Miroslav Lísa[1]
Rumen Denev[2]
Michal Holčapek[1]

[1]Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic
[2]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

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Research Article

Retention behavior of isomeric triacylglycerols in silver-ion HPLC: Effects of mobile phase composition and temperature

A systematic study of the retention behavior of isomeric triacylglycerols (TGs) in silver-ion HPLC on a ChromSpher Lipids column has been performed between 10 to 40°C using the most widespread hexane- and dichloromethane-based mobile phases. The randomization of mono-acyl TG standards and the random esterification of glycerol with fatty acids are employed to produce mixtures of TG isomers. The mobile phase composition has no influence on the general retention pattern, but significant differences in the retention order of double bond (DB) positional isomers in hexane and dichloromethane mobile phases are described and compared with the previous literature data. Saturated TGs with fatty acyl chain length from C7:0 to C22:0 are partially separated using the hexane mobile phase but not at all with the dichloromethane mobile phase. The hexane mobile phase enables at least partial resolution of TG regioisomers with up to seven DBs, while the resolution of only ALA/AAL and ALnA/AALn isomers is achieved with the dichloromethane mobile phase. The effect of temperature differs significantly depending on the mobile phase composition. Retention times of TGs increase with increasing temperature in the hexane mobile phase, while an opposite effect is observed for the dichloromethane mobile phase.

Keywords: Double bond positional isomers / Regioisomers / Silver-ion HPLC / *Trans* isomers / Triacylglycerol
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1 Introduction

Triacylglycerols (TGs) are one of the most important and widespread derivatives of natural fatty acids [1]. They are primary metabolites with great importance for the normal physiological function of all living organisms. TGs serve as the main energy supplies and important food components essential for the human diet. Their structure is rather simple (triesters of fatty acids and glycerol), but they represent one of the most complex natural mixtures due to the large variety of esterified fatty acyls. Natural samples may contain 3^n different TG species, where n represents the number of fatty acyls in the sample. This number includes all possible isomers of TGs with various fatty acyl chain lengths, number, position and *cis*/*trans* configuration of double bonds (DBs), and different positions of fatty acyl chains on the glycerol backbone (regioisomers and enantiomers). All of the possible isomers of TGs are not present in real samples because only certain combinations are preferred in each organism. Nevertheless, natural mixtures are usually very complex and the detailed analysis of the isomers is rather challenging [2].

The most powerful technique in the characterization of TG composition is HPLC, providing a separation of most TG isomers. Coupling with atmospheric pressure chemical ionization (APCI) MS [3–9] enables an unambiguous identification of TG species differing in the fatty acyl chain composition without the necessity of identical standards. Moreover, relative abundances of fragment ions can be used for the differentiation of TG regioisomers ($R^1R^1R^2$ *vs.* $R^1R^2R^1$) [3–5, 10] because experimental results show a lower preference of neutral loss of fatty acids from the *sn*-2 position compared to *sn*-1 and *sn*-3 positions providing the $[M+H-R^2COOH]^+$ fragment ion with a lower relative abundance than statistically expected. Nonaqueous RP HPLC mode provides the separation of TGs based on different fatty acyl chain lengths and degree of saturation, which can be applied even for very complex samples containing tens to hundreds of TG

Correspondence: Dr. Miroslav Lísa, Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 53210 Pardubice, Czech Republic
E-mail: Miroslav.Lisa@upce.cz
Fax: +420-46-603-7068

Abbreviations: A, arachidic acid (C20:0); **APCI**, atmospheric pressure chemical ionization; **DBs**, double bonds; **E**, elaidic acid (Δ*9t*-C18:1); **L**, linoleic acid (Δ*9, 12*-C18:2); **Ln**, linolenic acid (Δ*9, 12, 15*-C18:3); **γLn**, γ-linolenic acid (Δ*6, 9, 12*-C18:3); **O**, oleic acid (Δ*9*-C18:1); **Pe**, petroselinic acid (Δ*6*-C18:1); ***t*Pe**, *trans*-petroselinic acid (Δ*6t*-C18:1); **TG**, triacylglycerol; **Va**, vaccenic acid (Δ*11t*-C18:1); ***c*Va**, *cis*-vaccenic acid (Δ*11*-C18:1)

species [6, 8, 9, 11, 12]. Silver-ion HPLC is widely used for the separation of TGs based on the formation of weak reversible charge-transfer complexes between the π electrons of DBs and silver ions immobilized on the stationary phase [13, 14]. Retention patterns are similar for all lipids containing fatty acyls irrespective of the silver-ion chromatography technique, i.e. the retention is governed by the number, configuration, and positions of DBs in the fatty acyl chains [13–15]. In general, TGs are retained in order of increasing unsaturation number, compounds containing *trans* DBs are eluted significantly earlier than *cis* DB compounds [16–18], and compounds with conjugated DBs are eluted prior to compounds with methylene-interrupted DBs [13]. Furthermore, DB positional isomers [7, 19] and TG regioisomers [16, 18, 19] can also be separated using silver-ion HPLC. The good orthogonality of silver-ion and nonaqueous RP chromatographic modes is used in 2D HPLC for the characterization of very complex TG mixtures [18, 20]. The separation of TG enantiomers differing in positions of fatty acyls on the *sn*-1 and *sn*-3 positions ($R^1R^1R^2$ *vs.* $R^2R^1R^1$) has been achieved on a cellulose-tris-(3,5-dimethylphenylcarbamate) column with a hexane/2-propanol gradient [21].

The chromatographic resolution of TGs using silver-ion HPLC is affected by three factors: column properties, mobile phase composition, and column temperature. Nowadays, almost all silver-ion HPLC–MS experiments are based on the strong cation-exchanger type of silver-ion columns prepared in the laboratory or the single commercially available ChromSpher Lipids column with comparable performance. The mobile phase composition strongly affects the chromatographic resolution of TGs using silver-ion HPLC and careful optimization of solvent composition and gradient steepness can significantly improve the separation including the resolution of regioisomers. Two types of mobile phases are most frequently used in silver-ion HPLC. Chlorinated solvents, such as dichloromethane or dichloroethane with the addition of other polar modifiers at low concentrations, typically acetonitrile, acetone, or methanol [7, 22–25]. The second type is a hexane-based mobile phase with acetonitrile as the polar modifier [16, 17, 26, 27]. Hexane/acetonitrile mobile phases have the unique property to enable the resolution of TG regioisomers, which has not been reported so far for chlorinated mobile phases. The disadvantage of these systems is the low solubility of acetonitrile in hexane and low stability of mobile phases [28], which causes a lower reproducibility of retention times. This drawback can be significantly reduced by the addition of a small amount of 2-propanol into the mobile phase [4, 18, 19, 29, 30] or by the substitution of acetonitrile for butyronitrile [28] providing more stable mobile phases and reproducible retention times. In addition to these two main types, some other solvent combinations have also been reported, for example, toluene- [31, 32] and acetone-based mobile phases [33], but the results do not differ significantly.

