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Regioisomeric Characterization of Triacylglycerols Using Silver-Ion HPLC/MS and Randomization Synthesis of Standards

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Silver-ion normal-phase high-performance liquid chromatography (HPLC) provides a superior separation selectivity for lipids differing in the number and position of double bonds in fatty acid chains including the resolution of triacylglycerol (TG) regioisomers under optimized conditions. Our silver-ion HPLC method is based on the coupling of three columns in the total length of 75 cm and a new mobile phase gradient consisting of hexane−acetonitrile−2-propanol which provides better resolution and also reproducibility in comparison to previously used mobile phases. In our work, the chemical interesterification (randomization) of single-acid TG standards is used for the generation of regioisomeric series of TGs, because it provides a random distribution of fatty acids in TGs at well-defined concentration ratios. The baseline separation of regioisomeric TG pairs containing up to three double bonds and the partial separation of TG regioisomers with four to seven double bonds are reported for the first time. Our silver-ion high-performance liquid chromatography/mass spectrometry (HPLC/MS) method is applied for the regioisomeric characterization of complex samples of plant oils and animal fat, where the results clearly demonstrate different preference of *sn*-2 occupation in plants (mainly unsaturated fatty acids) versus animal fat (mainly saturated fatty acids).

Triacylglycerols (TGs) are the main constituents of plant oils[1−3] and animal fats[4] characterized by the total carbon number (CN), the type and stereospecific position of fatty acids, and the number, position, and configuration of double bonds (DBs) in acyl chains. Fatty acids are digested in the human or animal organism with the assistance of the stereospecific lipases, where fatty acids from *sn*-1 and *sn*-3 position are cleaved first yielding 2-monoacylglycerols. The stereospecific analysis of TGs is a long-standing and challenging problem in the lipid analysis, but it is of high importance due to the different availability of fatty acids for the human body depending on *sn* positions. Concerning the overall characterization of TGs in complex natural mixtures, the best results are obtained with nonaqueous reversed-phase (NARP) high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS), where the highest number of identified TGs has been reported for the coupling of chromatographic columns in the total length of 45 cm and the mobile phase gradient consisting of acetonitrile−2-propanol.[5−7] The retention depends on the equivalent carbon number (ECN) defined as the total CN in all acyl chains minus 2 times the number of DBs, but the optimized NARP systems can resolve most TGs even within one ECN group with the exception of positional isomers. The partial separation of regioisomers in NARP mode is feasible only with multiple column coupling and very long retention times in the range of 100−200 min,[8,9] which is not practical for routine analysis.

The main possibilities of regiospecific analysis of TGs are the following: (1) silver-ion HPLC,[10−13] (2) MS,[5−7] (3) enzymatic hydrolysis (e.g., pancreatic lipase, phospholipase A_2)[14] followed by some analytical technique (e.g., silver-ion HPLC), and (4) derivatization[15] followed by chiral HPLC (complicated, laborious, not suitable for complex mixtures). Silver-ion normal-phase HPLC is a powerful technique for the separation of lipids differing in the number and position of DBs. The principle of this method is based on the capability of unsaturated organic compounds to create weak reversible complexes with transition metals, such as a silver ion. Silver ions are integrated into the silica stationary phase (preferably on the basis of ion-exchange mechanism) interacting with π electrons of DBs during the sample elution throughout the chromatographic column. The retention of each sample compound primarily depends

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on the number of DBs, but it is also affected by the steric availability of DBs for the interaction with silver ions. The retention increases with increasing number of DBs with the secondary separation according to the position and geometry of DBs. Off-line or online two-dimensional (2D) HPLC of relatively complementary separation modes in NARP and silver-ion HPLC is a highly promising method for the analysis of TGs.[16,17]

The different ratios of $[M + H - R_iCOOH]^+$ fragment ions in atmospheric pressure chemical ionization (APCI) mass spectra of positional isomers (regioisomers) of TGs was first reported in 1996[18] and later on applied for HPLC/APCI-MS characterization of prevailing fatty acids in *sn*-2 position in plant oils.[19] All MS approaches are based on the fact that the neutral loss of fatty acid from the *sn*-2 position yields the fragment ion with a lower relative abundance compared to cleavages from the side *sn*-1/3 positions. It is often applied for the assignment of prevailing fatty acids in the *sn*-2 position, but for the quantitative determination of *sn*-2 occupation the calibration curves for mixtures of both regioisomers have to be measured[20−23] using the same instrument and ionization technique. APCI is the most frequently used ionization technique for TG analysis due to their nonpolar character, but electrospray ionization (ESI) can be applied as well due to the formation of ammonium adducts.[24−26]

If the TG has different fatty acids in *sn*-1 and *sn*-3 positions, then the carbon atom in the *sn*-2 position becomes a chiral center. Common analytical techniques working in a nonchiral environment cannot differentiate between *sn*-1 and *sn*-3 enantiomers; therefore, they are generally treated as equivalent due to the lack of suitable analytical techniques for their analysis.[3,5,19,21] No chiral separation of intact TGs have been reported so far with the exception of synthetic TGs containing very different fatty acids C8:0 versus C22:5 or C22:6,[27] which is not a combination occurring in nature. Due to the absence of any official recommendation for the designation of *sn*-1/3 isomers, we list them in the order of decreasing masses in accordance with our previously proposed rule,[20] e.g., OLP but not PLO. In case of isobaric fatty acids in *sn*-1/3 positions, the more common fatty acid is listed first, e.g., LnOγLn but not γLnOLn.

