linoleic (Δ*9,12*-C18:2); Ln, linolenic (Δ*9,12,15*-C18:3); γLn, gamma-linolenic (Δ*6,9,12*-C18:3); A, arachidic (C20:0).

■ RESULTS AND DISCUSSION

Synthesis of TG Standards. Two different synthetic procedures are applied for the preparation of TG isomers in this work as shown in Figure 1. The randomization reaction (Method 1, Figure 1A) of monoacyl TG standards catalyzed by sodium methoxide is used for the synthesis of all enantiomeric and regioisomeric TGs in one mixture based on our previously developed randomization procedure.[26] During the randomization process, individual fatty acyls in TGs are randomly distributed by inter- and intraesterification on the glycerol skeleton providing TGs with a random combination of fatty acyls including their regioisomers and enantiomers (Table 1). Figure 2A shows a chiral HPLC separation of randomization mixture prepared from AAA, OOO, and LnLnLn standards. The randomization reaction provides an equimolar mixture of all TG isomers due to the random distribution of fatty acyls without any visible preference of individual fatty acids. Various randomization mixtures of TGs containing fatty acyl chain lengths with 16, 18, and 20 carbon atoms and the number of DBs from 0 to 3 have been prepared. These mixtures have been applied for the optimization of chiral HPLC method and the characterization of retention behavior of TG isomers (Figure 2A and Supporting Information Figures S-1 and S-2).

Although the randomization reaction is very simple and fast method for the synthesis of both types of isomers, it provides only their mixture and individual enantiomers have to be identified based on retention times of individual standards. Figure 1B shows a scheme of the synthesis of TG enantiomers based on the stereospecific esterification of free hydroxyl groups in 1,2- and 2,3-isopropylidene-*sn*-glycerols with protected hydroxyl groups in *sn*-1,2 or *sn*-2,3 positions, respectively. In the first step, the free hydroxyl group of isopropylidene is esterified with selected fatty acid (R^1) catalyzed by DMAP and DCC coupling agents. Then, protected hydroxyl groups are hydrolyzed using TFA acid at low temperature (−20 °C) yielding optically pure 1-MG

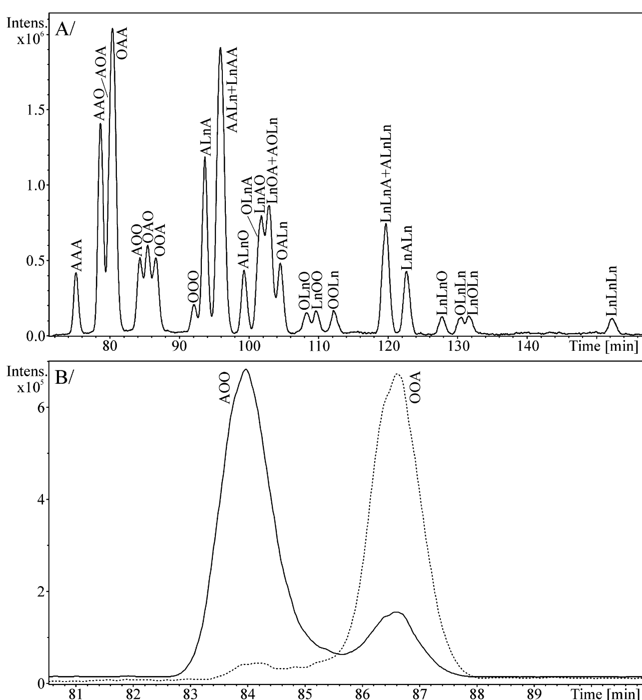

Figure 2. Comparison of chiral HPLC/APCI-MS chromatograms of synthesized triacylglycerol standards: A/mixture of all triacylglycerol isomers prepared by the randomization reaction of AAA, OOO, and LnLnLn standards according to Method 1, B/overlay of chromatograms of AOO and OOA enantiomers synthesized by the stereospecific esterification of 2,3- and 1,2-isopropylidene-*sn*-glycerols according to Methods 2 and 4, respectively. HPLC conditions: two Lux Cellulose-1 columns (250 mm × 4.6 mm, 3 μm, Phenomenex) connected in series, flow rate 1 mL/min, column temperature 35 °C, gradient 0 min −90% A + 10% B, 180 min 60% A + 40% B, where A is hexane and B is a mixture of hexane-2-propanol (99:1, v/v).

