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Regioisomeric analysis of triacylglycerols using silver-ion liquid chromatography–atmospheric pressure chemical ionization mass spectrometry: Comparison of five different mass analyzers

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ABSTRACT

Silver-ion high-performance liquid chromatography (HPLC) coupled to atmospheric pressure chemical ionization mass spectrometry (APCI-MS) is used for the regioisomeric analysis of triacylglycerols (TGs). Standard mixtures of TG regioisomers are prepared by the randomization reaction from 8 mono-acid TG standards (tripalmitin, tristearin, triarachidin, triolein, trielaidin, trilinolein, trilinolenin and tri-*gamma*-linolenin). In total, 32 different regioisomeric doublets and 11 triplets are synthesized, separated by silver-ion HPLC using three serial coupled chromatographic columns giving a total length of 75 cm. The retention of TGs increases strongly with the double bond (DB) number and slightly for regioisomers having more DBs in *sn*-1/3 positions. DB positional isomers (linolenic *vs.* γ-linolenic acids) are also separated and their reverse retention order in two different mobile phases is demonstrated. APCI mass spectra of all separated regioisomers are measured on five different mass spectrometers: single quadrupole LC/MSD (Agilent Technologies), triple quadrupole API 3000 (AB SCIEX), ion trap Esquire 3000 (Bruker Daltonics), quadrupole time-of-flight micrOTOF-Q (Bruker Daltonics) and LTQ Orbitrap XL (Thermo Fisher Scientific). The effect of different types of mass analyzer on the ratio of $[M+H-R_iCOOH]^+$ fragment ions in APCI mass spectra is lower compared to the effect of the number of DBs, their position on the acyl chain and the regiospecific distribution of acyl chains on the glycerol skeleton. Presented data on $[M+H-R_iCOOH]^+$ ratios measured on five different mass analyzers can be used for the direct regioisomeric determination in natural and biological samples.

1. Introduction

Plant oils, animal fats and fish oils are natural sources of TGs in the human diet. They are important for our nutrition, as they supply energy, essential fatty acids (FAs), fat soluble vitamins, sterols, etc. [1]. The complexity of natural and biological TG mixtures is tremendous, since they may contain hundreds of different TGs. TGs are characterized by the total carbon number (CN), the number, position and configuration (*cis*/*trans*) of DBs in FA acyl chains and the stereospecific position of FAs on the glycerol skeleton (*sn*-1, 2 or 3). The knowledge of stereospecific positions of individual FAs is highly important, because human pancreatic lipases (secreted into the duodenum) or lipoprotein lipases (active in chylomicrons and very low density lipoproteins), preferentially catalyze the hydrolysis of FAs in *sn*-1/3 positions, which results in the different bioavailability of FAs depending on their *sn*-position. The severe problem in the regioisomeric analysis of TGs is the lack of standards. A limited range of standards is commercially available, but they are quite expensive. Randomization (*i.e.*, chemical interesterification) is a common industrial process used for the modification of physicochemical properties of TG mixtures in food and cosmetic products [1]. In our previous work [2], we have modified this procedure for the use in the micro scale range (milligram amounts), which overcomes the lack of standards by the synthesis of standard regioisomeric mixtures at well defined composition. The randomization reaction yields a truly random distribution of statistically expected regioisomers.

The most powerful separation technique used in the detailed characterization of complex TG mixtures is non-aqueous reversed-phase (NARP) HPLC [3–8], but unfortunately it is mostly not convenient for the separation of regioisomers with the exception of extremely long retention times up to 200 min causing peak broadening [9]. Silver-ion normal phase HPLC can be applied for the separation of TGs into groups differing in the DB number

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[10–13], but it does not provide the same separation efficiency as NARP mode and it also suffers from a lower reproducibility. Unlike NARP, it provides a baseline resolution for regioisomers with up to three DBs and the partial resolution for highly unsaturated TGs [2]. The problem with the reproducibility in silver-ion systems can be partially solved by using a modified composition of commonly used hexane–acetonitrile mobile phases with the addition of 2-propanol to improve the mutual miscibility and therefore also the reproducibility. The principle of silver-ion chromatography is based on the formation of weak reversible complexes of π electrons from DBs with silver ions embedded in the stationary phase during the sample elution through the chromatographic column [11,12]. Two dimensional combination of NARP and silver-ion systems [10,14–17] provides quite good orthogonality in the analysis of TGs and therefore high peak capacity.

The main analytical techniques applicable for the regiospecific analysis of TGs are the following: (1) silver-ion HPLC [2,15–20], (2) MS [22–25], (3) enzymatic reactions [26], and (4) nuclear magnetic resonance (NMR) [27]. Each technique has certain limitations. NMR provides an absolute answer based on characteristic chemical shifts, but the practical applicability is severely limited by the need of pure compounds in milligram amounts. Enzymes with various levels of selectivity towards stereospecific positions or particular FA lengths are well known, but their application in a quantitative manner is often not as straightforward, because exact values of enzymatic stereoselectivity may not be known and also depends on experimental conditions. Furthermore, other analytical techniques (*e.g.*, HPLC) are required to detect products of enzymatic hydrolysis.

The ability of MS to distinguish regioisomers is known from the pioneering work published already in 1964 using electron ionization [28], which was 30 years later confirmed with APCI [29]. HPLC/APCI-MS is now often applied for characterization of prevailing FAs in the *sn*-2 position in complex natural mixtures [4–6,19–25]. Other ionization techniques, like electrospray ionization [30–35] or matrix-assisted laser desorption/ionization [36], can be used for the same purpose as well with the assistance of adduct formation with small cations (*e.g.*, ammonium, sodium or silver). Nevertheless, APCI is the most convenient ionization due to non-polar character of TGs, good compatibility with non-aqueous mobile phases and good sensitivity. All MS approaches rely on the fact that the neutral loss of FA from the middle *sn*-2 position is less energetically favored in comparison to cleavages from the side *sn*-1/3 positions [22–25,28,29]. This knowledge is often applied for the estimation of prevailing FA in the *sn*-2 position, but regioisomeric standards are essential for the quantitative determination [37–40].

The goal of this work is a systematic study of regioisomeric analysis of TGs based on our previously optimized silver-ion HPLC method and measurements of $[M+H-R_iCOOH]^+$ fragment ion ratios on five different types of mass analyzers. For this study, a broad range of regioisomeric standard mixtures is synthesized using the micro scale randomization procedure. This extensive data set measured in three different laboratories is used for the generalization of the fragmentation behavior in APCI mass spectra and the retention behavior in silver-ion chromatography, where the distribution of DBs in acyl chains has the highest importance.

2. Experimental

2.1. Chemicals

Acetonitrile, acetone, 2-propanol, methanol and hexane (HPLC grade solvents) and sodium methoxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Standards of tripalmitin (PPP, C16:0), tristearin (SSS, C18:0), triolein (OOO, Δ9-C18:1), trielaidin (EEE, Δ9trans-C18:1), trilinolein (LLL, Δ9,12-C18:2), trilinolenin (LnLnLn, Δ9,12,15-C18:3), tri-*gamma*-linolenin (γLnγLnγLn, Δ6,9,12-C18:3), triarachidin (AAA, C20:0), model mixtures of TG standards GLC#435 (all saturated single-acid TGs from C7:0 to C22:0) and GLC#437 (C16:0, C18:0, Δ9-C18:1, Δ9,12-C18:2, Δ9,12,15-C18:3 and C20:0) were purchased from Nu–ChekPrep (Elysian, MN, USA).