The chemical interesterification (so-called randomization) has been used in many industrial applications in fat and oil processing.[28] It changes physicochemical properties of natural oils with the assistance of fatty acids already present in TGs in a given oil or fat. The degree of change is based on the reaction temperature, reaction time, and catalysts used. The most common catalyst in this process is sodium methoxide, but it is possible to use bases, acids, and some metal ions as well. Sodium methoxide has the highest reactivity, but on the other hand, it is very sensitive to any trace of water, which stops the reaction due to the hydrolysis of sodium methoxide. In the first reaction step, the bonds between glycerol and fatty acids are cleaved yielding a mixture of diacylglycerols (DGs), monoacylglycerols (MGs), and fatty acids. These species subsequently undergo the interesterification reactions providing a random distribution of fatty acids in newly formed TGs.

The main goal of our work is the development of a silver-ion HPLC/MS method applicable for the separation and quantitation of regioisomeric ratios of TGs in complex mixtures. TG regioisomeric pairs are separated by the optimized silver-ion HPLC method, identified based on characteristic differences in their APCI mass spectra, and quantified according to the ratio of chromatographic peak areas. The randomization procedure plays an important role in generating the series of regioisomeric standards. Applications to complex natural samples of plant oils and animal fats containing different TG regioisomers are demonstrated.

EXPERIMENTAL SECTION

Materials. Acetonitrile, 2-propanol, methanol, ethanol, propionitrile, ethylacetate, hexane (solvents are HPLC gradient grade), and sodium methoxide were purchased from Sigma-Aldrich (St. Louis, MO). Standards of tripalmitin (PPP, C16:0), triolein (OOO, Δ9-C18:1), trilinolein (LLL, Δ9,12-C18:2), and trilinolenin (LnLnLn, Δ9,12,15-C18:3) were purchased from Nu-ChekPrep (Elysian, MN). Palm and olive oils were purchased from Augustus Oil Limited (Hampshire, England).

Sample Preparation. An amount of 10−15 g of the sample (sunflower or blackcurrant seeds, fat tissue from pig) was weighed, and then seeds were carefully crushed in a mortar to fine particles, whereas fat tissue was crushed in a homogenizer. Then 15 mL of hexane was added, and this mixture was stirred occasionally for 15 min. The solid particles were filtered out using a course filter paper, and the extract was filtered again using a fine filter (0.45 μm). From the filtered extract, hexane was evaporated using a mild stream of nitrogen to yield pure plant oil or animal fat.

Randomization. Amounts of 50 mg of each TG standard and 100 mg of sodium methoxide were weighed into a dry boiling flask with the addition of 2 mL of hexane dried with molecular sieves. The mixture was heated for 30 min in a water bath under the reflux condenser. The reaction temperature was kept constant at 75 °C. Then, the mixture was extracted with water and three times with 1 mL of methanol to remove sodium methoxide. The hexane phase containing randomized analyte was injected into the silver-ion HPLC system.

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Silver-Ion HPLC. HPLC was performed on a liquid chromatograph Agilent 1200 series (Agilent Technology, Waldbronn, Germany). The final HPLC method for analyses of plant oils and animal fats used the following conditions: three silver-ion chromatographic columns ChromSpher Lipids (250 mm × 4.6 mm, 5 μm, Varian, Palo Alto, CA) connected in series, the flow rate of 1 mL/min, the injection volume of 1 μL, column temperature of 25 °C, and the mobile phase gradient of 0 min 100% A, 140 min 61% A + 39% B, where A is a mixture of hexane−2-propanol−acetonitrile (99.8:0.1:0.1, v/v/v) and B is the mixture of hexane−2-propanol−acetonitrile (98:2:2, v/v/v). The mobile phase was prepared fresh every day before analyses. Silver-ion columns were conditioned at 50 μL/min of the initial mobile phase composition overnight and at 1 mL/min for 1 h before the first analysis. The injector needle was washed with the mobile phase before each injection. The chromatographic system is equilibrated between injections for 30 min. The hybrid quadrupole time-of-flight (QqTOF) analyzer micrOTOF-Q (Bruker Daltonics, Bremen, Germany) with positive-ion APCI was used in the mass range m/z of 50−1200 with the following tuning parameters: flow of the nebulizing and drying gas 5 and 3 L/min, respectively, temperatures of the drying gas and APCI heater 300 and 400 °C, respectively. Reconstructed ion current chromatograms were used to support the identification and quantitation of coeluting peaks.

Fatty Acid Abbreviations. M, myristic (C14:0); C15:0, pentadecanoic; Po, palmitoleic (Δ9-C16:1); P, palmitic (C16:0); Mo, margaroleic (Δ9-C17:1); Ma, margaric (C17:0); St, stearidonic (Δ6,9,12,15-C18:4); Ln, α-linolenic (Δ9,12,15-C18:3); γLn, γ-linolenic (Δ6,9,12-C18:3); L, linoleic (Δ9,12-C18:2); O, oleic (Δ9-C18:1); S, stearic (C18:0); C20:2, eicosadienoic (Δ11,14-C20:2); G, gadoleic (Δ9-C20:1); A, arachidic (C20:0); B, behenic (C22:0); C23:0, tricosanoic (C23:0); Lg, lignoceric (C24:0).