(Methods 2 and 3) or 3-MG (Methods 4 and 5). These MGs are esterified in the next step with another fatty acid (R^2, Methods 2 and 4) providing TG enantiomers. TG enantiomers composed from two different fatty acyls are prepared by this procedure (Figure 2B). Mixed-acyl TG enantiomers are obtained, if two or more fatty acids are added in the second esterification step (Methods 3 and 5). These TGs (Figure 3) are composed from selected fatty acyl (R^1) in *sn*-1 (Method 3) or *sn*-3 (Method 5) positions and randomly distributed fatty acyls (R^2 + R^3) on remaining *sn*-2,3 or *sn*-1,2 hydroxyl groups, respectively. The set of 8 mixtures of enantiomeric mixed-acyl TGs (Figure 3 and Supporting Information Figures S-3–S-5) has been prepared by this procedure from stearic, oleic, linoleic, and linolenic acids as C18 fatty acids with 0−3 DBs covering a wide range of most common combinations of fatty acids in natural TG samples. Individual fatty acids are first specifically esterified in *sn*-1 or *sn*-3 position and after the deprotection step an equimolar mixture of selected fatty acids is added for the random esterification of remaining hydroxyl groups. Table 2 shows a composition of synthesized mixtures of enantiomeric mixed-acyl TGs using this procedure (Methods 3 and 5).

A special attention have to be given to reaction conditions to prevent the fatty acyl migration during the synthesis. Low temperature of deprotection step is crucial for the prevention of fatty acyl migration on the glycerol skeleton. If deprotection temperature is higher or reagents are not properly cooled before the reaction, increased rate of fatty acyl migration is observed, which may result in the racemic mixture of both isomers. The optical purity of synthesized TG enantiomers under optimized reaction conditions is sufficient to obtain retention times of individual isomers and in most cases it is better than 93%.

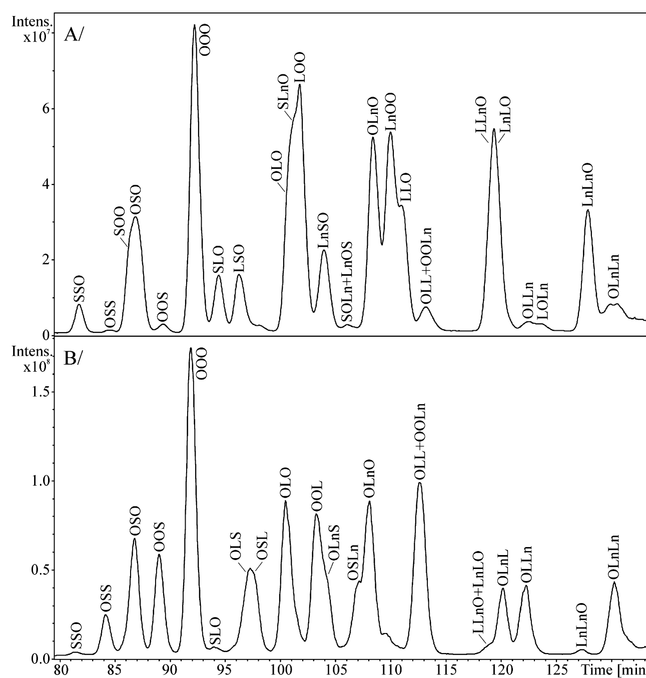

Figure 3. Chiral HPLC/APCI-MS chromatograms of synthesized mixtures of enantiomeric triacylglycerols of R^1R^2O type (A/Method 5) and OR^2R^3 type (B/Method 3), where R^i are randomly distributed stearic (S), oleic (O), linoleic (L) and linolenic (Ln) acyls in *sn*-1/2 or *sn*-2/3 positions. HPLC conditions are identical as for Figure 2.