2.2. Randomization synthesis of regioisomeric TG mixtures

Standards of mixed-acid triacylglycerols ($R_1R_1R_2$, $R_1R_2R_2$, etc.) were prepared from mono-acid triacyglycerols ($R_1R_1R_1$) using the randomization procedure described recently [2]. The mixture of three mono-acid TGs were weighed (15 mg of each TG) into a dry boiling flask together with 90 mg of sodium methoxide and 2 mL of hexane dried with molecular sieves. The mixture was heated for 30 min in a water bath under reflux. 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 analytes was used for HPLC analyses. The same procedure was applied for the preparation of all randomization mixtures. The full list of synthesized TG regioisomers used in this study is shown in Table 1. Ratios of $[M+H-R_iCOOH]^+$ fragment ions shown in Tables 2 and 3 represent mean values of at least four points (mostly six points) with their standard deviations. Table 2 lists the data on all measured regioisomeric doubles including $R_1R_2R_3$ type TGs containing two different FAs with identical masses (O and E, Ln and γLn), while Table 3 shows the data on regioisomeric triplets.

2.3. Silver-ion HPLC

The final silver-ion HPLC method used for analyses of randomization mixtures is as follows. Three silver-ion chromatographic

Table 1
List of components of randomization mixtures and synthesized TG regioisomers (certain combinations in doublets occur multiple times).

Standards used for randomization	Regioisomers						
	Doublets						Triplets
PPP + OOO + LLL	POP/OPP	OOP/OPO	PLP/LPP	LLP/LPL	OOL/OLO	OLL/LOL	OLP/LOP/OPL
PPP + OOO + LnLnLn	POP/OPP	OOP/OPO	PLnP/LnPP	OLnO/OOLn	LnLnP/LnPLn	OLnLn/LnOLn	OLnP/LnOP/OPLn
PPP + LLL + LnLnLn	PLP/LPP	PLnP/LnPP	LLP/LPL	LnLnP/LnPLn	LLnL/LLLn	LLnLn/LnLLn	LLnP/LnLP/LPLn
SSS + OOO + LLL	SOS/SSO	SLS/SSL	SOO/OSO	SLL/LSL	OOL/OLO	OLL/LOL	SLO/SOL/OSL
SSS + OOO + LnLnLn	SOS/SSO	SOO/OSO	SLnS/SSLn	OLnO/OOLn	SLnLn/LnSLn	OLnLn/LnOLn	SLnO/SOLn/OSLn
AAA + OOO + LLL	AOA/AAO	ALA/AAL	AOO/OAO	ALL/LAL	OOL/OLO	OLL/LOL	ALO/AOL/OAL
AAA + OOO + LnLnLn	AOA/AAO	AOO/OAO	ALnA/AALn	OLnO/OOLn	ALnLn/LnALn	OLnLn/LnOLn	ALnO/AOLn/OALn
AAA + LLL + LnLnLn	ALA/AAL	ALnA/AALn	ALL/LAL	ALnLn/LnALn	LLnL/LLLn	LLnLn/LnLLn	ALnL/ALLn/LALn
OOO + LLL + LnLnLn	OOL/OLO	OLL/LOL	OLnO/OOLn	LLnL/LLLn	OLnLn/LnOLn	LLnLn/LnLLn	OLnL/OLLn/LOLn
PPP + OOO + EEE	PEP/EPP	POP/OPP	EEP/EPE	OOP/OPO	EEO/EOE	OEO/OOE	OEP/EOP/EPO
OOO + LnLnLn + γLnγLnγLn	OLnO/OOLn	OγLnO/OOγLn	OLnLn/LnOLn	OγLnγLn/γLnOγLn	LnLnγLn/LnγLnLn	γLnLnγLn/ γLnγLnLn	OLnγLn/OγLnLn/ LnOγLn

Table 2
Ratios of fragment ions for TG regioisomeric doublets recorded on five different mass spectrometers after silver-ion HPLC separation (see Section 2 for detailed conditions).