In silver-ion HPLC, the effect of temperature is rather complex and is still not fully understood. The change of temperature influences the strength of silver–DB complex, but it is also assumed to strengthen the complex between the silver ion and acetonitrile in the mobile phase [16]. The increase of retention times of TGs with increasing temperature has been described in hexane/acetonitrile mobile phases [16, 19, 34], which is rather unusual in HPLC. Species with higher numbers of *cis* DBs are affected more strongly [16, 24]. On the other hand, the temperature decrease from 20 to 0°C causes the lower retention of TGs in hexane/acetonitrile, but it significantly increases with a further decrease of temperature [34]. However, a comprehensive study of temperature effects depending on the mobile phase composition has not been performed so far.

The goal of our work is the systematic study of the retention behavior of TGs in silver-ion HPLC affected by hexane- and dichloromethane-based mobile phases and by the column temperature. The set of TG standards comprising a large number of isomers is prepared using the random transesterification of TG standards and the random esterification of glycerol by a mixture of selected fatty acids. The retention behavior of all synthesized TG isomers using different mobile phases and temperatures is described and discussed.

2 Materials and methods

2.1 Reagents and solvents

Acetonitrile, 2-propanol (HPLC gradient grade), dichloromethane, methanol and hexane (HPLC grade), glycerol, sodium methoxide, 4-dimethylaminopyridine, and *N,N′*-dicyclohexylcarbodiimide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The model mixture of TG standards GLC#435 (all saturated mono-acyl TGs from C7:0 to C22:0), tripalmitin (PPP, C16:0), tristearin (SSS, C18:0), tripetroselinin (PePePe, Δ*6*-C18:1), triolein (OOO, Δ*9*-C18:1), trielaidin (EEE, Δ*9t*-C18:1), tri-*cis*-vaccenin (*c*Va*c*Va*c*Va, Δ*11*-C18:1), trilinolein (LLL, Δ*9, 12*-C18:2), trilinolenin (LnLnLn, Δ*9, 12,15*-C18:3), tri-γ-linolenin (γLnγLnγLn, Δ*6,9,12*-C18:3), triarachidin (AAA, C20:0) and petroselinic (Δ*6*-C18:1), *trans*-petroselinic (Δ*6t*-C18:1), *cis*-vaccenic (Δ*11*-C18:1) and vaccenic (Δ*11t*-C18:1) acids were purchased from Nu–ChekPrep (Elysian, MN, USA). All solvents and reagents used for HPLC analyses and synthetic procedures were dried with molecular sieves.

2.2 Synthesis of TG standards

2.2.1 Random transesterification of TGs (Method 1)

TG regioisomers were prepared from mono-acyl TG standards (OOO, LLL, etc.) using the randomization procedure described earlier [19]. Briefly, 10 mg of each TG standard and 50 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 at 75°C for 30 min in a water bath under reflux. Then, the mixture was extracted

Table 1. TG composition of mixtures used for silver-ion HPLC–APCI-MS experiments, initial compounds and methods used for their preparation

Mixture	Initial compounds	Method	TG composition[a)]
Mixture 1	Saturated TGs from C7:0 to C22:0, PePePe, OOO, EEE, *c*Va*c*Va*c*Va, LLL, LnLnLn, γLnγLnγLn	Mixture of TG standards	Saturated TGs from C7:0 to C22:0, EEE, *c*Va*c*Va*c*Va, OOO, PePePe, LLL, LnLnLn, γLnγLnγLn
Mixture 2	AAA, OOO, LnLnLn	Method 1	AAA, AOA, AAO, AOO, OAO, ALnA, OOO, AALn, ALnO, AOLn, OALn, OLnO, OOLn, ALnLn, LnALn, OLnLn, LnOLn, LnLnLn
Mixture 3	OOO, LLL, LnLnLn	Method 1	OOO, OLO, OOL, OLL, LOL, OLnO, OOLn, LLL, OLnL, OLLn, LOLn, LLnL, LLLn, OLnLn, LnOLn, LLnLn, LnLLn, LnLnLn
Mixture 4	AAA, LLL, LnLnLn + OOO	Method 1 (OOO added after reaction)	AAA, ALA, AAL, ALnA, OOO, AALn, ALL, LAL, ALnL, ALLn, LALn, LLL, ALnLn, LnALn, LLnL, LLLn, LLnLn, LnLLn, LnLnLn
Mixture 5	AAA, OOO, LLL	Method 1	AAA, AOA, AAO, ALA, AAL, AOO, OAO, ALO, AOL, OAL, OOO, ALL, LAL, OLO, OOL, OLL, LOL, LLL
Mixture 6	EEE, OOO	Method 1	EEE, EOE, OEE, OOE, OEO, OOO
Mixture 7	Pe, *t*Pe, glycerol	Method 2	*t*Pe*t*Pe*t*Pe, *t*Pe*t*Pe*t*Pe, Pe*t*Pe*t*Pe, PePe*t*Pe, Pe*t*PePe, PePePe
Mixture 8	*c*Va, Va, glycerol	Method 2	VaVaVa, Va*c*VaVa, *c*VaVaVa, *c*Va*c*VaVa, *c*VaVa*c*Va, *c*Va*c*Va*c*Va

a) Listed according to retention times in the hexane mobile phase.

with water and methanol to remove sodium methoxide. The hexane phase containing the synthesized TGs was evaporated using a gentle stream of nitrogen and redissolved in hexane at the appropriate concentration before HPLC–MS analysis.

2.2.2 Random esterification of glycerol (Method 2)

A total of 15 mg of each fatty acid, 9.5 mg of glycerol, 13 mg of 4-dimethylaminopyridine, and 22 mg of *N,N'*-dicyclohexylcarbodiimide were weighed into a dry vial with the addition of 1 mL of dichloromethane dried with molecular sieves. The mixture was stirred at room temperature for 1.5 h. Then, TGs were extracted with hexane, the solvent was evaporated using a gentle stream of nitrogen, and the residue was redissolved in hexane to an appropriate concentration before HPLC–MS analysis.

2.3 Silver-ion HPLC–APCI-MS

Silver-ion HPLC–MS experiments were performed on a liquid chromatograph Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany) coupled to an ion-trap mass analyzer Esquire 3000 (Bruker Daltonics, Bremen, Germany). Positive-ion APCI was used in the mass range m/z 50–1200 with the following parameters: pressure of the nebulizing gas 50 psi, drying gas flow rate 3 L/min, temperature 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 of coeluting peaks.

A ChromSpher Lipids column (250 × 4.6 mm, 5 μm, Agilent Technologies) with a flow rate of 1 mL/min and injection volume of 1 μL was used for all silver-ion HPLC experiments with the following gradients:

(i) Hexane-based mobile phase: 0 min 100% A, 60 min 52% A + 48% 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).
(ii) Dichloromethane-based mobile phase: 0 min 100% A, 60 min 33% A + 67% B, where A is dichloromethane and B is dichloromethane/acetonitrile (90:10, v/v).