RESULTS AND DISCUSSION

Optimization of Silver-Ion HPLC of TGs. It is well-known that the silver-ion chromatography suffers from a lower reproducibility of retention times in comparison to reversed-phase systems.[29] In the present work, the maximum attention has been paid to the optimization of chromatographic performance in terms of regioselectivity, reproducibility, and robustness. The most important parameters are the selection of column type and mobile phase composition. The optimization of mobile phase composition starts from the hexane−acetonitrile system which is the most widespread system used in silver-ion HPLC[13,30] providing the best results concerning the regioisomeric resolution.[12] The critical problem of this frequently used mobile phase is a low mutual miscibility of these solvents and the evaporative changes of prepared mobile phases, which significantly contributes to the reproducibility problems.[29] This knowledge has driven us to test the different mobile phase compositions with the goal to achieve a better separation selectivity and reproducibility of retention times for TGs. Extensive experiments with the combination of different polar modifiers (2-propanol, ethanol, propionitrile, and ethylacetate) in hexane[31] have shown that it is not easy to improve the selectivity of the hexane−acetonitrile systems. Almost comparable resolution is obtained with hexane−propionitrile systems, where the reproducibility problems are not encountered (in agreement with ref 32), but the serious drawback of this method is the toxicity of propionitrile. We discourage the use of propionitrile because of the health hazard in the laboratory. Other tested systems show poor regioisomeric selectivity. On the basis of these experiments, we have decided to add the third solvent to the hexane−acetonitrile system to improve the mutual miscibility while keeping the chromatographic performance, which has been achieved with the hexane−acetonitrile−2-propanol system. Then, the optimization

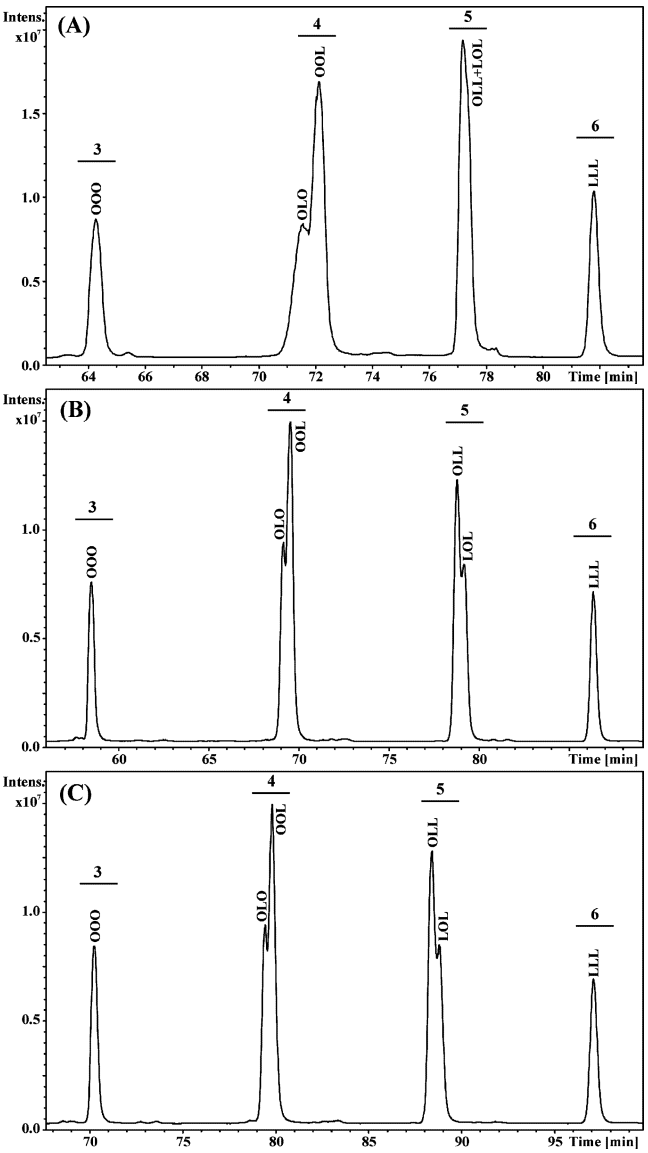

Figure 1. Effect of separation temperature on silver-ion HPLC/APCI-MS analysis of TGs in the randomization mixture of OOO/LLL: (A) 15, (B) 25, and (C) 40 °C. Other chromatographic conditions are described in the Experimental Section.

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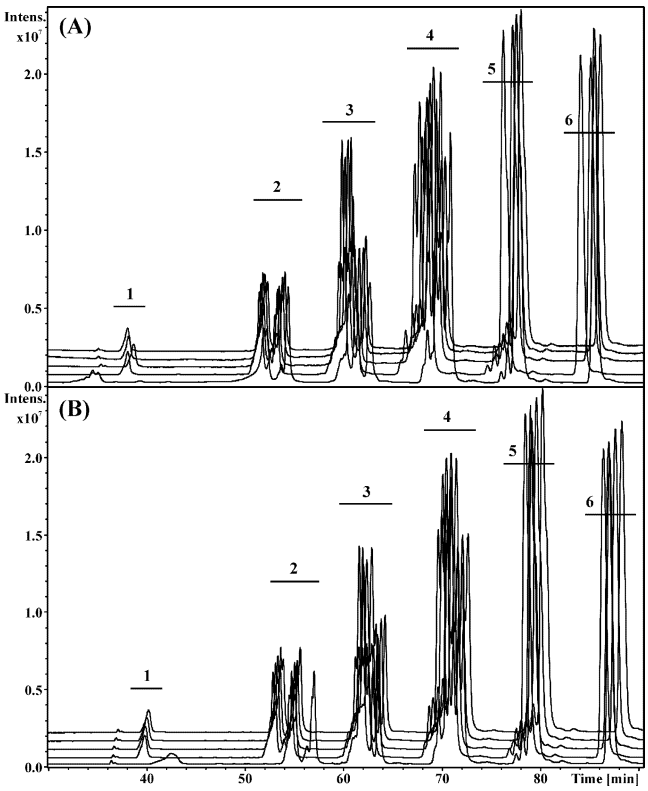

Figure 2. Separation reproducibility for sunflower oil analysis: (A) five consecutive injections in the first day; (B) five consecutive injections in the second day. Chromatographic conditions are described in the Experimental Section.