TG[a]	DB	Ratio of fragment ions $[R_1R_1]^+/[R_1R_2]^+$	Orbitrap (Thermo)	QqTOF (Bruker)	Ion trap (Bruker)	Single quadrupole (Agilent)	Triple quadrupole (AB SCIEX)
PEP	1	$[PP]^+/[EP]^+$	(30 ± 2):100	(32 ± 4):100	(25 ± 1):100	(28 ± 1):100	(26 ± 1):100
EPP		$[PP]^+/[EP]^+$	(94 ± 1):100	(85 ± 3):100	(93 ± 4):100	(86 ± 1):100	(88 ± 2):100
AOA		$[AA]^+/[AO]^+$	(31 ± 4):100	(27 ± 3):100	(31 ± 2):100	(27 ± 2):100	(30 ± 3):100
AAO		$[AA]^+/[AO]^+$	(94 ± 2):100	(87 ± 6):100	(79 ± 4):100	(78 ± 2):100	(80 ± 4):100
SOS		$[SS]^+/[SO]^+$	(27 ± 3):100	(40 ± 6):100	(33 ± 4):100	(35 ± 1):100	(38 ± 1):100
SSO		$[SS]^+/[SO]^+$	(90 ± 3):100	(94 ± 4):100	(91 ± 5):100	(86 ± 2):100	(80 ± 2):100
POP		$[PP]^+/[OP]^+$	(32 ± 1):100	(33 ± 5):100	(23 ± 2):100	(26 ± 1):100	(32 ± 2):100
OPP		$[PP]^+/[OP]^+$	(72 ± 3):100	(80 ± 5):100	(89 ± 6):100	(83 ± 3):100	(84 ± 1):100
EEP	2	$[EE]^+/[EP]^+$	(66 ± 1):100	(58 ± 3):100	(57 ± 1):100	(52 ± 1):100	(59 ± 2):100
EPE		$[EE]^+/[EP]^+$	(29 ± 2):100	(19 ± 1):100	(19 ± 1):100	(18 ± 1):100	(19 ± 1):100
EOP		$[OE]^+/[EP]^+$	(92 ± 2):100	(88 ± 4):100	(80 ± 5):100	(53 ± 1):100	(63 ± 1):100
OEP		$[OE]^+/[EP]^+$	(70 ± 1):100	(67 ± 2):100	(65 ± 3):100	(52 ± 1):100	(61 ± 1):100
OPE		$[OE]^+/[EP]^+$	(18 ± 1):100	(26 ± 1):100	(20 ± 1):100	(19 ± 1):100	(19 ± 1):100
ALA		$[AA]^+/[AL]^+$	(45 ± 3):100	(40 ± 6):100	(45 ± 2):100	(37 ± 2):100	(43 ± 1):100
AAL		$[AA]^+/[AL]^+$	(67 ± 3):100	(94 ± 3):100	(63 ± 3):100	(93 ± 2):100	(91 ± 5):100
SLS		$[SS]^+/[SL]^+$	(44 ± 1):100	(44 ± 2):100	(42 ± 3):100	(37 ± 1):100	(27 ± 1):100
SSL		$[SS]^+/[SL]^+$	(61 ± 1):100	(95 ± 3):100	(81 ± 3):100	(94 ± 3):100	(90 ± 4):100
PLP		$[PP]^+/[LP]^+$	(44 ± 3):100	(39 ± 1):100	(39 ± 2):100	(38 ± 2):100	(45 ± 1):100
LPP		$[PP]^+/[LP]^+$	(84 ± 3):100	(87 ± 2):100	(77 ± 5):100	(96 ± 1):100	(85 ± 2):100
AOO		$[OO]^+/[AO]^+$	(54 ± 1):100	(57 ± 4):100	(53 ± 1):100	(56 ± 2):100	(66 ± 1):100
OAO		$[OO]^+/[AO]^+$	(18 ± 2):100	(19 ± 3):100	(17 ± 1):100	(17 ± 1):100	(22 ± 1):100
SOO		$[OO]^+/[SO]^+$	(60 ± 4):100	(70 ± 5):100	(50 ± 5):100	(54 ± 1):100	(55 ± 1):100
OSO		$[OO]^+/[SO]^+$	(21 ± 3):100	(22 ± 2):100	(17 ± 1):100	(19 ± 1):100	(18 ± 1):100
OOP		$[OO]^+/[OP]^+$	(51 ± 1):100	(62 ± 2):100	(64 ± 1):100	(54 ± 2):100	(56 ± 1):100
OPO		$[OO]^+/[OP]^+$	(16 ± 1):100	(20 ± 1):100	(21 ± 1):100	(19 ± 1):100	(20 ± 1):100
ALnA	3	$[AA]^+/[ALn]^+$	(44 ± 1):100	(30 ± 2):100	(37 ± 1):100	(35 ± 2):100	(41 ± 2):100
AALn		$[AA]^+/[ALn]^+$	(81 ± 3):100	(86 ± 1):100	(92 ± 2):100	(89 ± 3):100	(89 ± 2):100
SLnS		$[SS]^+/[SLn]^+$	(42 ± 1):100	(30 ± 2):100	(36 ± 2):100	(34 ± 3):100	(25 ± 1):100
SSLn		$[SS]^+/[SLn]^+$	(82 ± 3):100	(87 ± 2):100	(83 ± 6):100	(82 ± 4):100	(83 ± 2):100
PLnP		$[PP]^+/[LnP]^+$	(47 ± 3):100	(49 ± 4):100	(32 ± 2):100	(32 ± 2):100	(41 ± 3):100
LnPP		$[PP]^+/[LnP]^+$	(92 ± 4):100	(97 ± 1):100	(77 ± 6):100	(86 ± 3):100	(88 ± 1):100
ALL	4	$[LL]^+/[AL]^+$	(83 ± 3):100	(73 ± 1):100	(89 ± 2):100	(83 ± 3):100	(86 ± 2):100
LAL		$[LL]^+/[AL]^+$	(31 ± 1):100	(30 ± 1):100	(36 ± 5):100	(32 ± 2):100	(37 ± 1):100
SLL		$[LL]^+/[SL]^+$	(77 ± 4):100	(75 ± 5):100	(90 ± 2):100	(80 ± 2):100	(89 ± 2):100
LSL		$[LL]^+/[SL]^+$	(23 ± 2):100	(16 ± 2):100	(25 ± 3):100	(11 ± 1):100	(34 ± 1):100
LLP		$[LL]^+/[LP]^+$	(91 ± 3):100	(81 ± 1):100	(78 ± 2):100	(77 ± 3):100	(78 ± 5):100
LPL		$[LL]^+/[LP]^+$	(39 ± 1):100	(33 ± 1):100	(48 ± 2):100	(29 ± 2):100	(33 ± 2):100
OLO		$[OO]^+/[OL]^+$	(46 ± 2):100	(45 ± 4):100	(32 ± 3):100	(43 ± 1):100	(36 ± 1):100
OOL		$[OO]^+/[OL]^+$	(60 ± 2):100	(88 ± 4):100	(71 ± 6):100	(68 ± 2):100	(77 ± 2):100
OLL	5	$[LL]^+/[OL]^+$	(89 ± 2):100	(81 ± 4):100	(88 ± 7):100	(71 ± 2):100	(79 ± 1):100
LOL		$[LL]^+/[OL]^+$	(46 ± 2):100	(34 ± 4):100	(42 ± 3):100	(36 ± 2):100	(40 ± 1):100
OLnO		$[OO]^+/[OLn]^+$	(29 ± 5):100	(23 ± 2):100	(24 ± 2):100	(26 ± 1):100	(26 ± 2):100
OOLn		$[OO]^+/[OLn]^+$	(72 ± 2):100	(76 ± 4):100	(67 ± 5):100	(64 ± 2):100	(79 ± 2):100
OOγLn		$[OO]^+/[O\gamma Ln]^+$	100:(41 ± 1)	100:(51 ± 2)	100:(50 ± 1)	100:(44 ± 1)	100:(49 ± 2)
ALnLn	6	$[LnLn]^+/[ALn]^+$	(81 ± 3):100	(89 ± 4):100	(90 ± 3):100	(86 ± 1):100	(91 ± 2):100
LnALn		$[LnLn]^+/[ALn]^+$	(36 ± 3):100	(31 ± 2):100	(37 ± 2):100	(31 ± 2):100	(44 ± 1):100
SLnLn		$[LnLn]^+/[SLn]^+$	(78 ± 1):100	(83 ± 1):100	(87 ± 3):100	(80 ± 2):100	(86 ± 2):100
LnSLn		$[LnLn]^+/[SLn]^+$	(30 ± 1):100	(32 ± 1):100	(48 ± 2):100	(29 ± 2):100	(29 ± 1):100
LnLnP		$[LnLn]^+/[LnP]^+$	(84 ± 3):100	(78 ± 3):100	(92 ± 7):100	(86 ± 1):100	(76 ± 1):100
LnPLn		$[LnLn]^+/[LnP]^+$	(33 ± 3):100	(28 ± 2):100	(45 ± 3):100	(29 ± 1):100	(37 ± 2):100
LLnL	7	$[LL]^+/[LLn]^+$	(38 ± 4):100	(36 ± 3):100	(41 ± 4):100	(44 ± 3):100	(44 ± 1):100
LLLn		$[LL]^+/[LLn]^+$	(68 ± 6):100	(68 ± 5):100	(67 ± 4):100	(71 ± 2):100	(74 ± 2):100
OLnLn		$[LnLn]^+/[OLn]^+$	(87 ± 2):100	(79 ± 2):100	(88 ± 4):100	(86 ± 2):100	(81 ± 3):100
LnOLn		$[LnLn]^+/[OLn]^+$	(38 ± 3):100	(34 ± 2):100	(43 ± 3):100	(32 ± 2):100	(45 ± 2):100
γLnOγLn		$[\gamma Ln\gamma Ln]^+/[O\gamma Ln]^+$	(10 ± 1):100	(11 ± 1):100	(18 ± 3):100	(12 ± 1):100	(15 ± 1):100
LLnLn	8	$[LnLn]^+/[LLn]^+$	(75 ± 4):100	(61 ± 2):100	(73 ± 5):100	(62 ± 3):100	(75 ± 2):100
LnLLn		$[LnLn]^+/[LLn]^+$	(46 ± 1):100	(36 ± 4):100	(44 ± 2):100	(44 ± 2):100	(47 ± 1):100

[a] TGs within double bond groups are sorted according to their retention times.

columns ChromSpher Lipids (250 mm × 4.6 mm, 5 μm, Varian, Palo Alto, CA, USA) were connected in series, the flow rate was set at 1 mL/min, the injection volume was 1 μL, the column temperature was 25 °C, and the mobile phase gradient was: 0 min – 100% A, 140 min – 61% A + 39% B, where A is the 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 (96:2:2, v/v/v). The mobile phase was prepared fresh every day. 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 was equilibrated between injections at least for 30 min.

2.4. Mass spectrometers used for comparative study

Positive-ion APCI and the mass range *m*/*z* of 50–1200 were used in all cases.

Table 3
Ratios of fragment ions for TG regioisomeric triplets recorded on five different mass spectrometers after silver-ion HPLC separation (see Section 2 for detailed conditions).