Table 2. Composition of Enantiomeric Triacylglycerols in Mixtures Synthesized by Stereospecific Esterification of 1,2- and 2,3-Isopropylidene-*sn*-glycerols According to Method 3 and 5 Described in Figure 1

general triacylglycerol formula[a]	composition of triacylglycerols[b]
SR^2R^3	SSS; SSO/SOS; SOO; SSL/SLS; SLO/SOL; SLnS/SSLn; SLnO/SOLn; SLL; SLnL/SLLn; SLnLn
R^1R^2S	SSS; SOS/OSS; OOS; SLS/LSS; OLS/LOS; SLnS/LnSS; OLnS/LnOS; LLS; LLnS/LnLS; LnLnS
OR^2R^3	OSS; OSO/OOS; OOO; OLS/OSL; OLO/OOL; OLnS/OSLn; OLnO/OOLn; OLL; OLnL/OLLn; OLnLn
R^1R^2O	SSO; SOO/OSO; OOO; SLO/LSO; OLO/LOO; SLnO/LnSO; OLnO/LnOO; LLO; LLnO/LnLO; LnLnO
LR^2R^3	LSS; LSO/LOS; LOO; LLS/LSL; LLO/LOL; LLnS/LSLn; LLnO/LOLn; LLL; LLnL/LLLn; LLnLn
R^1R^2L	SSL; SOL/OSL; OOL; SLL/LSL; OLL/LOL; SLnL/LnSL; OLnL/LnOL; LLL; LnLL/LLnL; LnLnL
LnR^2R^3	LnSS; LnSO/LnOS; LnOO; LnLS/LnSL; LnLO/LnOL; LnLnS/LnSLn; LnLnO/LnOLn; LnLL; LnLnL/LnLLn; LnLnLn
R^1R^2Ln	SSLn; SOLn/OSLn; OOLn; SLLn/LSLn; OLLn/LOLn; SLnLn/LnSLn; OLnLn/LnOLn; LLLn; LLnLn/LnLLn; LnLnLn

[a] R^1, R^2, R^3 = different fatty acyls in *sn*-1, 2, 3 positions including S, stearic; O, oleic; L, linoleic; and Ln, linolenic acyls. [b] Sorted according to retention times in our chiral HPLC/APCI-MS method.

Chiral HPLC/APCI-MS Analysis. The chiral polysaccharide column packed with cellulose-tris-(3,5-dimethylphenylcarba-

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mate) selector has been used for this study as the most promising chiral stationary phase for the separation of nonpolar TG enantiomers based on literature[48,49] and manufacturers information. The separation in normal-phase mode is selected due to nonpolar character of TGs and their low solubility in polar solvents typical for the reversed-phase mode. The careful optimization of chromatographic conditions has been done with the goal to achieve the best separation of chiral isomers (regardless the analysis time) including the optimization of column length, separation temperature, mobile phase and gradient composition. The change of separation temperature (Supporting Information Figure S-6) does not show any significant trend in the chromatographic resolution of TGs unlike to NARP-HPLC analysis of TGs, where the chromatographic resolution increases with decreasing separation temperature.[9] The best separation of individual isomers is achieved at 35 °C (Supporting Information Figure S-6C). Hexane-based mobile phases with acetonitrile, 2-propanol and their mixtures have been tested (Supporting Information Figures S-7 and S-8). The hexane-2-propanol mobile phase (Supporting Information Figure S-7A) shows the best separation of TG isomers. Hexane−acetonitrile−2-propanol (Supporting Information Figure S-7B) and hexane−acetonitrile (Supporting Information Figure S-8) phases provide similar chromatographic separation as hexane-2-propanol mobile phase for TG isomers with 5−7 DBs, but enantiomeric pairs with 1 to 3 DBs are not separated at all.