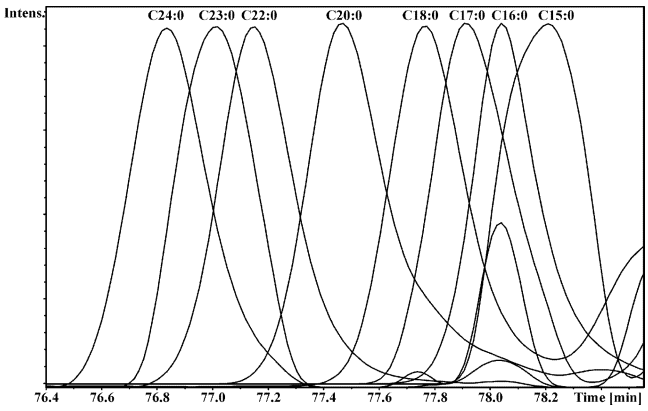

Figure 3. Effect of the fatty acid chain length on the retention in silver-ion HPLC/APCI-MS analysis of TGs in randomized sunflower oil shown by reconstructed chromatograms of $[XL]^+$ ions for XLL type TGs, where X is saturated fatty acid from C15:0 to C24:0.

of gradient conditions has been performed[31] resulting in the final method described in the Experimental Section.

The commercial silver-ion ChromSpher Lipids column provides the best performance and separation selectivity in the lipid analysis based on our previous experiences and the literature data as well.[12,13,16,17,25] An increased length of chromatographic column can improve the chromatographic resolution, as demonstrated earlier in NARP[20] or silver-ion[13] separations of TGs. Unlike NARP systems, the back pressure is not a limiting factor here, because the mobile phases typically consist of hexane with a low percentage of polar modifier. The limiting factors are mainly long retention times associated with the extended column length and

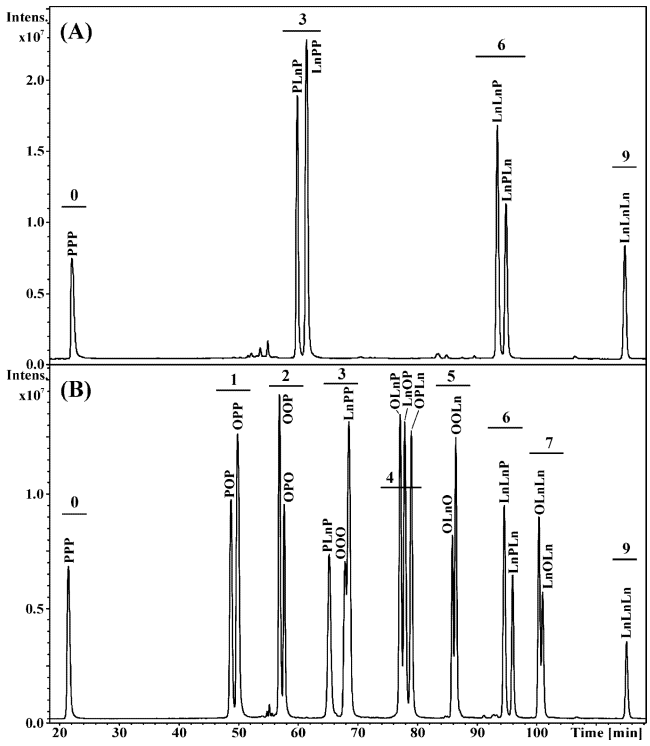

Figure 4. Silver-ion HPLC/APCI-MS analysis of randomization mixtures: (A) PPP/LnLnLn; (B) PPP/OOO/LnLnLn.

peak-broadening effects for multiple column coupling. On the basis of these considerations, the comparison of one, two, and three 25 cm ChromSpher Lipids columns connected in series has been performed (Supporting Information Figure S1). Retention times are approximately doubled and tripled for coupling of two or three columns but not exactly due to different retention characteristics of individual commercial columns.

The chromatographic resolution of lipids in NARP systems is significantly affected by temperature; generally, an improvement is observed at lower temperature. Contrary to NARP chromatographic systems, the retention in silver-ion HPLC increases with increased temperature (Figure 1). Peak shapes at low temperature (e.g., 15 °C in Figure 1A) are distorted. There is almost no visible difference in the resolution between 25 °C (Figure 1B) and 40 °C (Figure 1C), but the analysis time is shorter for 25 °C. Moreover, 40 °C is the maximum recommended temperature by the manufacturer; hence, 25 °C is selected as optimum temperature for further experiments.

Maximum attention has been paid to the right chromatographic practice to reduce the fluctuation of retention times, which involves mainly the following precautions. Mobile phases are prepared every day fresh using solvents dried with molecular sieves and kept in tightly closed bottles to avoid evaporation. All measurements are performed in a single block of several weeks on the same system without changing between normal- and reversed-phase systems. Before any measurement, the columns are conditioned using the low flow rate of initial gradient composition (50 μL/min) overnight and the standard flow rate for 1 h before the analysis.