HPLC experiments were performed at 10, 20, 25, 30, and 40°C for hexane and 10, 25, and 40°C for dichloromethane mobile phases. The column was conditioned 45 min before each analysis to achieve a good reproducibility.

3 Results and discussion

3.1 Silver-ion HPLC–APCI-MS analysis of TGs

A wide range of TG species was tested in this work using silver-ion HPLC in an effort to cover the retention behavior of naturally occurring TGs in plant and animal tissues including all possible isomers, that is, TGs with various fatty acyl chain lengths, number, position and *cis*/*trans* configuration

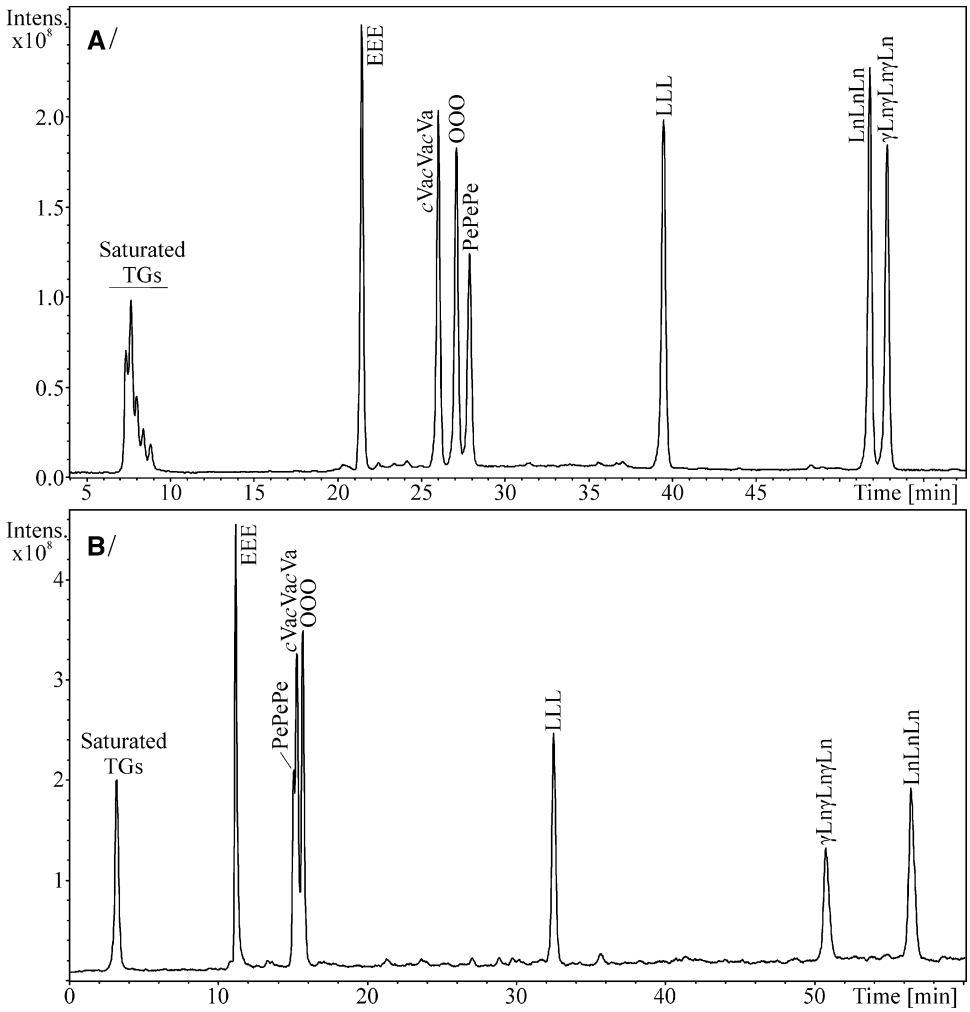

Figure 1. Effect of fatty acyl chain lengths, degree of unsaturation, DB configuration and position (Mixture 1) on the retention behavior of TGs in silver-ion HPLC–APCI-MS analysis: A/ hexane and B/ dichloromethane mobile phases. HPLC conditions: ChromSpher Lipids column (250 × 4.6 mm, 5 μm, Agilent Technologies), flow rate 1 mL/min, column temperature 25°C, gradient A/ 0 min 100% A, 60 min 52% A + 48% 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), B/ 0 min 100% A, 60 min 33% A + 67% B, where A is dichloromethane and B is dichloromethane/acetonitrile (90:10, v/v).

of DBs, and different positions of fatty acyl chains on the glycerol skeleton (regioisomers). For this purpose, two synthetic procedures (the randomization of TGs and the random esterification of glycerol by fatty acids) have been applied to produce isomeric mixed-acyl TGs (Table 1). The randomization reaction (Method 1) of mono-acyl TG standards (AAA, OOO, etc.) catalyzed by sodium methoxide [19] provides an equimolar mixture of all possible TG isomers including regioisomers and enantiomers (Table 1) due to the random distribution of individual fatty acyls on the glycerol skeleton during the randomization process. The random esterification of glycerol by the mixture of selected fatty acids (Method 2) is used in cases when corresponding TG standards are not commercially available. This method provides an equimolar mixture of all possible TG isomers due to the random distribution of fatty acyls on the glycerol skeleton without any noticeable preference of individual fatty acids (Table 1).

Two different silver-ion HPLC methods using the most widespread hexane- and dichloromethane-based mobile phases and a ChromSpher Lipids column have been developed in this work. The hexane mobile phase is used according to our previously developed method [19], which enables the separation of TGs with different degrees of unsaturation, DB positional isomers and also TG regioisomers. This mobile phase contains acetonitrile as a polar modifier with the addition of 2-propanol. In this method, 2-propanol in the mobile phase does not influence the retention behavior of TGs as shown in Supporting Information Fig. S1, but it increases the miscibility of hexane and acetonitrile and improves the reproducibility of retention times [19]. Acetonitrile is also used as the polar modifier in the dichloromethane mobile phase. The presence of acetonitrile in both mobile phases partly eliminates the influence of modifier on the retention behavior of TGs so that mainly the effects of hexane and dichloromethane solvents should be observed. An identical silver-ion HPLC column is used for experiments with both mobile phases to guarantee the same properties of the column. First, the column was used in the hexane mobile phase, then properly flushed and used in the dichloromethane mobile phase. The column properties are significantly changed after use with dichloromethane in the mobile phase, which were tested with selected hexane experiments. The original performance of the column was reached after two days of conditioning with hexane mobile phase. Column temperatures