TG[a]	DB	Ratio of fragment ions $[R_1R_3]^+/[R_1R_2]^+/[R_2R_3]^+$	Orbitrap (Thermo)	QqTOF (Bruker)	Ion trap (Bruker)	Single quadrupole (Agilent)	Triple quadrupole (AB SCIEX)
ALO	3	$[AO]^+/[AL]^+/[OL]^+$	(42 ± 3):(75 ± 1):100	(56 ± 1):(83 ± 2):100	(50 ± 4):(84 ± 2):100	(71 ± 2):(81 ± 1):100	(49 ± 2):(74 ± 2):100
AOL		$[AL]^+/[AO]^+/[OL]^+$	(30 ± 2):(84 ± 3):100	(38 ± 1):(95 ± 3):100	(36 ± 2):(91 ± 4):100	(38 ± 2):(94 ± 1):100	(32 ± 2):(73 ± 2):100
OAL		$[OL]^+/[AO]^+/[AL]^+$	(46 ± 2):100:(80 ± 2)	(46 ± 1):100:(79 ± 3)	(54 ± 1):100:(89 ± 1)	(44 ± 2):100:(81 ± 3)	(53 ± 2):100:(91 ± 4)
SLO		$[SO]^+/[SL]^+/[OL]^+$	(48 ± 2):(67 ± 2):100	(62 ± 2):(86 ± 1):100	(42 ± 1):(77 ± 3):100	(56 ± 3):(91 ± 2):100	(46 ± 2):(86 ± 1):100
SOL		$[SL]^+/[SO]^+/[OL]^+$	(33 ±2):(87 ± 2):100	(49 ± 1):(95 ± 1):100	(38 ± 2):(88 ± 4):100	(44 ± 2):(89 ± 1):100	(43 ± 1):(90 ± 1):100
OSL		$[OL]^+/[SO]^+/[SL]^+$	(54 ± 2):100:(98 ± 1)	(46 ± 1):100:(81 ± 1)	(58 ± 4):100:(92 ± 4)	(25 ± 2):100:(63 ± 1)	(36 ± 1):100:(75 ± 1)
OLP		$[OP]^+/[OL]^+/[LP]^+$	(55 ± 1):100:(73 ± 1)	(72 ± 1):100:(84 ± 1)	(40 ± 5):100:(56 ± 4)	(56 ± 2):100:(78 ± 2)	(48 ± 2):100:(70 ± 2)
LOP		$[LP]^+/[OL]^+/[OP]^+$	(39 ± 2):100:(95 ± 3)	(53 ± 5):100:(97 ± 1)	(29 ± 2):100:(83 ± 3)	(39 ± 1):100:(91 ±1)	(35 ± 1):100:(85 ± 4)
OPL		$[OL]^+/[OP]^+/[LP]^+$	(47 ± 2):100:(77 ± 3)	(58 ± 1):100:(85 ± 2)	(75 ± 4):100:(89 ± 3)	(46 ± 1):100:(74 ± 3)	(64 ± 1):100:(89 ± 2)
ALnO	4	$[AO]^+/[ALn]^+/[OLn]^+$	(52 ± 1):(88 ± 1):100	(54 ± 2):(96 ± 4):100	(54 ± 4):(88 ± 1):100	(55 ± 1):(94 ± 1):100	(56 ± 3):(93 ± 2):100
AOLn		$[ALn]^+/[AO]^+/[OLn]^+$	(37 ± 1):100:(82 ± 2)	(42 ± 4):100:(76 ± 1)	(43 ± 2):100:(84 ± 1)	(38 ± 1):100:(88 ± 2)	(40 ± 1):100:(84 ± 1)
OALn		$[OLn]^+/[AO]^+/[ALn]^+$	(43 ± 3):(83 ± 1):100	(48 ± 3):(79 ± 4):100	(47 ± 3):(88 ± 3):100	(45 ± 1):(90 ± 2):100	(42 ± 2):(86 ± 2):100
SLnO		$[SO]^+/[SLn]^+/[OLn]^+$	(54 ± 2):100:(97 ± 2)	(44 ± 1):100:(90 ± 2)	(42 ± 2):100:(96 ± 2)	(50 ± 4):100:(95 ± 2)	(50 ± 1):100:(86 ± 2)
SOLn		$[SLn]^+/[SO]^+/[OLn]^+$	(41 ± 2):100:(88 ± 3)	(43 ± 2):100:(91 ± 4)	(58 ± 1):100:(97 ± 2)	(42 ± 3):100:(91 ± 1)	(52 ± 2):100:(90 ± 2)
OSLn		$[OLn]^+/[SO]^+/[SLn]^+$	(41 ± 3):100:(82 ± 2)	(36 ± 2):100:(91 ± 3)	(51 ± 2):100:(85 ± 2)	(50 ± 1):100:(96 ± 1)	(47 ± 2):100:(93 ± 3)
OLnP		$[OP]^+/[OLn]^+/[LnP]^+$	(55 ± 2):100:(91 ± 1)	(60 ± 5):100:(93 ± 1)	(49 ± 2):100:(70 ± 2)	(54 ± 3):100:(96 ± 1)	(46 ± 2):100:(90 ± 2)
LnOP		$[LnP]^+/[OLn]^+/[OP]^+$	(36 ± 1):(90 ± 3):100	(33 ± 1):(95 ± 4):100	(50 ± 1):(88 ± 3):100	(44 ± 1):(86 ± 1):100	(35 ± 2):(92 ± 2):100
OPLn		$[OLn]^+/[OP]^+/[LnP]^+$	(55 ± 2):100:(86 ± 1)	(49 ± 2):100:(92 ± 4)	(61 ± 2):100:(81 ± 4)	(44 ± 2):100:(95 ± 1)	(50 ± 1):100:(90 ± 2)
ALnL	5	$[AL]^+/[ALn]^+/[LLn]^+$	(70 ± 2):(88 ± 2):100	(79 ± 1):(90 ± 2):100	(48 ± 5):(77 ± 5):100	(63 ± 1):(69 ± 2):100	(50 ± 3):(64 ± 1):100
ALLn		$[ALn]^+/[AL]^+/[LLn]^+$	(51 ± 2):(91 ± 1):100	(66 ± 4):(93 ± 3):100	(46 ± 4):(82 ± 2):100	(54 ± 3):(81 ± 4):100	(35 ± 2):(57 ± 1):100
LALn		$[LLn]^+/[AL]^+/[ALn]^+$	(87 ± 2):100:(95 ± 1)	(49 ± 1):100:(83 ± 1)	(64 ± 1):100:(81 ± 4)	(49 ± 2):100:(82 ± 2)	(75 ± 2):100:(85 ± 2)
LLnP		$[LP]^+/[LLn]^+/[LnP]^+$	(45 ± 1):100:(92 ± 2)	(78 ± 3):100:(88 ± 6)	(36 ± 2):100:(57 ± 2)	(63 ± 1):100:(70 ± 1)	(50 ± 2):100:(62 ± 2)
LnLP		$[LnP]^+/[LLn]^+/[LP]^+$	(47 ± 1):100:(92 ± 1)	(44 ± 3):100:(82 ± 3)	(42 ± 2):100:(80 ± 2)	(57 ± 2):100:(82 ± 2)	(42 ± 2):100:(80 ± 2)
LPLn		$[LLn]^+/[LP]^+/[LnP]^+$	(38 ± 2):100:(87 ± 1)	(47 ± 1):100:(93 ± 2)	(67 ± 3):100:(89 ± 2)	(47 ± 1):100:(82 ± 2)	(73 ± 2):100:(89 ± 3)
OLnL	6	$[OL]^+/[OLn]^+/[LLn]^+$	(71 ± 1):(88 ± 3):100	(62 ± 3):(95 ± 4):100	(54 ± 4):(71 ± 4):100	(63 ± 3):(84 ± 3):100	(55 ± 2):(72 ± 2):100
OLLn		$[OLn]^+/[OL]^+/[LLn]^+$	(70 ± 4):(82 ± 2):100	(54 ± 3):(94 ± 2):100	(73 ± 5):(84 ± 5):100	(70 ± 1):(87 ± 3):100	(79 ± 2):(92 ± 2):100
LOLn		$[LLn]^+/[OL]^+/[OLn]^+$	(61 ± 1):100:(90 ± 5)	(56 ± 2):100:(78 ± 1)	(76 ± 2):100:(87 ± 2)	(69 ± 3):100:(93 ± 2)	(83 ± 3):100:(86 ± 4)

[a] TGs within double bond groups are sorted according to their retention times.