A remarkable improvement in the reproducibility of retention times has been achieved in comparison to traditional hexane–acetonitrile mobile phases previously used in our laboratory. The

Table 1. Basic APCI-MS Fragmentation Characteristics of Analyzed Regioisomeric TGs in Randomization Mixtures

TG	DB[a]	ratio of $[M + H - R_iCOOH]^+$ ions[b]
POP	1	$[OP]^+/[PP]^+ = 100:33$
OPP		$[OP]^+/[PP]^+ = 100:80$
PLP	2	$[LP]^+/[PP]^+ = 100:39$
LPP		$[LP]^+/[PP]^+ = 100:87$
OOP		$[OP]^+/[OO]^+ = 100:62$
OPO		$[OP]^+/[OO]^+ = 100:20$
PLnP	3	$[LnP]^+/[PP]^+ = 100:49$
LnPP		$[LnP]^+/[PP]^+ = 100:97$
OLP		$[OL]^+/[OP]^+/[LP]^+ = 100:72:84$
LOP		$[OL]^+/[OP]^+/[LP]^+ = 100:97:53$
OPL		$[OL]^+/[OP]^+/[LP]^+ = 58:100:85$
LLP	4	$[LP]^+/[LL]^+ = 100:81$
LPL		$[LP]^+/[LL]^+ = 100:33$
OLO		$[OL]^+/[OO]^+ = 100:45$
OOL		$[OL]^+/[OO]^+ = 100:88$
OLnP		$[OLn]^+/[OP]^+/[LnP]^+ = 100:60:93$
LnOP		$[OLn]^+/[OP]^+/[LnP]^+ = 95:100:33$
OPLn		$[OLn]^+/[OP]^+/[LnP]^+ = 49:100:92$
OLL	5	$[OL]^+/[LL]^+ = 100:81$
LOL		$[OL]^+/[LL]^+ = 100:34$
LLnP		$[LLn]^+/[LP]^+/[LnP]^+ = 100:78:88$
LPLn		$[LLn]^+/[LP]^+/[LnP]^+ = 44:100:82$
LnLP		$[LLn]^+/[LP]^+/[LnP]^+ = 100:93:47$
OLnO		$[OLn]^+/[OO]^+ = 100:23$
OOLn		$[OLn]^+/[OO]^+ = 100:76$
OLnL	6	$[OL]^+/[OLn]^+/[LLn]^+ = 62:95:100$
OLLn		$[OL]^+/[OLn]^+/[LLn]^+ = 94:54:100$
LOLn		$[OL]^+/[OLn]^+/[LLn]^+ = 100:78:56$
LnLnP		$[PLn]^+/[LnLn]^+ = 100:78$
LnPLn		$[PLn]^+/[LnLn]^+ = 100:28$
LLnL	7	$[LLn]^+/[LL]^+ = 100:36$
LLLn		$[LLn]^+/[LL]^+ = 100:68$
OLnLn		$[OLn]^+/[LnLn]^+ = 100:79$
LnOLn		$[OLn]^+/[LnLn]^+ = 100:34$
LLnLn	8	$[LLn]^+/[LnLn]^+ = 100:36$
LnLLn		$[LLn]^+/[LnLn]^+ = 100:61$

[a] DB, number of double bonds. [b] Listed values correspond to the arithmetic mean of at least three consecutive measurements.

Table 2. Peak Area Ratios of TG Regioisomeric Groups in Silver-Ion HPLC/MS after the Randomization of Equal Amounts of PPP, OOO, LLL, and LnLnLn

TG randomization mixture	TG regioisomeric group[a]	peak area ratio
OOO/PPP	OOP/OPO	65:35
	OPP/POP	63:37
OOO/LLL	OOL/OLO	68:32
	OLL/LOL	62:38
OOO/LnLnLn	OOLn/OLnO	66:34
	OLnLn/LnOLn	65:35
LLL/PPP	LLP/LPL	61:39
	LPP/PLP	60:40
LLL/LnLnLn	LLLn/LLnL	68:32
	LLnLn/LnLLn	62:38
LnLnLn/PPP	LnLnP/LnPLn	60:40
	LnPP/PLnP	61:39
OOO/LnLnLn/PPP	OLnP/LnOP/OPLn	33:34:33
OOO/LLL/PPP	OLP/LOP/OPL	29:35:36
OOO/LLL/LnLnLn	OLnL/OLLn/LOLn	30:33:37

[a] *sn*-1 and *sn*-3 positions are not differentiated in this study, e.g., OOP and POO are treated as equivalent.

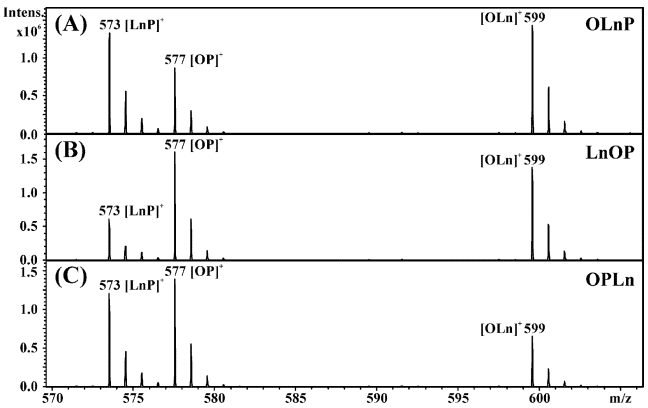

Figure 5. Positive-ion APCI mass spectra of (A) OLnP, (B) LnOP, and (C) OPLn in a zoomed region of *m/z* 570−605 with diacylglycerol fragment ions for peaks from the randomization mixture shown in Figure 4B.