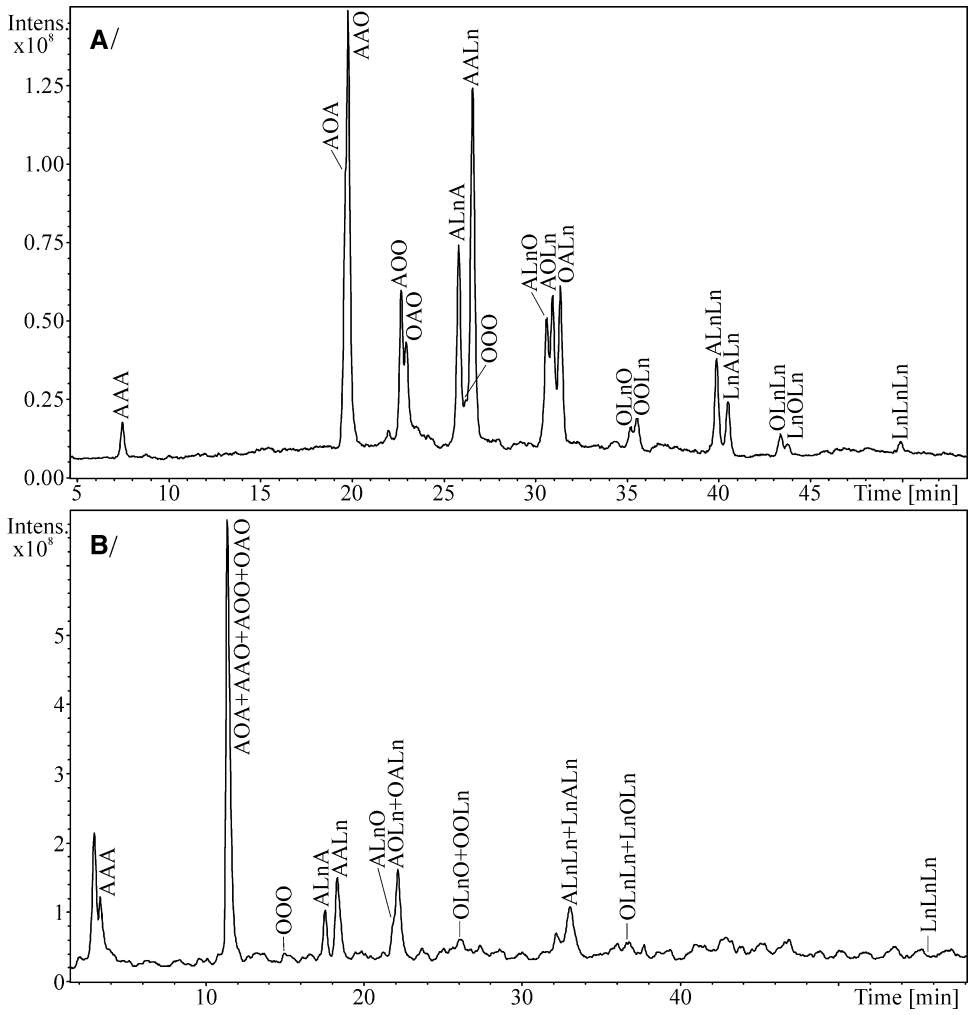

Figure 2. Comparison of silver-ion HPLC–APCI-MS chromatograms of synthesized TGs (Mixture 2) prepared by the randomization reaction of AAA, OOO, and LnLnLn standards according to Method 1: A/ hexane and B/ dichloromethane mobile phases. HPLC conditions are the same as stated for Fig. 1.

in the range from 10 to 40°C were used for testing the temperature effect on the retention behavior of TGs. This temperature range is commonly used for silver-ion HPLC experiments and it is dictated by the lower limit of the column thermostat used for these experiments and the upper limit of column stability recommended by the manufacturer.

Individual TGs are identified based on their APCI full mass spectra. Protonated molecules $[M+H]^+$ or ammonium adducts $[M+NH_4]^+$ are used for the determination of TG molecular weights, while fatty acyl composition is determined from $[M+H-R^iCOOH]^+$ fragment ions formed by neutral losses of fatty acids from the glycerol skeleton. TG regioisomers are identified based on the lower relative abundance of $[M+H-R^2COOH]^+$ fragment ion formed by the neutral loss of fatty acid from the *sn*-2 position, which is energetically less preferred compared to the *sn*-1 and *sn*-3 positions. TG enantiomers differing between the *sn*-1 and *sn*-3 fatty acyl chains cannot be resolved by silver-ion HPLC or identified by MS and require the use of chiral column [21]. Fatty acyls in *sn*-1 and *sn*-3 positions are ordered according to their decreasing molecular weight, for example, TG with arachidic and oleic acyls in the outer *sn*-1/3 positions and linoleic acyl in the *sn*-2 position is annotated with arachidic acyl in the *sn*-1 position as ALO (not OLA), because arachidic acyl has higher molecular weight (MW = 312) compared to oleic acyl (MW = 282). The APCI mass spectra of TGs in both hexane and dichloromethane mobile phases are almost identical, but significantly worse S/N is observed due to the increased baseline for dichloromethane given by a lower compatibility of chlorinated solvents with MS detection compared to hexane (based on our experience).

3.2 Effect of mobile phase composition

Figures 1–5 and Supporting Information Figs. S2–S4 show silver-ion HPLC–APCI-MS chromatograms of prepared TG mixtures using hexane (A/) and dichloromethane (B/) mobile phases at a column temperature of 25°C. In both silver-ion HPLC systems, general retention patterns are valid, that is, TGs are retained in order of increasing number of DBs, *trans* isomers elute prior to *cis* isomers and DB positional isomers are also separated. Nevertheless, large differences between both mobile phases are observed. Figure 1 shows