(1) Quadrupole time-of-flight (QqTOF) analyzer micrOTOF-Q (Bruker Daltonics, Bremen, Germany) – flow rate of the nebulizing and drying gas 5 and 3 L/min, respectively, temperatures of the drying gas and APCI heater 300 °C and 400 °C, respectively. QqTOF was coupled to an Agilent 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany).
(2) Ion trap (IT) analyzer Esquire 3000 (Bruker Daltonics, Bremen, Germany) – pressure of the nebulizing gas 70 psi, drying gas flow rate 3 L/min, temperatures of the drying gas and APCI heater were 350 °C and 400 °C. The ion trap was coupled to the Agilent 1200 Series liquid chromatograph.
(3) Single quadrupole (Q) LC/MSD (Agilent Technologies, Waldbronn, Germany) – pressure of the nebulizing gas 50 psi, drying gas flow rate 7 L/min, temperatures of the drying gas and APCI heater were 300 °C and 400 °C. The Q was coupled to the Agilent 1100 Series liquid chromatograph.
(4) Triple quadrupole (QqQ) API 3000 (AB SCIEX, Foster City, CA, USA) – pressure of the nebulizing and curtain gas 15 psi and 6 psi, respectively, and temperature of APCI 400 °C. The QqQ was coupled to the Waters 2690 Alliance liquid chromatograph (Waters, Milford, MA, USA).
(5) LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA,USA) – flow rate of the sheath and auxiliary gases 55 and 25 arbitrary units, respectively, vaporizer temperature 300 °C and capillary temperature 200 °C. The Orbitrap was coupled to the Rheos Allegro quaternary gradient UHPLC pump (Thermo

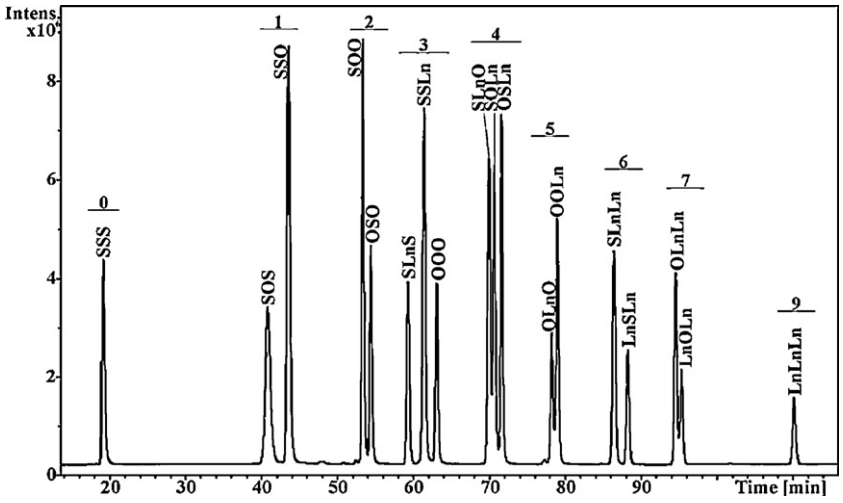

Fig. 1. Silver-ion HPLC/APCI-MS chromatogram of the randomization mixture prepared from tristearin (SSS, C18:0), triolein (OOO, Δ9-C18:1) and trilinolenin (LnLnLn, Δ9,12,15-C18:3), see Section 2 for conditions. Numbers correspond to the double bond number.

Fisher Scientific), HTSPAL autosampler (CTC Analytics AG, Zwingen, Switzerland), and DeltaChromTM CTC 100 column oven (Watrex, Prague, Czech Republic).

2.5. Notation of TGs and FAs

TGs are annotated using initials of trivial names of FAs arranged according to their *sn*-1, *sn*-2 and *sn*-3 positions. Positions *sn*-1 and *sn*-3 cannot be distinguished by silver-ion HPLC, therefore the order in these side positions follow decreasing masses in accordance with our previously established convention [2–5,7,9,10,23,38], *i.e.*, OLP but not PLO. If FA residues have identical masses, then sn-1 position is used for more common FA, *e.g.*, LnOγLn but not γLnOLn, OPE but not EPO. Abbreviations of FAs used in this work: P, palmitic (C16:0); S, stearic (C18:0); O, oleic (Δ9-C18:1); E, elaidic (Δ9t-C18:1); L, linoleic (Δ9,12-C18:2); Ln, linolenic (Δ9,12,15-C18:3); γLn, *gamma*-linolenic (Δ6,9,12-C18:3); A, arachidic (C20:0).

3. Results and discussion

The first problem in the regioisomeric analysis of TGs is the use of appropriate regioisomeric standards. Some TG regioisomers are commercially available from Larodan (Malmö, Sweden), but their prices are rather high. In our previous work [2], we have developed a method for the preparation of regioisomeric mixtures based on the well known randomization procedure used in food and cosmetic industries for the production of lipid mixtures with required properties, called functional lipids. Table 1 lists 11 performed randomization reactions providing 32 different doublets and 11 triplets of regioisomeric TGs. The synthesis has required a total reaction time of 11 h (30 min for preparation + 30 min for reaction = 1 h * 11 reactions) and the total analytical time is 55 h (2 h for the chromatographic run, 2 analyses per sample, 1 h for the system equilibration = 5 h * 11 reactions); in total it is about 66 h of working time. This laborious synthetic part of the work is essential