reproducibilities (Figure 2) for three selected peaks with 2 (PLP), 4 (PLL), and 6 (LLL) DBs are acceptable now, as demonstrated on relative standard deviations of retention times of 0.4%, 1.0%, and 0.7% for 1 day measurements and 1.9%, 1.7%, and 1.4% for 2 days measurements. In hexane−acetonitrile mobile phases used in our laboratory previously, the 1 day reproducibility for these three peaks was 7.4%, 6.8%, and 5.2%. Some shifts in retention times can occur on longer time scale, but they can be efficiently eliminated by the use of the relative retention $r = (t_{R,TG} - t_M)/(t_{R,std} - t_M)$, as listed in Supporting Information Table S1. The retention in silver-ion mode is governed mainly by the DB number, but certain separation also occurs for TGs differing only in the length of the fatty acid chain (see Figure 3). The retention order of TGs within these groups with the constant DB number in sunflower oil analysis is the following: for XLL type (where X is the saturated fatty acid), LgLL < C23:0LL < BLL < ALL < SLL < LLMa < LLP < LLC15:0 < LLM; for XLO type, LgLO < C23:0LO < BLO < ALO < SLO < OLMa < OLP; for XLS and XLP types, LgLS < BLS + LgLP < ALS + BLP < SLS + ALP < SLP < PLP, etc. The reconstructed ion current traces for diacylglycerol fragment ions of XLL series are overlaid in Figure 3 to illustrate possible separation according to the chain length. Nonlabeled minor peaks in this figure correspond to mass interferences from other coeluting TGs (not XLL series) with identical diacylglycerol fragment ions. Retention characteristics of all TGs in particular DB groups identified in plant oils (sunflower, blackcurrant, olive, and palm) and animal fat (lard) are summarized in Supporting Information Table S1. Differences in retention times of logical XLL series are approximately 0.4 min per two methylene groups (see Figure 3). The retention order of regioisomers follows the rule that more DBs in *sn*-1/3 positions mean a stronger interaction with silver ions embedded in the stationary phase and hence higher retention in comparison with regioisomers having the same unsaturations

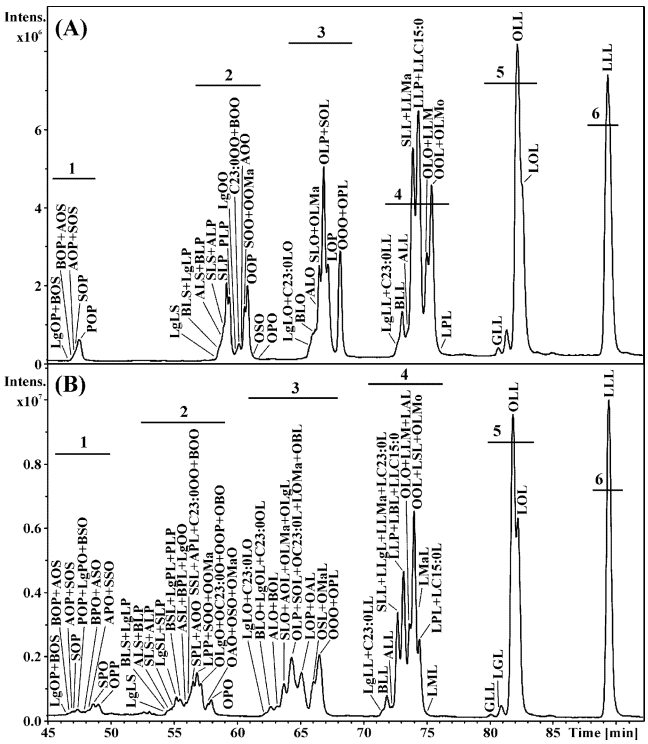

Figure 6. Silver-ion HPLC/APCI-MS analysis of (A) sunflower oil and (B) randomized sunflower oil.

in the middle *sn*-2 position. The probable explanation is a better steric availability of the DB in *sn*-1/3 positions. Regioisomers of XYO/YXO and XYL/YXL types (X and Y are saturated fatty acids) and saturated TGs are not differentiated.

Randomization of TG Mixtures and Their Separation. The chemical interesterification of fatty acids in TGs (randomization) is a known process used in industrial processing of oils and fats to modify physicochemical properties of food products.[28] In our work, the randomization is applied for the generation of standard mixtures of regioisomers (Figure 4), which are too expensive or often not commercially available at all. First, the randomization reaction has been optimized[31] resulting in a robust and reproducible procedure (see the Experimental Section for conditions). Theoretically, the randomization of binary mixtures of equal amounts of single-acid TGs $R_1R_1R_1$ and $R_2R_2R_2$ should provide eight combinations of TGs at identical concentrations: $R_1R_1R_1$, $R_1R_2R_1$, $R_1R_1R_2$, $R_2R_1R_1$, $R_1R_2R_2$, $R_2R_2R_1$, $R_2R_1R_2$, and $R_2R_2R_2$. In practice, enantiomers $R_1R_1R_2$ versus $R_2R_1R_1$ and $R_1R_2R_2$ versus $R_2R_2R_1$ cannot be resolved in a nonchiral environment; therefore, we obtain the following concentration ratios (in parentheses) for initial TGs and two regioisomeric pairs: $R_1R_1R_1$ (1), $R_1R_2R_1$ (1), $R_1R_1R_2 + R_2R_1R_1$ (2), $R_1R_2R_2 + R_2R_2R_1$ (2), $R_2R_1R_2$ (1), $R_2R_2R_2$ (1). The theoretical calculation fits well with experimental results for randomization reactions, as illustrated in Figure 4A and Table 2 for binary randomization mixtures, which confirms that the chemical interesterification is really a random process applicable for the generation of regioisomeric standards at defined concentration ratios. Figure 4B shows the chromatogram of the ternary randomization mixture of OOO, LnLnLn, and PPP providing six regioisomeric doublets at concentration ratios 2:1 and one triplet OLnP/LnOP/OPLn with identical concentrations. This way the standard mixtures of TG regioisomers are generated for the optimization of regioisomeric silver-ion separation. Chromatograms of randomization mixtures (Figure 5 and Supporting Information Figures S2 and S3) also demonstrate the stereoselectivity of our HPLC method providing the baseline separation for regioisomers containing up to three DBs and at least partial separation for TGs with four to eight DBs. As a rule, the bigger the difference in the DB number of fatty acids means better separation of corresponding TG regioisomers, e.g., the baseline separation of P/L and P/Ln regioisomers is relatively easily achieved (Figure 5 and Supporting Information Figure S2). The example of partial resolution of LnOLn/OLnLn regioisomers (Figure 4B) containing seven DBs shows that the number of DBs is probably not a limiting factor for the regioisomeric separation, but the critical requirement for the successful separation of highly polyunsaturated regioisomeric TGs is the difference in the DB number between fatty acids. Polyunsaturated TGs containing fatty acids differing only by one DB are critical pairs for regioisomeric separation, e.g., O/L (peak splitting) and L/Ln (only peak shoulder) pairs. Fatty acids differing by two and more DBs are well-separated for TGs containing up to four DBs and partially for five and more DBs. Some improvement may be expected with further extension of column length at the expense of very long retention times and also rather high costs of multiple columns.