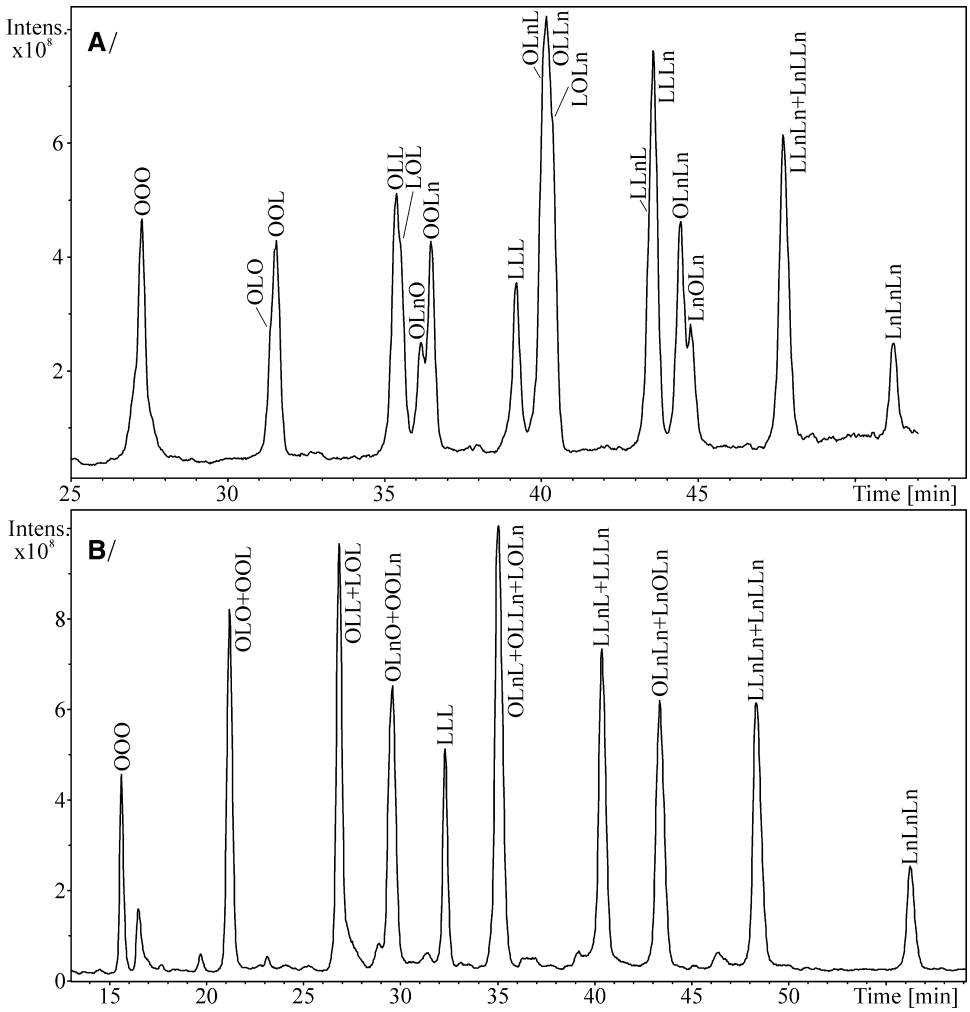

Figure 3. Comparison of silver-ion HPLC–APCI-MS chromatograms of synthesized TGs (Mixture 3) prepared by the randomization reaction of OOO, LLL, and LnLnLn standards according to Method 1: A/ hexane and B/ dichloromethane mobile phase. HPLC conditions are the same as stated for Fig. 1.

effects of different fatty acyl chain lengths, degree of unsaturation, DB configuration and position on the retention behavior of TGs in silver-ion HPLC using hexane (Fig. 1A) and dichloromethane (Fig. 1B) mobile phases. Significant differences are observed mainly for the separation of DB positional isomers. In the hexane mobile phase, retention times of DB positional isomers with C18:1 and C18:3 acyls increase with decreasing distance between the first DB and the carbonyl, that is, *c*Va*c*Va*c*Va < OOO < PePePe (Δ*11* < Δ*9* < Δ*6*) and LnLnLn < γLnγLnγLn (Δ*9, 12, 15* < Δ*6,9,12)*, respectively. The retention order of isomers is changed on using the dichloromethane mobile phase to PePePe < *c*Va*c*Va*c*Va < OOO (Δ*6* < Δ*11* < Δ*9*) and γLnγLnγLn < LnLnLn (Δ*6,9,12* < Δ*9, 12, 15*). The retention order of C18:3-TG isomers using our dichloromethane mobile phase corresponds to data achieved with chlorinated mobile phases, such as dichloromethane/1,2-dichloroethane/acetone/acetonitrile [7], but also with acetone/acetonitrile [35] (Table 2). All experiments in this work differ only in hexane and dichloromethane because the silver-ion HPLC column is identical, acetonitrile is the modifier in both mobile phases and 2-propanol does not influence the retention order of TGs (Supporting Information Fig. S1). Therefore, the change of retention order of DB positional isomers in this work is probably because of the different influence of hexane and dichloromethane on silver ion–DB complexation. In general, the change of retention order of DB positional isomers between hexane- and dichloromethane-based mobile phases cannot be unambiguously ascribed to the change of mobile phase solvent only, but this phenomenon is probably given by a more complex mechanism, as demonstrated for the separation of DB positional isomers of fatty acid phenacyl esters using different mobile phases (Table 2). For example, the change of retention order of some C20:1 and C18:3 fatty acid isomers has been demonstrated after the change of modifier from acetonitrile to 2-propanol [36], that is, the retention order of C20:1 fatty acid isomers is Δ13 < Δ11 < Δ5 < Δ8 using hexane/dichloromethane/acetonitrile *vs.* Δ13 < Δ11 < Δ8 < Δ5 using hexane/dichloromethane/2-propanol or the retention order of C18:3 isomers Δ9, 12, 15 < Δ6, 9, 12 using dichloromethane/acetonitrile *vs.* Δ6, 9, 12 < Δ9, 12, 15 using dichloromethane/2-propanol mobile phase. On the other hand, the retention order of C18:3 fatty acid isomers using dichloromethane/acetonitrile (Δ9, 12, 15 < Δ6, 9, 12) does

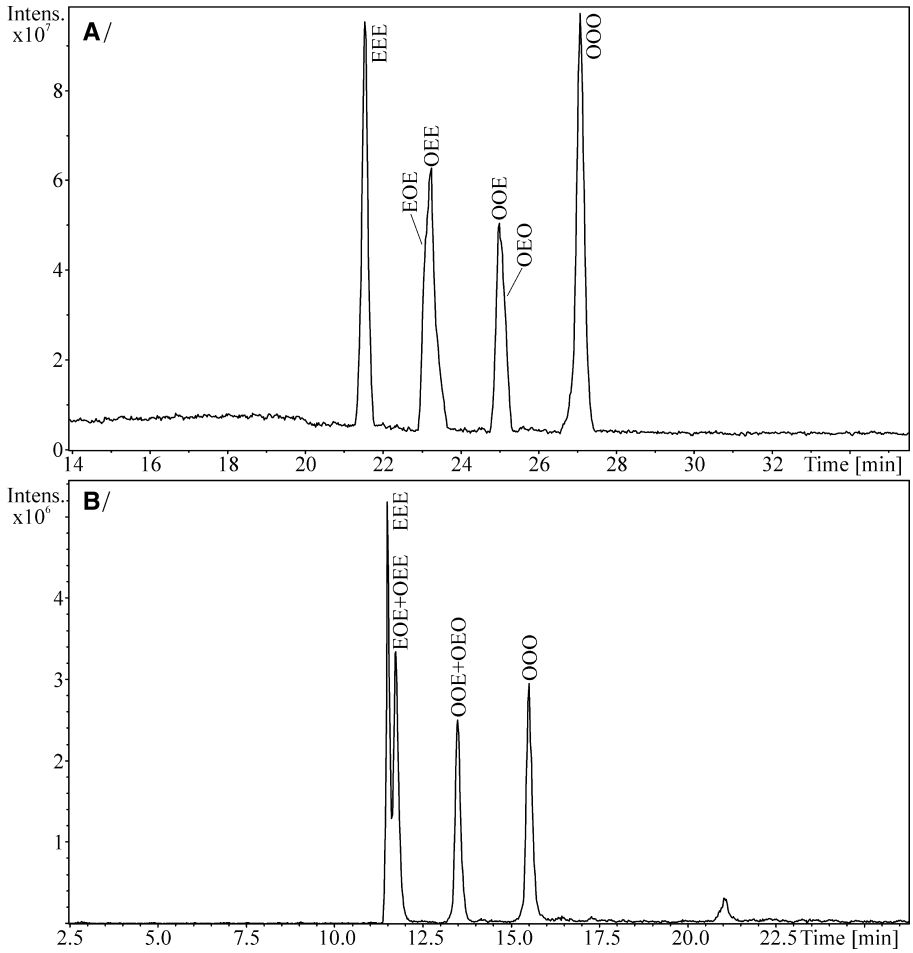

Figure 4. Comparison of silver-ion HPLC–APCI-MS chromatograms of synthesized TGs (Mixture 6) prepared by the randomization reaction of OOO and EEE standards according to the Method 1: A/ hexane and B/ dichloromethane mobile phases. HPLC conditions the same as stated for Fig. 1.