The concentration of 2-propanol in the mobile phase and gradient steepness strongly influence retention times of TGs and also their chromatographic resolution. The final change of 2-propanol concentration in our gradient is 0.1%/hour, therefore, the precision of mobile phase preparation is especially important to achieve a good reproducibility of retention times of TGs using chiral HPLC. The column conditioning for 60 min with a flow rate of 1 mL/min is used between two runs, because it also shows a significant effect on the chromatographic reproducibility. The final chiral HPLC method provides retention times of TGs (Supporting Information Table S-1) with a standard deviation typically lower than 1.5 min among different days, which is still acceptable for the analysis in normal-phase mode and retention times up to 150 min.

Molecular weights and esterified fatty acyls of TGs are identified based on both protonated molecules and fragment ions in their positive-ion APCI mass spectra. TG regioisomers and enantiomers provide the same APCI mass spectra, but differences in relative abundances of fragment ions can be used for the differentiation of regioisomers (Supporting Information Figure S-9) based on well-known fact that the neutral loss of fatty acid from *sn*-2 position is less preferred.[6,14,28,34] For example, the ratio of fragment ions $[OO]^+/[AO]^+ = 15/100$ for OAO regioisomer (Supporting Information Figure S-9A) is lower due to the loss of arachidic acid from the *sn*-2 position compared to 43/100 for AOO (Supporting Information Figure S-9B) and 40/100 for OOA (Supporting Information Figure S-9C) enantiomers. On the other hand, negligible differences in the range of several percent are observed for the relative abundance of fragment ions of enantiomers, which is not sufficient for their differentiation and is attributed to common experimental variation of relative abundances, for example, $[OO]^+/[AO]^+ = 43 \pm 5/100$ for AOO (Supporting Information Figure S9-B) and $40 \pm 6/100$ for OOA (Supporting Information Figure S9-C) (data obtained from 4 consecutive runs). No measurable differences in ratios of relative abundances of protonated molecules vs fragment ions are observed for TG isomers. For this reason, individual TG enantiomers are synthesized for the determination of their retention order.

Retention Behavior of TGs in Chiral HPLC. Figure 4 shows the chiral HPLC/APCI-MS analysis of monoacyl TG

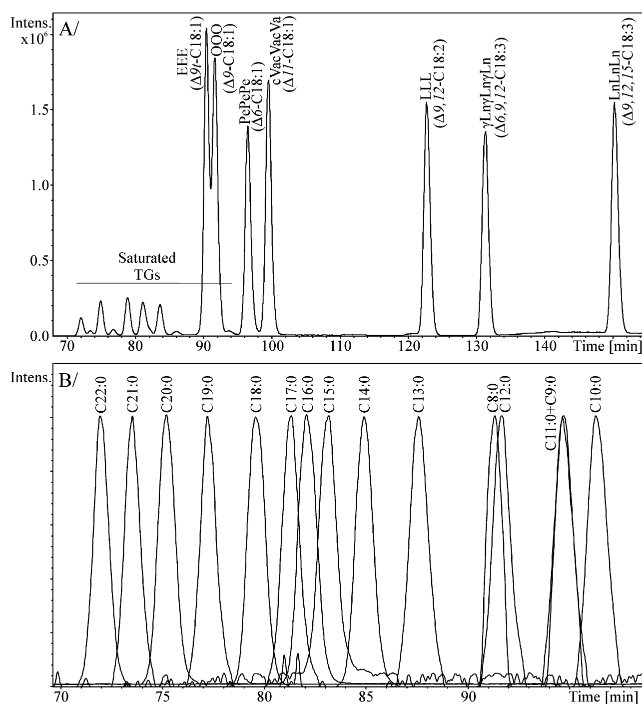

Figure 4. Effect of degree of unsaturation, double bonds configuration and position on the retention behavior of triacylglycerols in the chiral HPLC/APCI-MS analysis. A/Mixture of monoacyl triacylglycerol standards with saturated (from C8:0 to C22:0), monounsaturated (E, elaidic; O, oleic; Pe, petroselinic; cVa, *cis*-vaccenic), diunsaturated (L, linoleic), and triunsaturated (Ln, linolenic; γLn, gamma linolenic) fatty acyls, B/reconstructed ion current chromatograms of diacylglycerol fragment ions $[R^1R^1]^+$ of saturated triacylglycerols. HPLC conditions are identical as for Figure 2.