APCI Mass Spectra of Regioisomeric TGs. The randomization is used for the generation of regioisomeric mixtures of TGs, which can be subsequently separated in silver-ion HPLC, and APCI mass spectra of separated regioisomers can be recorded regardless the physical absence of pure regioisomeric standards. Figure 5 shows the differences in mass spectra of regioisomeric triplet OLnP/LnOP/OPLn, which is chromatographically separated unlike all previous papers. APCI mass spectra shown in Figure 5 correspond to the spectra of pure standards. The advantage of silver-ion HPLC/MS determination of regioisomers is the fact that regioisomers do not differ in their relative responses (see ratios in Table 2); hence, peak area ratios correspond to concentration ratios. Table 1 lists exact ratios of DG fragment ions obtained only from randomization experiments to be sure that these results are not affected by possible coelutions common in complex natural samples. Two MS approaches are used for *sn*-2 fatty acid characterization. The simple approach just determines the prevailing fatty acid in the middle *sn*-2 position based on lower relative abundance of the corresponding DG fragment ion,[5,16,19,17,20,21,25] but it has been demonstrated recently[34–37] that the type of fatty acids (mainly DB number and positions) affects the relative abundances of corresponding DG fragment ions. More exact approach is based on the construction of calibration curves using identical standards of regioisomeric pairs mixed at different ratios,[21–23,26,33] but the precision of this determination may be affected by certain fluctuations of fragment ion ratios. Ratios of DG fragment ions shown in Table 1 can be applied for NARP-HPLC determination of regioisomeric ratios measured on the same instrument, because the calibration curves are linear and

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Table 3. Calculation of Theoretical TG Composition after the Randomization of TGs in Sunflower Oil (n is the Number of Fatty Acids, R_i is Variable Fatty Acid, and x_{R_i} is the Weight Fraction of R_i)[a]

	no. of TGs		relative concentration of TGs[b]	
type[b]	theoretical	sunflower oil	theoretical	sunflower oil
$R_1R_1R_1$	n	6	$x_{R_1}^3$	LLL (23.3), OOO (1.2), PPP (0.05), SSS (0.01), BBB (<0.01), AAA (<0.01)
$R_1R_1R_2$	$n(n-1)$	30	$3x_{R_1}^2x_{R_2}$	OLL (26.0), OOL (9.7), LLP (8.7), SLL (5.8), BLL (1.3), OOP (1.2), LPP (1.1), SOO (0.80), ALL (0.56), SSL (0.49), OPP (0.41), BOO (0.18), SSO (0.18), SPP (0.09), AOO (0.08), SSP (0.06), BBL (0.02), BPP (0.02), APP (0.01), BSS (0.01), BBO (0.01), ASS (<0.01), BBP (<0.01), SBB (<0.01), BBA (<0.01), AAO (<0.01), AAL (<0.01), AAP (<0.01), AAS (<0.01), BAA (<0.01)
$R_1R_2R_3$	$[n(n-1)(n-2)]/6$	20	$6x_{R_1}x_{R_2}x_{R_3}$	OLP (6.5), SLO (4.4), SLP (1.5), BLO (0.97), SOP (0.55), ALO (0.41), BLP (0.32), SLB (0.22), ALP (0.14), BOP (0.12), ASL (0.09), BSO (0.08), AOP (0.05), BSP (0.03), ASO (0.03), BAL (0.02), BAO (0.01), ASP (0.01), BAP (<0.01), BAS (<0.01)
total	$[n(n+1)(n+2)]/6$	56		sum of TGs is 96.91%; the rest corresponds to TGs containing trace fatty acids

[a] Analyzed sunflower oil has the following weight fractions in % of main fatty acids (ref 5): linoleic (L) 61.52%, oleic (O) 22.94%, palmitic (P) 7.69%, stearic (S) 5.15%, behenic (B) 1.14%, arachidic (A) 0.49%, and the rest (1.07%) corresponds to trace fatty acids. [b] Stereospecific configuration is not distinguished in this table.

Table 4. Relative Peak Areas of Main TGs in Sunflower Oil before the Randomization Compared with the Experimental and Theoretical Relative Concentrations after the Randomization

	relative peak areas [%]		
TG	before randomization (exptl)	after randomization (exptl)	after randomization (theor)
SLP	1.8	0.7	0.5
PLP	1.6	0.6	0.4
LPP/SOO	0/1.1	0.9/0.8	0.7/0.5
OOP	1.9	1.0	0.8
OSO	0.1	0.4	0.3
OPO	0.1	0.4	0.4
SLO	2.4	1.5	1.5
OLP/SOL	2.0/4.3	1.8/2.7	1.5/2.2
OBL/LOP	0/3.1	0.4/2.5	0.3/2.2
OSL	0	1.4	1.5
OOO/OPL	3.7/0.1	1.5/2.7	1.2/2.2
BLL	1.6	0.9	0.9
SLL	7.5	4.1	3.9
LLP	9.7	5.8	5.8
OLO	3.0	3.6	3.2
OOL	6.5	7.7	6.5
LPL	0.4	3.0	2.9
OLL	16.4	16.0	17.3
LOL	4.9	9.4	8.7
LLL	17.2	20.0	23.3