not correspond to the retention order of C18:3-TG isomers using our dichloromethane/acetonitrile mobile phase (Δ6, 9, 12 < Δ9, 12, 15), but to the hexane/acetonitrile/2-propanol mobile phase (Δ9, 12, 15 < Δ6, 9, 12). Moreover, other results show the identical retention order of C18:1 fatty acid isomers in 1,2-dichloroethane/dichloromethane/acetonitrile [24] and hexane/dichloromethane/2-propanol [37] mobile phases with acetonitrile and 2-propanol as modifiers, that is, Δ11 < Δ9 < Δ6, which also corresponds to the retention order of C18:1-TGs using our hexane/acetonitrile/2-propanol mobile phase.

The chromatographic behavior of TGs with different fatty acyl chain lengths varied in both silver-ion systems. While fully saturated mono-acyl TGs containing C7:0 to C22:0 acyls are partly separated in the hexane mobile phase (Fig. 1A), no separation is observed in the dichloromethane mobile phase (Fig. 1B). Retention times of saturated TGs increase with the decreasing number of carbon atoms in fatty acyl chains in the hexane mobile phase (Supporting Information Fig. S5), which is consistent with previously published data [4, 19, 38]. The separation of TG species without DBs using silver-ion HPLC has been ascribed to a normal-phase effect of silica-based silver-ion HPLC columns [38]. This phenomenon is probably suppressed by the higher polarity of dichloromethane and a different interaction with the stationary phase, because no separation of saturated species in dichloromethane mobile phase appeared using the same silver-ion HPLC column as for hexane experiments.

The dichloromethane mobile phase is less suitable for the separation of TG regioisomers, because only ALA/AAL and ALnA/AALn regioisomers (Fig. 2B, Supporting Information Figs. 2B and 3B) are separated in synthesized mixtures. In the hexane mobile phase, regioisomers with up to seven DBs (OLnLn/LnOLn) are at least partially resolved (Figs. 2A, 3A, Supporting Information Figs. 2A and 3A). Better separation of TG regioisomers is observed for TGs containing fatty acyl chains with substantial differences in DB number, for example, ALA/AAL isomers ($R = 0.97$ at 25°C) are partially but clearly resolved, while LLnLn/LnLLn isomers elute in a single peak ($R = 0$) (Table 3). In general, the separation of TG regioisomers with a combination of fatty acyl chains of higher unsaturation like O/L or L/Ln is very difficult [17, 19] and they are observed as broader peaks with shoulders in this work, for example, OLO/OOL and OLL/LOL or LLnL/LLLn and LLnLn/LnLLn (Fig. 3A). As a rule of thumb, regioisomers with fatty acyl chains with higher number of DBs in the *sn*-1 and *sn*-3 positions are retained more strongly, for example, ALnO (one DB in the *sn*-1 and *sn*-3 positions) < AOLn (three DBs) < OALn (four DBs) (Fig. 2A). The separation

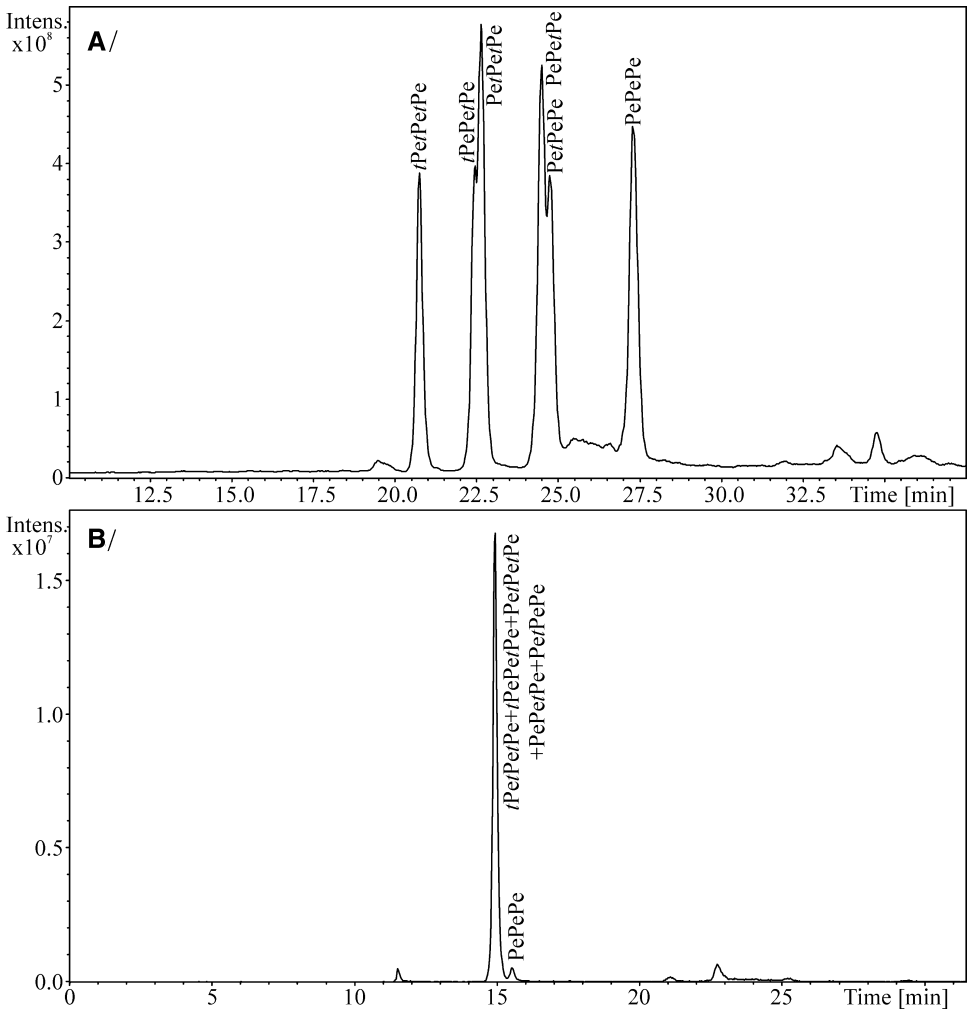

Figure 5. Comparison of silver-ion HPLC–APCI-MS chromatograms of synthesized TGs (Mixture 7) prepared by the random esterification of glycerol with Pe and *t*Pe fatty acid standards according to Method 2: A/ hexane and B/ dichloromethane mobile phases. HPLC conditions are the same as stated for Fig. 1.

of TG regioisomers with *cis* and *trans* configuration of DBs strongly depends on their positions in fatty acyl chains. Thus, while *t*PePe*t*Pe/Pe*t*Pe*t*Pe and PePe*t*Pe/Pe*t*PePe isomers (Δ*6*) are partially resolved at 25°C (Fig. 5A), no separation of E/O (Δ*9*) (Fig. 4A) and Va/*c*Va (Δ*11*) (Supporting Information Fig. S4A) isomers is observed. Individual TG regioisomers are identified based on relative ratios of $[M+H-R^{i}COOH]^{+}$ fragment ions. These ratios are similar for both mobile phases, for example, the ratio of $[AA]^{+}/[ALn]^{+} = 61{:}100$ for ALnA and 100:83 for AALn in the hexane mobile phase compared to 52:100 and 94:100 in the dichloromethane mobile phase, respectively.