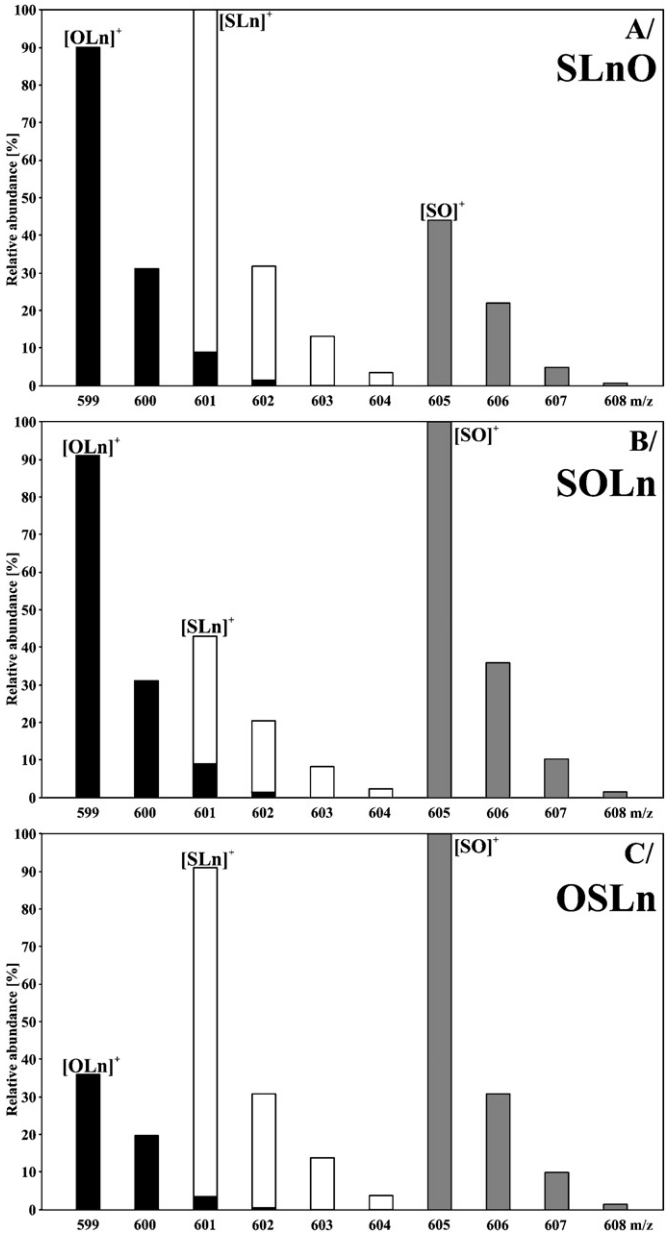

Fig. 2. Normalized relative abundances of $[M+H-R_iCOOH]^+$ fragment ions in APCI mass spectra of regioisomeric triplet SLnO (A), SOLn (B) and OSLn (C) obtained from the chromatogram shown in Fig. 1.

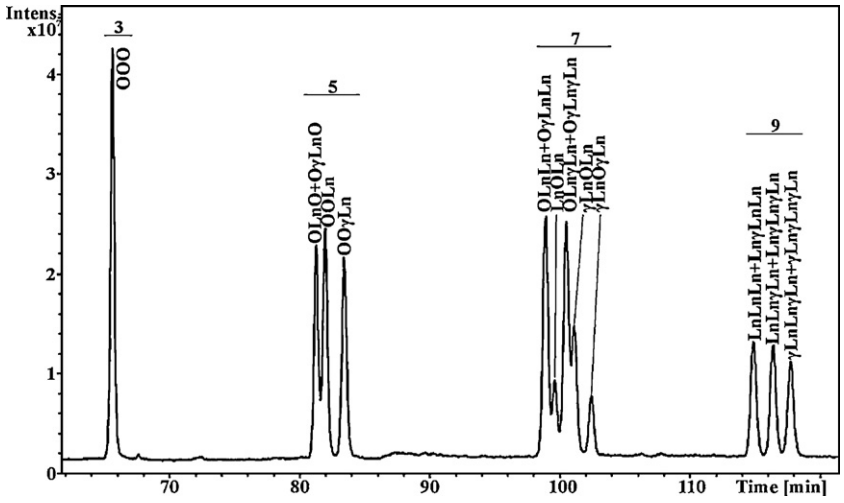

Fig. 3. Silver-ion HPLC/APCI-MS chromatogram of the randomization mixture prepared from triolein (OOO, Δ9-C18:1), trilinolenin (LnLnLn, Δ9,12,15-C18:3) and tri-γ-linolenin (γLnγLnγLn, Δ6,9,12-C18:3), see Section 2 for conditions. Numbers correspond to the double bond number.

for a systematic study of retention and fragmentation behavior of regioisomeric TGs.

Fig. 1 shows a typical chromatogram of the randomization mixture of three mono-acid TGs (SSS, OOO and LnLnLn) with the baseline resolution of most peaks, while some of them have only partial resolution (*e.g.*, OLnO/OOLn, OLnLn/LnOLn) but still sufficient for reliable peak integration. Fig. 2 shows regions of APCI mass spectra with diacylglycerol fragments of the regioisomeric triplet SLnO (A), SOLn (B) and OSLn (C). For the identification of *sn*-2 FA, contributions of A + 2 and A + 3 isotopes should be subtracted from $[M+H-R_iCOOH]^+$ fragment ions with *m*/*z* values higher by two units, *e.g.*, A + 2 isotopic peak from $[OLn]^+$ fragment ion contributes to the peak of $[SLn]^+$ at *m*/*z* 601. Tables 2 and 3 show raw data without A + 2 isotopic subtraction for all measured regioisomers. The neutral loss of FA from the middle *sn*-2 position is less preferred, therefore the smallest peak among $[M+H-R_iCOOH]^+$ ions corresponds to the neutral loss from *sn*-2 position, *e.g.*, the smallest peak of $[SO]^+$ in Fig. 2A confirms that Ln is in the *sn*-2 position (SLnO). DBs in outer *sn*-1/3 positions have a stronger effect on the retention probably caused by a better steric availability of DBs for interaction with the silver-ions embedded in the stationary phase. The same retention pattern is valid for all studied regioisomers which can be expressed as a simple rule of thumb: more DBs in outer *sn*-1/3 positions result in a slightly higher retention in silver-ion HPLC.

A highly complex retention pattern is observed in the case of TGs containing isomers differing only in the position of DBs (Fig. 3), such as linolenic (Δ9,12,15-C18:3) and γ-linolenic (Δ6,9,12-C18:3) acids. When the only difference between two TGs is in the DB positions for *sn*-2 FA (OLnO *vs.* OγLnO), then no visible chromatographic separation is observed (Fig. 3). On the other hand, the same difference occurring in *sn*-1/3 positions (OOLn *vs.* OOγLn) leads to a very good resolution with the difference of retention times about 1.6 min. The identification of the individual peaks in Fig. 3 is based on the combination of several known facts: (A) the knowledge of retention behavior of TGs containing Ln based on the measurement of other randomization mixtures, (B) differences in fragment ion ratios for *sn*-1,3 *vs.* *sn*-2, (C) differences in $[M+H]^+/[M+H-R_iCOOH]^+$ ratios between Ln and γLn, (D) differences in retention times between Ln and γLn, (E) agreement of statistical ratios of peak areas in individual DB groups and the theoretical calculation, *e.g.*, the identification of the DB = 7 group in Fig. 3 is as follows: OLnLn/LnLnO + OγLnLn/LnγLnO (theoretical relative abundance 4; experimental 4), LnOLn (1; 1.1), OLnγLn/γLnLnO + OγLnγLn/γLnγLnO (4; 4), γLnOLn/LnOγLn (2; 2),

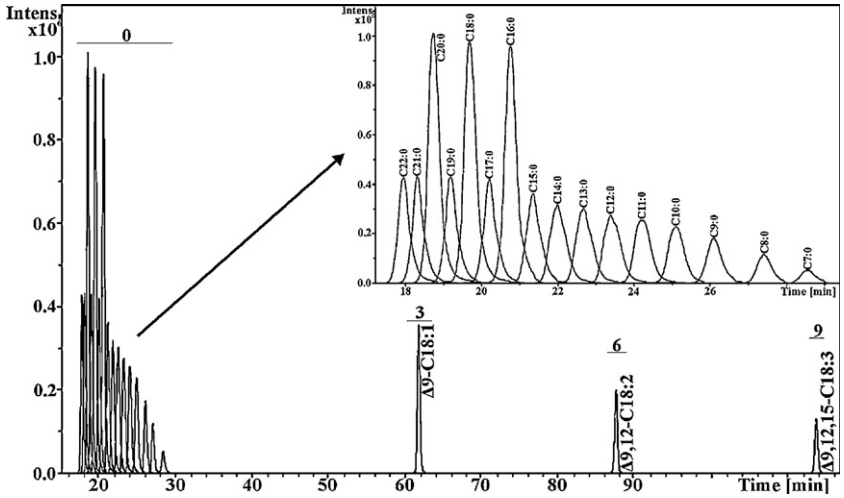

Fig. 4. Silver-ion HPLC/APCI-MS chromatogram of all mono-acid TG standards. Numbers correspond to the double bond number. Inset zoom shows the resolution of saturated TGs from C7:0 to C22:0. Concentrations of all standards are identical except for C16:0, C18:0 and C20:0 with doubled concentrations.