standards with saturated fatty acyls from C8:0 to C22:0 and C18 unsaturated fatty acyls containing 1 to 3 DBs with the different cis-/trans-configuration (Δ9-C18:1-OOO/EEE) and the position (C18:1-PePePe/OOO/cVacVacVa and C18:3-γLnγLnγLn/LnLnLn) of DBs. In general, the retention of TGs is governed by the number of DBs and their retention times increase with increasing number of DBs in fatty acyls. Retention times of TGs also strongly depend on cis-/trans-configuration of DBs, positions of DBs and lengths of fatty acyl chains. EEE (t_R = 90.2 min) with trans-configuration of DBs have slightly lower retention time compared to OOO (t_R = 91.7 min) with cis-configuration, which is the same pattern as observed for silver-ion HPLC,[13,21,23] but the difference between retention times of both isomers is significantly lower. All DB positional isomers with C18:1 and C18:3 fatty acyls are baseline separated, but their retention order cannot be predicted as the dependence on the increasing or decreasing distance of the first DB from the carbonyl group unlike to other chromatographic modes. In silver-ion HPLC, TG positional isomers elute in the order *Δ11*-C18:1 < *Δ9*-C18:1 < *Δ6*-C18:1 and *Δ9,12,15*-C18:3 < *Δ6,9,12*-C18:3 (ref 28 and our unpublished

data) compare to Δ*9*-C18:1 < Δ*6*-C18:1 < Δ*11*-C18:1 using chiral HPLC. Moreover, the reversed order of Δ*6,9,12*-C18:3 < Δ*9,12,15*-C18:3 positional isomers compared to Δ*9*-C18:1 < Δ*6*-C18:1 isomers is observed in the chiral HPLC. TGs are also partially separated according to fatty acyl chain lengths, as clearly demonstrated on the separation of saturated TGs (Figure 4B). Their retention times increase with decreasing fatty acyl chain length from C22:0 to C10:0 fatty acyls, but the retention order of shorter TGs is reversed, that is, C8:0 < C9:0 < C10:0. Unusual chromatographic behavior of TG positional isomers and TGs with different fatty acyl chain lengths is probably caused by the combination of different retention mechanisms in the chiral HPLC given by the column containing nonpolar chiral selector coated on silicagel stationary phase, where mechanisms of nonaqueous reversed-phase and normal-phase modes together with chiral interactions are combined. The accurate characterization of contributions of individual retention mechanism would require more systematic study on different chiral columns.