Minor changes in the retention order of TGs in both systems can be observed inside TG groups with the same number of DBs. Groups of TGs containing one and two DBs are not resolved in the dichloromethane mobile phase compared to the hexane mobile phase (Fig. 2 and Supporting Information Fig. S3). OOO elutes between ALnA and AALn isomers in hexane (Fig. 2A and Supporting Information Fig. S2A), while it elutes ahead of these TGs in the dichloromethane mobile phase (Fig. 2B and Supporting Information Fig. S2B). Similarly, ALL/LAL isomers elute ahead of OLO/OOL isomers in hexane (Supporting Information Fig. S3A) compared to OLO/OOL isomers eluting ahead of ALL/LAL isomers in the dichloromethane mobile phase (Supporting Information Fig. S3B). Significant differences can be observed among the separation of *cis*/*trans* TG isomers with the different position of DBs using the dichloromethane mobile phase. While E/O isomers are resolved using the dichloromethane mobile phase (except from their regioisomers) (Fig. 4B), most of the *t*Pe/Pe isomers (Fig. 5B) and Va/*c*Va isomers (Supporting Information Fig. S4B) co-elute in one peak. The chromatographic resolution of TGs using the dichloromethane mobile phase is usually better than in the hexane mobile phase at these conditions (Table 3), but the dichloromethane mobile phase does not provide any resolution of most TG regioisomers, saturated TGs and some *cis*/*trans* TG isomers.

3.3 Effect of temperature

The effect of column temperature on the van't Hoff plots and relative retention times of synthesized mixtures using

Table 2. Influence of the mobile phase composition on the retention order of DB positional isomers of mono-acyl triacylglycerols (TGs) and fatty acid phenacyl esters (FAPEs)

Mobile phase composition	Species	Retention order of double bond positional isomers	Ref.
Hexane/acetonitrile/2-propanol	C18:1-TG	$\Delta11 < \Delta9 < \Delta6$	
Dichloromethane/acetonitrile	C18:1-TG	$\Delta6 < \Delta11 < \Delta9$	
1,2-Dichloroethane/dichloromethane/acetonitrile	C18:1-FAPE	$\Delta11 < \Delta9 < \Delta6$	[24]
Hexane/dichloromethane/2-propanol	C18:1-FAPE	$\Delta11 < \Delta9 < \Delta6$	[37]
Hexane/dichloromethane/acetonitrile	C20:1-FAPE	$\Delta13 < \Delta11 < \Delta5 < \Delta8$	[36]
Hexane/dichloromethane/2-propanol	C20:1-FAPE	$\Delta13 < \Delta11 < \Delta8 < \Delta5$	[36]
Hexane/acetonitrile/2-propanol	C18:3-TG	$\Delta9, 12, 15 < \Delta6, 9, 12$	
Dichloromethane/acetonitrile	C18:3-TG	$\Delta6, 9, 12 < \Delta9, 12, 15$	
Dichloromethane/1,2-dichloroethane/acetone/acetonitrile	C18:3-TG	$\Delta6, 9, 12 < \Delta9, 12, 15$	[7]
Acetone/acetonitrile	C18:3-TG	$\Delta6, 9, 12 < \Delta9, 12, 15$	[35]
Dichloromethane/acetonitrile	C18:3-FAPE	$\Delta9, 12, 15 < \Delta6, 9, 12$	[36]
Dichloromethane/2-propanol	C18:3-FAPE	$\Delta6, 9, 12 < \Delta9, 12, 15$	[36]

Table 3. Chromatographic resolution of selected TG pairs containing various combinations of fatty acyls (Sa—saturated, Mo—monounsaturated, D—diunsaturated, and T—triunsaturated fatty acyls) using silver-ion HPLC with hexane and dichloromethane mobile phases at different temperatures

TG pairs		Chromatographic resolution[a)]							
		Hexane					Dichloromethane		
		10°C	20°C	25°C	30°C	40°C	10°C	25°C	40°C
Sa/Sa	SSS/PPP	0.65	0.51	0.69	0.56	0.67	0	0	0
Mo/Mo	EEE/OOO	4.29	5.97	7.82	8.32	12.47	10.12	9.42	9.42
T/T	γLnγLnγLn/LnLnLn	1.38	1.07	1.27	1.23	1.43	4.06	4.36	4.36
Sa/Mo	AOA/AAO	1.15	1.62	0.64	0.25	0.49	0	0	0
	OOA/OAO	0.30	0.31	0.44	0.59	0.61	0	0	0
Sa/D	ALA/AAL	0.59	0.57	0.97	0.82	0.76	1.14	1.12	0
	ALL/LAL	0.41	0.50	0.35	0.41	0.51	0	0	0
Sa/T	ALnA/LnAA	0.60	0.71	0.73	0.67	0.68	0.89	0.82	0.80
	ALnLn/LnALn	0.54	0.69	0.32	0.49	0.43	0	0	0
Mo/Di	OLO/OOL	0	0	0	0	0	0	0	0
	OLL/LOL	0	0	0	0	0	0	0	0
Mo/T	OLnO/OOLn	0.38	0.37	0.77	0.40	0.37	0	0	0
	OLnLn/LnOLn	0.41	0.26	0.36	0.37	0.29	0	0	0
D/T	LLnL/LLLn	0	0	0	0	0	0	0	0
	LLnLn/LnLLn	0	0	0	0	0	0	0	0

a) Chromatographic resolution $R = 2\ (t_{RB} - t_{RA})/(w_A + w_B)$, where t_R are retention times of the TGs and w their peak widths.