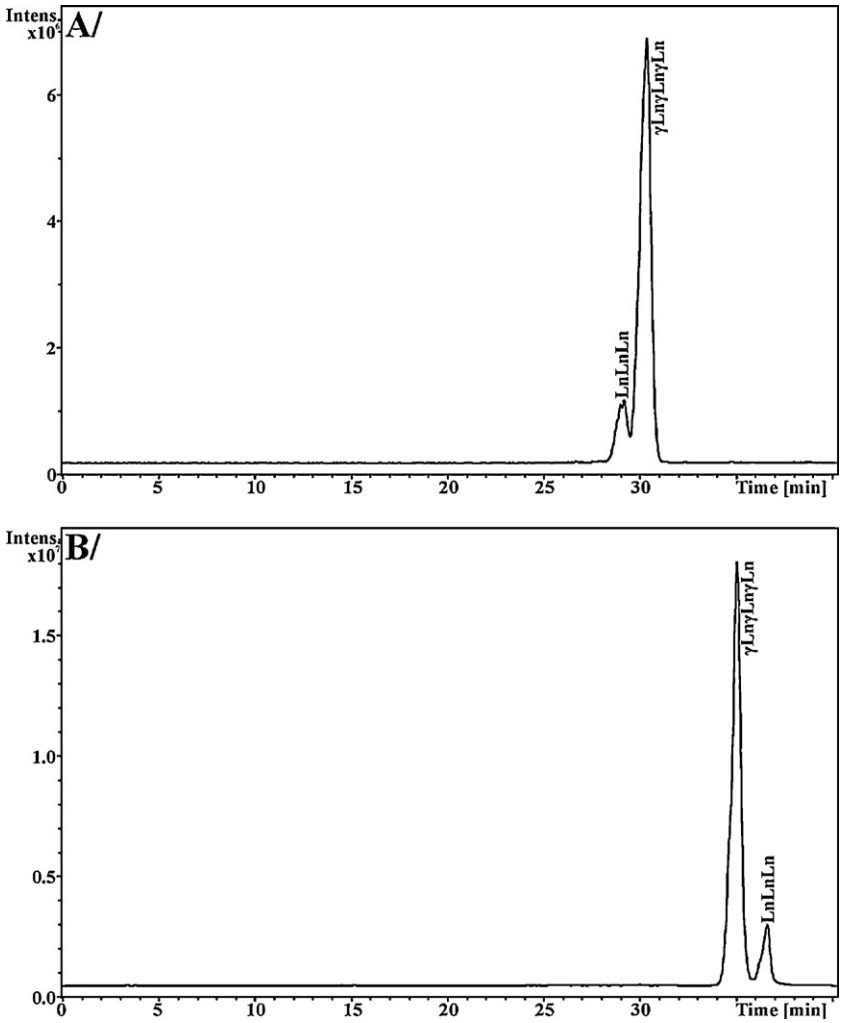

Fig. 5. Comparison of retention behavior of LnLnLn and γLnγLnγLn in two different mobile phase systems: (A) 0 min – 100% A, 60 min – 50% A + 50% B, where A is hexane–2-propanol–acetonitrile (99.8:0.1:0.1, v/v/v) and B is hexane–2-propanol–acetonitrile (96:2:2, v/v/v) [2] and (B) 0 min – 100% acetone, 5 min – 100% acetone, 30 min – 94% acetone + 7% acetonitrile, 40 min – 89% acetone + 11% acetonitrile [20]. All other experimental conditions are identical as in Section 2.

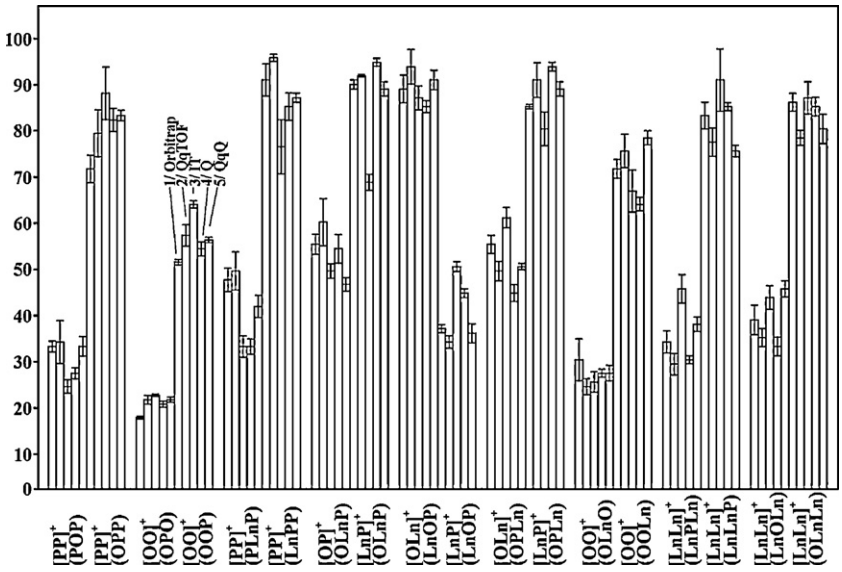

Fig. 6. Plots of relative abundances of $[R_1R_1]^+$ related to $[R_1R_2]^+$ fragment ions (100%) with their standard deviations for the randomization mixture PPP/OOO/LnLnLn measured on five different instruments: (1) LTQ Orbitrap XL (Thermo Fisher Scientific), (2) quadrupole time-of-flight micrOTOF-Q (Bruker Daltonics), (3) ion trap Esquire 3000 (Bruker Daltonics), (4) single quadrupole LC/MSD (Agilent Technologies) and (5) triple quadrupole API 3000 (AB SCIEX).

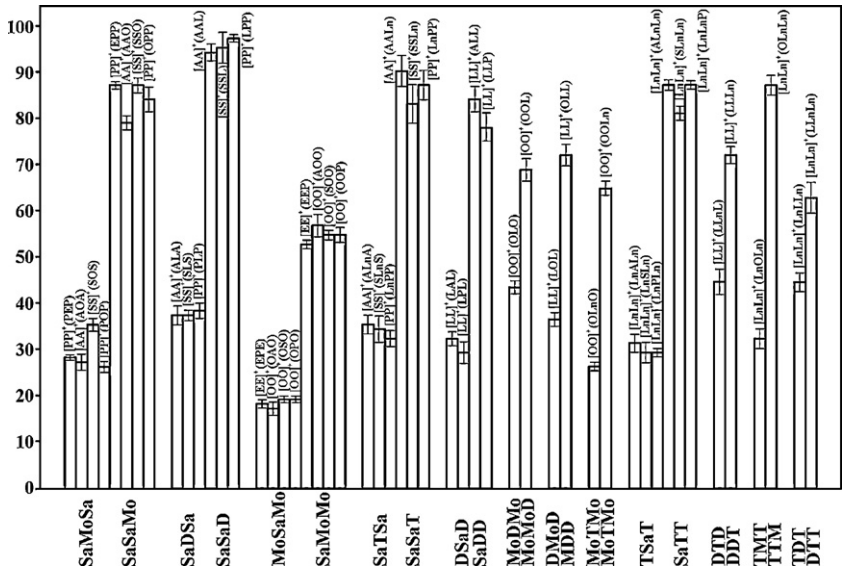

Fig. 7. Plots of relative abundances of $[R_1R_1]^+$ related to $[R_1R_2]^+$ fragment ions (100%) with their standard deviations for randomization mixtures measured on the single quadrupole mass spectrometer; annotation of *x* axis: Sa – saturated, Mo – monounsaturated, D – diunsaturated, T – triunsaturated; annotation of particular columns correspond to individual $[M+H-R_2COOH]^+$ ions fragmented from TGs written in parentheses.