The separation of TG enantiomers using chiral HPLC is governed by the number of DBs of esterified fatty acyls in *sn*-1 and *sn*-3 positions without significant influence of fatty acyl in *sn*-2 position. In the case of enantiomeric pairs containing only one DB in *sn*-1 and *sn*-3 positions (i.e., combination of oleic and saturated fatty acyls), enantiomers with DB in *sn*-1 position have higher retention times compared to *sn*-3 position. For example, enantiomer OOA (86.5 min) with oleic acid in *sn*-1 position has higher retention time than AOO (84.3 min) isomer (Figure 2**A**). The effect of the number of DBs in *sn*-2 position on the separation of enantiomers can be demonstrated on the enantiomeric pair OLnA (101.0 min) and ALnO (98.9 min), where linolenic acyl with 3 DBs in *sn*-2 position has no significant effect on the retention of isomers and their difference in retention times (Δ 2.1 min) is the same as for OOA/AOO pair (Δ 2.2 min). TGs with three and more DBs in outer positions have the reversed retention order of isomers. The enantiomer with fatty acyl containing more DBs in *sn*-3 position has higher retention time compared to *sn*-1 position, for example, OALn (t_R = 104.3 min) with linolenic acid in *sn*-3 position has higher retention time compared to LnAO (101.2 min), OOLn (112.5 min) compared to LnOO (109.3 min), etc. All enantiomers are at least partially separated except for some TGs having combinations of saturated and di- (linoleic) or triunsaturated (linolenic) fatty acyls in *sn*-1 and *sn*-3 positions, without visible separation, that is, LPP/PPL, LOP/POL, LLP/PLL, AALn/LnAA, LnOA/AOLn and LnLnA/ALnLn enantiomeric pairs. On the other hand, TGs with the combination of monounsaturated and saturated fatty acyls in *sn*-1/3 positions are well separated, for example, AAO (78.6 min)/OAA (80.3), AOO (84.3)/OOA (86.5), etc. The fatty acyl chain length has also a partial effect on the separation of enantiomers, as illustrated on TG enantiomers containing palmitic or stearic acyls in the combination with linoleic acyl (Supporting Information Figure S-2). PPL/LPP and LLP/PLL enantiomeric pairs (Supporting Information Figure S-2B) are not separated at all unlike to SSL/LSS and SLL/LLS pairs (Supporting Information Figure S-2A), which are partially separated. The complex retention behavior can be demonstrated on enantiomeric pairs composed from three fatty acyls having identical fatty acyl composition, but in different stereochemical positions. For example, enantiomers in the randomization mixture prepared from AAA, OOO, and LnLnLn elute in the order ALnO (98.9 min, higher number of DB in *sn*-3 position) < OLnA (101.0, *sn*-1), LnAO (101.2, *sn*-1) < OALn (104.3, *sn*-3), and LnOA (102.8, *sn*-1) = AOLn (*sn*-3) (Figure 2**A**). The retention behavior of TG enantiomers in the chiral HPLC is rather complex and cannot be generalized for all isomers, therefore the use of identical standards for the initial identification of all enantiomers is essential. For this reason, individual enantiomeric isomers presented in analyzed samples are synthesized in this work to confirm their retention order.

Analysis of Real Samples. The developed chiral HPLC/APCI-MS method is applied for the characterization of composition of TG isomers in natural samples represented by plant (hazelnut oil) and human (plasma) samples. Figure 5 shows chiral HPLC/APCI-MS chromatograms of hazelnut oil and human plasma samples with identified TG isomers. TGs marked by asterisk mean that the determination of enantiomer is not unambiguous due to the coelution of both isomers (e.g., LOP* and LLP* in hazelnut oil) or missing identical standard for low abundant TGs (e.g., SPP*, SPO*, and OSP* in the plasma sample). For these TGs, fatty acyls in *sn*-2 positions are identified based on APCI mass spectra and fatty acyls in *sn*-1 and *sn*-3 positions are arranged according to decreasing molecular masses, that is, LPP, LOP, LLP, etc. In total, 19 (hazelnut oil) and 26 (human plasma) TGs including enantiomers and regioisomers are identified. The number of identified TGs in analyzed samples is lower compared to our previous NARP-HPLC/MS and 2D-HPLC/MS methods, that is, 30 TG species have been identified in hazelnut oil NARP-HPLC/MS.[10] On the other hand, these methods do not provide any information about the enantiomeric composition. The lower number of identified TGs can be probably explained by the fact that small peaks of trace TGs are further resolved into individual stereoisomers resulting in the decrease of their