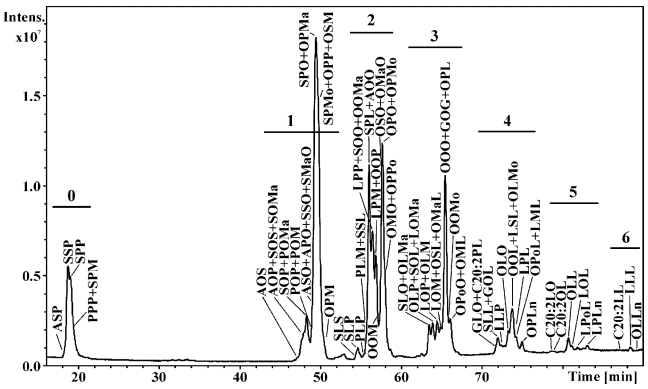

Figure 7. Silver-ion HPLC/APCI-MS analysis of lard.

Table 5. Comparison of Regioisomeric Occupation of the *sn*-2 Position for Saturated (Palmitic) and Unsaturated (Oleic and Linoleic) Fatty Acids in Plant Oil (Sunflower) and Animal Fat (Lard)

regioisomeric pair	sunflower oil	lard
POP/OPP	100/0	8/92
OOP/OPO	98/2	12/88
PLP/LPP	100/0	1/99
LLP/LPL	97/3	9/91
OLP/LOP/OPL	63/36/1	3/12/85

the effect of mobile phase on the ratio of DG fragment ions is negligible. This way the lack or high prices of pure regioisomers can be overcome.

Analysis of Complex Natural Mixtures. The randomization process has been applied for sunflower, blackcurrant, olive, and palm oils and lard as a representative of animal fats. Retention times of all identified TGs in these samples are summarized in Supporting Information Table S1. The comparison of chromatograms before and after randomization (Figure 6) is useful for the verification of *sn*-2 determination, because the *sn*-2 occupation is truly random after the interesterification, as shown in randomization reactions of TG standard mixtures (Figure 5 and Supporting Information Figures S2 and S3). Table 3 shows the calculation of theoretical TG composition in the randomized mixture[28] applied for the real sample of sunflower oil with known fatty acid composition.[5] To keep reasonable the complexity of this table, only six main fatty acids are included in the calculation, while remaining fatty acids form about 1%, and they are referred as trace fatty acids. This calculation provides 56 combinations of TGs (neglecting regioisomers in this table), but 27 of them have negligible concentrations (<0.1%). The comparison of initial TG composition of sunflower oil with experimental and theoretical TG composition after the randomization is illustrated in Table 4. Good correlation between theoretical and experimental values

confirms that this model is applicable for the calculation of TG concentrations after the randomization on the condition that the initial fatty acid composition is correctly determined.

The generally accepted opinion is that unsaturated fatty acids (mainly linoleic) preferentially occupy the *sn*-2 position in plant oils, whereas it is just opposite for animal fats, where unsaturated fatty acids are found mainly in *sn*-1/3 positions. The established method for regiospecific determination is the enzymatic hydrolysis of whole plant oils or animal fats, which provides overall information on *sn*-2 average preference for all TGs found in this sample. In our work, a representative plant oil (sunflower—Figure 6) and animal fat (lard—Figure 7) are compared concerning the quantitative data for *sn*-2 preferences of fatty acids in intact TGs. Table 5 lists regioisomeric ratios of TGs composed of three common fatty acids (palmitic, oleic, and linoleic) occurring both in plant and animal samples. This is a clear quantitative proof based on the silver-ion separation of intact TGs that *sn*-2 occupation for saturated versus unsaturated fatty acids is selective with opposite preferences for plants and animals. The interesting example is TG composed of P, O, and L, where the *sn*-2 occupation preference for sunflower oil is in the order of increasing DB number (OLP/LOP/OPL = 63/36/1), but it is just opposite for the lard sample (OLP/LOP/OPL = 3/12/85). Peak area ratios are obtained using the reconstruction of suitable ion currents considering masses of coeluting peaks. The data for other analyzed plant oils (palm, olive, and blackcurrant oils—data not shown) are in agreement with this conclusion. The knowledge of *sn*-2 preference in TGs is very important due to the bioavailability of particular fatty acids, because human lipases first cleave fatty acids in *sn*-1/3 positions and therefore fatty acids present in the *sn*-2 position can be worse accessible for the human organism.

CONCLUSIONS

Silver-ion chromatography is a powerful method for the analysis of TG regioisomers found in plant oils and animal fats. The optimization of HPLC conditions together with the coupling of three 25 cm silver-ion ChromSpher Lipids columns in series provides the best separation selectivity reported so far for the regioisomeric analysis of TGs enabling the determination of individual regioisomeric ratios for TGs found in natural samples. In this work, our HPLC/APCI-MS has enabled the identification of 196 TGs containing 0−11 DBs and fatty acid chain length from 14 to 24 carbon atoms. The comparison of representative plant oil (sunflower) and animal fat (lard) provides quantitative data (Table 5) for the preferential occupation of the *sn*-2 position by unsaturated fatty acids in plant oils and saturated fatty acids in animal fats. The randomization is used here as a new approach for the generation of regioisomeric standards of TGs. The randomization of selected plant oils shows that there is a clear preference of certain fatty acid combinations and regioisomeric order in TGs before the randomization process, whereas the TG composition after the randomization is truly random. The position of fatty acids on the glycerol skeleton is very important from the nutrition point of view, and the presented method can contribute to this area, because the absence of reliable quantitative methods applicable for complex natural and biological samples limits such research.

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