γLnOγLn (1; 0.9). The fragmentation behavior of TGs containing γLn is significantly different, as illustrated on the example of OOγLn (see Table 2), where the ratio of $[R_1R_1]^+/[R_1R_2]^+$ is reversed in comparison to all other measured TGs.

The retention in silver-ion chromatography increases with an increasing number of DBs. Optimized conditions can provide at least the partial separation even for saturated TGs, as shown in the chromatogram (Fig. 4) of standard mixtures containing saturated TGs with C7:0 to C22:0 and unsaturated TGs with C18:1, C18:2 and C18:3. If the response factor (RF) of C18:1 (triolein) is set to 1.00, then RFs of saturated TGs from C14:0 to C22:0 are in the range of 0.59–0.64 (relative difference is lower than 8%), the RF of LLL is 1.37 and of LnLnLn 1.86. Regioisomers have identical RFs. The best chromatographic resolution of regioisomers has been published by our method [2] using hexane–2-propanol–acetonitrile as the mobile phase and in separations published by Adlof [18] using conventional hexane–acetonitrile mobile phase. The addition of 2-propanol does not bring any improvement in the chromatographic resolution, but it improves the limited miscibility of hexane and acetonitrile and therefore yields much better intra- and inter-day reproducibility, which is a serious problem with hexane–acetonitrile mobile phases. It is interesting that the retention order of TGs containing Ln and γLn in our work is reversed in comparison to all published articles so far [11,20,41,42]. For that reason, we repeated the experiment [20] using the previously reported mobile phase composition. Fig. 5 confirms that the retention order of DB positional isomers strongly depends on the mobile phase composition and that the order of LnLnLn and γLnγLnγLn is really reversed in the two systems. Other interesting example of strong effect of DB position is the ratio of $[R_1R_1]^+/[R_1R_2]^+$ fragment ions, which is completely reversed in case of OOγLn unlike to all other regioisomers listed in Table 2.

The main goal of our inter-instrument comparison of APCI mass spectra of TG regioisomers has been to study to what extent are published ratios of $[M+H-R_iCOOH]^+$ ions [30,33,34,39,40] applicable for different instruments and laboratories. Five different types of mass analyzers from various manufacturers have been selected and all regioisomeric mixtures have been measured by a single person using identical conditions. Fig. 6 shows a visual comparison for some regioisomers (the full data set is shown in Tables 2 and 3). Mostly, differences among instruments are within the common experimental errors expressed by the standard deviation but not in all cases. These values of $[M+H-R_iCOOH]^+$ ratios can be used for quantitation without the need of silver-ion HPLC separation of regioisomers. For example, NARP-HPLC provides the separation of TGs but without regioisomeric distinction but by comparing the ratio of $[M+H-R_iCOOH]^+$ ions with those in Tables 2 and 3, the regioisomeric ratio can be determined. This assumption is based on the known fact [6,24,37–39] that calibration curves for regioisomeric mixtures are linear and therefore accurately determined $[M+H-R_iCOOH]^+$ ratios of pure regioisomers can be used for the construction of this linear dependence. We have not detected any dependence of $[M+H-R_iCOOH]^+$ ratios on the mobile phase composition. Measurements of regioisomeric standards on the same instrument should generate, of course, more accurate results, but this level of precision is often not required in the regioisomeric analysis, since the common practice often relies only on the determination of the prevailing FA in the middle *sn*-2 position based on the deviation from statistically expected ratios of $[M+H-R_iCOOH]^+$ ions.

Ratios of $[M+H-R_iCOOH]^+$ fragment ions strongly depend on the DB number and position in individual FAs, while the DB geometry and FA length have only minor influence (Fig. 7). The data measured on the quadrupole analyzer are grouped according to the unsaturation level of individual FAs, *i.e.*, saturated (Sa), monounsaturated (Mo), diunsaturated (D) and triunsaturated (T). Good correlation is observed among values in individual groups, especially considering the fact that differences in *cis*/*trans* isomerism (*e.g.*, PEP *vs.* POP in SaMoSa group, EPP *vs.* OPP in SaSaMo group) or high differences in alkyl chain lengths (*e.g.*, AOA *vs.* POP in SaMoSa group differs by eight carbon atoms) are neglected. On the other hand, the positions of DBs have a much stronger effect on $[M+H-R_iCOOH]^+$ ratios, as illustrated by the difference between LnOLn and γLnOγLn (32 *vs.* 12%, see Tables 2 and 3) in agreement with previous works [43–45]. Fig. 7 suggests that the data for a TG with a given unsaturation pattern can be generalized for other TGs with the same unsaturation pattern, which can solve the lack of unusual standards but still provide accurate regioisomeric determination. It can be concluded that the number and distribution of DBs have major effects on both retention behavior in silver-ion mode and ratios of

$[M+H-R_iCOOH]^+$ ions, while the alkyl chain length of FAs and the geometry of DBs have only very minor effects.

4. Conclusions

Possibilities and limitations of two basic approaches (silver-ion HPLC and ratios of $[M+H-R_iCOOH]^+$ fragment ions) in the regioisomeric analysis of TGs have been systematically studied. The randomization synthesis of a broad range of standard regioisomeric mixtures, their silver-ion HPLC separation and subsequent measurements of APCI mass spectra on five different mass analyzers (single quadrupole, triple quadrupole, ion trap, hybrid QqTOF and Orbitrap) produce an extensive data set for the generalization of retention and fragmentation behavior of regioisomeric TGs. The randomization enables the preparation of any regioisomeric combination, because mono-acid TG standards are commercially available for both common and unusual TGs. Silver-ion chromatograms show a clear separation of TGs into groups differing in the number of DBs. Our optimized silver-ion system also provides a good resolution inside DB groups, where the DB position and stereospecific position on the glycerol skeleton (*sn*-1/3 or 2) play an important role. DBs in outer *sn*-1/3 positions cause slightly stronger retention compared to *sn*-2 regioisomers. If the only difference between two TGs is in the position of DB (*e.g.*, linolenic *vs.* γ-linolenic acids), then no separation is observed for different DB positions occurring in *sn*-2 FA, while the same situation for *sn*-1/3 positions results in a good chromatographic resolution. The instrument type, for those instruments tested, has only minor effect on $[M+H-R_iCOOH]^+$ ratios, therefore published values can be used for the *sn*-2 identification and rough quantitation on other instruments as well. We recommend three alternative approaches for the TG regioisomeric analysis depending on the laboratory equipment and skills: (A) ratios of chromatographic peak areas in silver-ion HPLC (no need for regioisomeric standards due to the known retention order and identical response factors for regioisomers), (B) fragment ion ratios and (C) combination of both approaches which should provide the most reliable results.

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