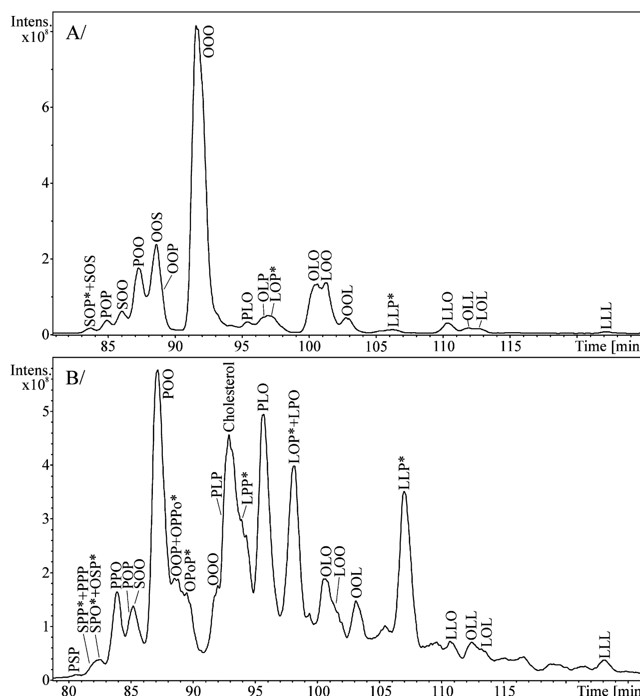

Figure 5. Chiral HPLC/APCI-MS chromatograms of: A/hazelnut oil, and B/human plasma samples. HPLC conditions are identical as for Figure 2. TGs marked by asterisk mean that the determination of enantiomer is not unambiguous because of the coelution of both isomers or missing identical standard for low abundant TGs.

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peak areas. In complex biological samples, these small peaks can be overlapped by peaks of more abundant TGs. The next step will be the use of two-dimensional HPLC (NARP × chiral), which should yield the same number of identified TGs together with their enantiomeric resolution. Ionization efficiencies of enantiomers are identical, therefore peak ratios of resolved enantiomers correspond to their concentration ratios.

Cholesterol and 12 cholesteryl esters (CEs) containing 9 fatty acyls are identified in TG fraction from the total lipid extract of human plasma sample (Supporting Information Figure S-10 and Table S-2) because of the coelution of cholesterol and CEs with TGs in the HILIC mode used for the fractionation. CE species are annotated by the carbon number (CN) and the number of DBs (CN:DB-CEs) without the identification of DB position in fatty acyl chains or the chirality of sterol part, which is not the goal of this work. Individual fatty acyls are identified based on the neutral loss of corresponding fatty acid providing the most abundant fragment ion in positive-ion APCI mass spectra of CEs (Supporting Information Figure S-11). The relative abundance of protonated molecules in APCI spectra of CEs strongly depends on the saturation degree of esterified fatty acyls and it is higher for unsaturated fatty acyls (4% for C18:2-CE and 10% for C20:4-CE), while $[M + H]^+$ ion is completely missing for monounsaturated and saturated fatty acyls. Molecular weights of these CEs are determined based on potassium adducts $[M + K]^+$. The adduct of molecule with the fragment ion m/z = 369 is also observed for CEs with unsaturated fatty acyls (e.g., Supporting Information Figure S-11). The most abundant CEs in human plasma are C18:2-CE, C18:1-CE, and C16:0-CE. This corresponds with the fatty acid composition of identified TGs, where TGs containing L, O, and P acyls are also the most abundant species. CEs with the identical fatty acyl composition in different retention times are detected (Supporting Information Table S-2), that is, two species of C16:1-CE (t_R = 16.0 and 17.3 min), C18:1-CE (16.6 and 17.8 min), and C18:2-CE (20.1 and 27.0 min), but without the identification of the isomerism (cholesterol enantiomers or positional isomer of DBs) due to the lack of identical standards.

The relative abundance of TG enantiomers and regioisomers in hazelnut oil and human plasma is listed in Table 3. The sum of peak areas from reconstructed ion chromatograms of fragment ions and protonated molecules is used for the determination of isomeric ratios, that is, the sum of $[M + H]^+$ at m/z 883 and fragment ions $[OO]^+$ at m/z 603 and $[OL]^+$ at m/z 601 is used for the determination of OLO, LOO, and OOL ratios. The ratio of coeluted enantiomers cannot be determined (e.g., LLP and PLL), because they provide the same fragment ions with comparable relative abundances. In the case of the coelution of enantiomers and regioisomers (e.g., POP and OPP in hazelnut oil and human plasma samples), the presence of regioisomer (OPP) in the peak can be confirmed or excluded based on the comparison of fragment ion ratio in the chromatographic peak with the ratio known for pure standards, that is, the ratio of fragment ions $[OP]^+/[PP]^+$ = 100:23 in hazelnut oil or 100:26 in plasma compared to 100:31 for POP and 100:75 for OPP standards exclude possible coelution of OPP isomer in the peak. If both isomers are present in the peak, the ratio of fragment ions is between these two values.

Table 3. Relative Ratios of Triacylglycerol Isomers in Hazelnut Oil and Human Plasma Samples Determined by Chiral HPLC/APCI-MS

triacylglycerol isomers	hazelnut oil	plasma
SSO/(SOS + OSS)	0/100 (SOS)[a]	0/0
PPO/(POP + OPP)	0/100 (POP)[a]	61/39 (POP)[a]
SOO/OSO/OOS	39/0/61	100/0/0
POO/(OPO + OOP)	54/46 (OOP)[a]	82/18 (OOP)[a]
PLP/(LPP + PPL)	0/0	23/77[b]
PLO/OLP/(POL + LOP + LPO)/OPL	27/29/44 (POL +LOP)[a,b]/0	55/0/45[b]/0
OLO/LOO/OOL	46/39/15	43/30/27
LPL/(LLP + PLL)	0/100[b]	0/100[b]
LLO/OLL/LOL	55/23/22	35/46/19

[a]Prevailing triacylglycerol isomer. [b]Relative ratios of these enantiomers cannot be determined due to the coelution.

TGs in both samples are composed mainly from P, O and L acyls with similar combinations in TGs, but their distribution in individual stereochemical positions is different (Table 3). The *sn*-2 position in hazelnut oil TGs is preferentially esterified by unsaturated fatty acyls, because no isomer with saturated fatty acyl in this position is identified. These results fit well with our previously published data on the sunflower oil using silver-ion HPLC.[15] Human plasma TGs contain both saturated and unsaturated fatty acyls in *sn*-2 position without a clear preference of one type. For example, groups of TG isomers PPO/POP/OPP = 61:39:0 and PLP/(LPP + PPL) = 23:77 show higher abundances of TGs containing saturated fatty acyls in *sn*-2 position (i.e., PPO and LPP+PPL) compared to groups POO/OPO/OOP = 82:0:18 and LPL/(LLP + PLL) = 0:100, where no isomers with saturated fatty acyls in *sn*-2 position are identified. Preferences in the esterification of fatty acyls in *sn*-1 and *sn*-3 positions are probably caused by the different selectivity of enzymes involved in the synthesis of TGs. Fatty acyls with the higher number of DBs are preferred in *sn*-1 position in hazelnut oil, while they are slightly preferred in *sn*-3 position in plasma, i.e., the ratio of enantiomers SOO/OOS = 39/61 in hazelnut oil and 100/0 in plasma, LOO/OOL = 39:15 and 30:27, and LLO/OLL = 55:33 and 35:46.

CONCLUSIONS

The first ever reported method for the routine chiral analysis of enantiomeric TGs in natural samples is presented here. This work opens new field of chiral lipidomic analysis, because differences between *sn*-1 and *sn*-3 positions have been neglected so far. Our chiral HPLC/APCI-MS method under optimized chromatographic conditions enables the separation of most TG enantiomers and regioisomers according to the DB composition of fatty acyls. Two different approaches are applied for the synthesis of TG isomers. The randomization reaction of monoacyl TG standards is applied for the preparation of mixtures of TG enantiomers and regioisomers, which are then used for the characterization of retention behavior. The synthesis of TG enantiomers even with mixed fatty acyls is developed for the determination of individual enantiomers using the stereospecific esterification of 1,2- or 2,3-isopropylidene-*sn*-glycerol. The optimized chiral HPLC/APCI-MS method is applied for the characterization of TG isomer composition in real samples represented by hazelnut oil and human plasma samples, where the preference of saturated or unsaturated fatty acyls in different positions is recognized. Unsaturated fatty acyls in *sn*-2 position and fatty acyls with a higher number of DBs in *sn*-1 position are preferred in hazelnut oil, while no significant preference of fatty acyl in *sn*-2 position and only moderate preference of fatty acyls with the higher number of DBs in *sn*-3 position is observed in plasma sample.

Our results show that natural distribution of fatty acids is not random, but different for various organisms.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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