hexane and dichloromethane mobile phases is shown in the Supporting Information (Supporting Information Charts S1–S9). Figures 6 and 7 show the effect of temperature on the retention times of mono-acyl TGs with fatty acyl chains differing in length, number, configuration and position of DBs using hexane and dichloromethane mobile phases (Supporting Information Table 1). The effect of temperature differs between the mobile phases. In the hexane mobile phase, retention times increase with higher temperature (in the range 10–40°C; Fig. 6), which is consistent with published data using a hexane/acetonitrile mobile phase [16, 34]. This phenomenon is rather unusual for most HPLC modes and has been explained by the fact that temperature induces changes in the stability of the acetonitrile–DB complex [16]. On the other hand, our dichloromethane mobile phase also contains acetonitrile as the modifier, but retention times of TGs decrease with increasing temperature (Fig. 7). This temperature effect has also been shown in the analysis of methyl and phenacyl esters of oleic acid using a 1,2-dichloroethane/dichloromethane/acetonitrile

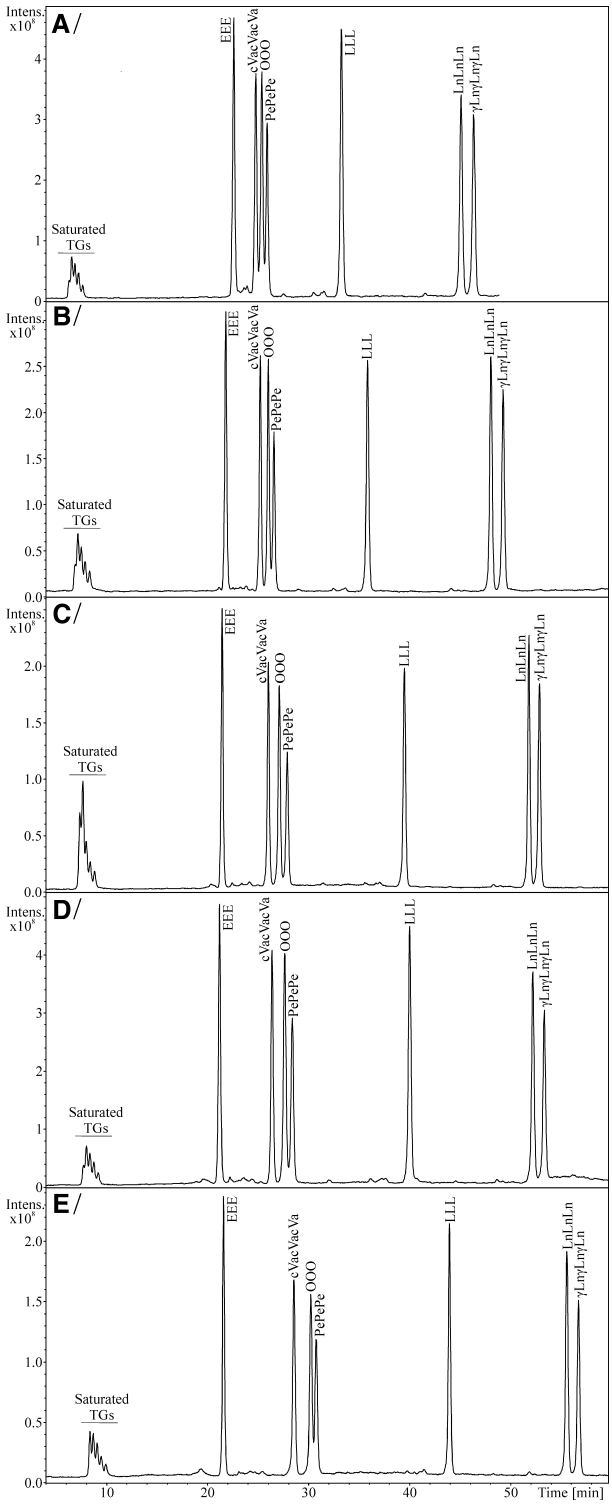

Figure 6. Effect of column temperature on the retention behavior of TGs with various fatty acyl chain lengths, degrees of unsaturation, DB configurations and positions (Mixture 1) in silver-ion HPLC–APCI-MS analysis using the hexane mobile phase: A/ 10, B/ 20, C/ 25, D/ 30, and E/ 40°C. HPLC conditions: ChromSpher Lipids column (250 × 4.6 mm, 5 μm, Agilent Technologies), flow rate 1 mL/min, gradient 0 min 100% A, 60 min 52% A + 48% 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).

mobile phase [24]. Therefore, the different retention behavior is probably not caused by acetonitrile, which is identical for both mobile phases, but mainly by the effect of hexane and dichloromethane solvents on the stationary phase.

In the hexane mobile phase, the effect of temperature is stronger for TGs with the higher number of DBs. For example, the retention time of LnLnLn changes from 46.9 min at 10°C to 56.8 min at 40°C compared to retention times of saturated TGs or EEE (*trans* TGs), which are almost identical at all temperatures (Fig. 6, Supporting Information Table S1). The chromatographic resolution of C18:1-TG isomers slightly increases with increasing temperature mainly for EEE, *c*Va*c*VacVa and OOO isomers (Table 3, Fig. 6). TGs with a higher number of DBs are also affected strongly in the dichloromethane mobile phase (Fig. 7), but the change of retention times is slightly lower compared to that in the hexane mobile phase, for example, the retention time of LnLnLn changes from 55.8 min at 10°C to 48.4 min at 40°C (Supporting Information Table S1). A stronger influence of temperature on the chromatographic resolution using the dichloromethane mobile phase is observed for C18:1-TG isomers, which are better resolved at higher temperature (Fig. 7).

The effect of temperature on the chromatographic resolution of TG regioisomers in the hexane mobile phase is significantly lower compared to the resolution of TGs with a different number of DBs (Supporting Information Fig. S6, Table 3). The chromatographic resolution of regioisomers with three and more DBs is almost identical at all temperatures (Table 3). The resolution of other isomers slightly increases at higher temperatures, except the AOA/AAO isomers, which have a higher resolution at 10 and 20°C (Table 3). A more significant effect of temperature on the chromatographic resolution of regioisomers is observed for *cis*/*trans* TG isomers (Fig. 8, Supporting Information Figs. S7 and S8). Their chromatographic resolution significantly improves when the temperature increases. While the *t*PePe*t*Pe/*t*Pe*t*PePe and PePe*t*Pe/Pe*t*PePe isomers are not resolved at 10°C (Fig. 8A), they are partially separated at 40°C (Fig. 8E). The EOE/EEO and OOE/OEO isomers (Supporting Information Fig. S7) are partially resolved at 40°C, but the VacVaVa/*c*VaVaVa and Va*c*VaVa/*c*VaVaVa isomers (Supporting Information Fig. S8) are not separated at all. Temperature does not affect the chromatographic resolution of the ALA/AAL and ALnA/LnAA isomers using the dichloromethane mobile phase, except for the separation of ALA/AAL isomers at 40°C without any resolution (Table 3).

4 Concluding remarks

A comprehensive study of the retention pattern of a large number of TG isomers containing the most common fatty acyl chains is demonstrated here using silver-ion HPLC on a ChromSpher Lipids column and the most widely used hexane- and dichloromethane-based mobile phases. The hexane-based mobile phase with 2-propanol and acetonitrile as modifiers enables the resolution of TG regioisomers with up to seven DBs. The dichloromethane mobile phase provides

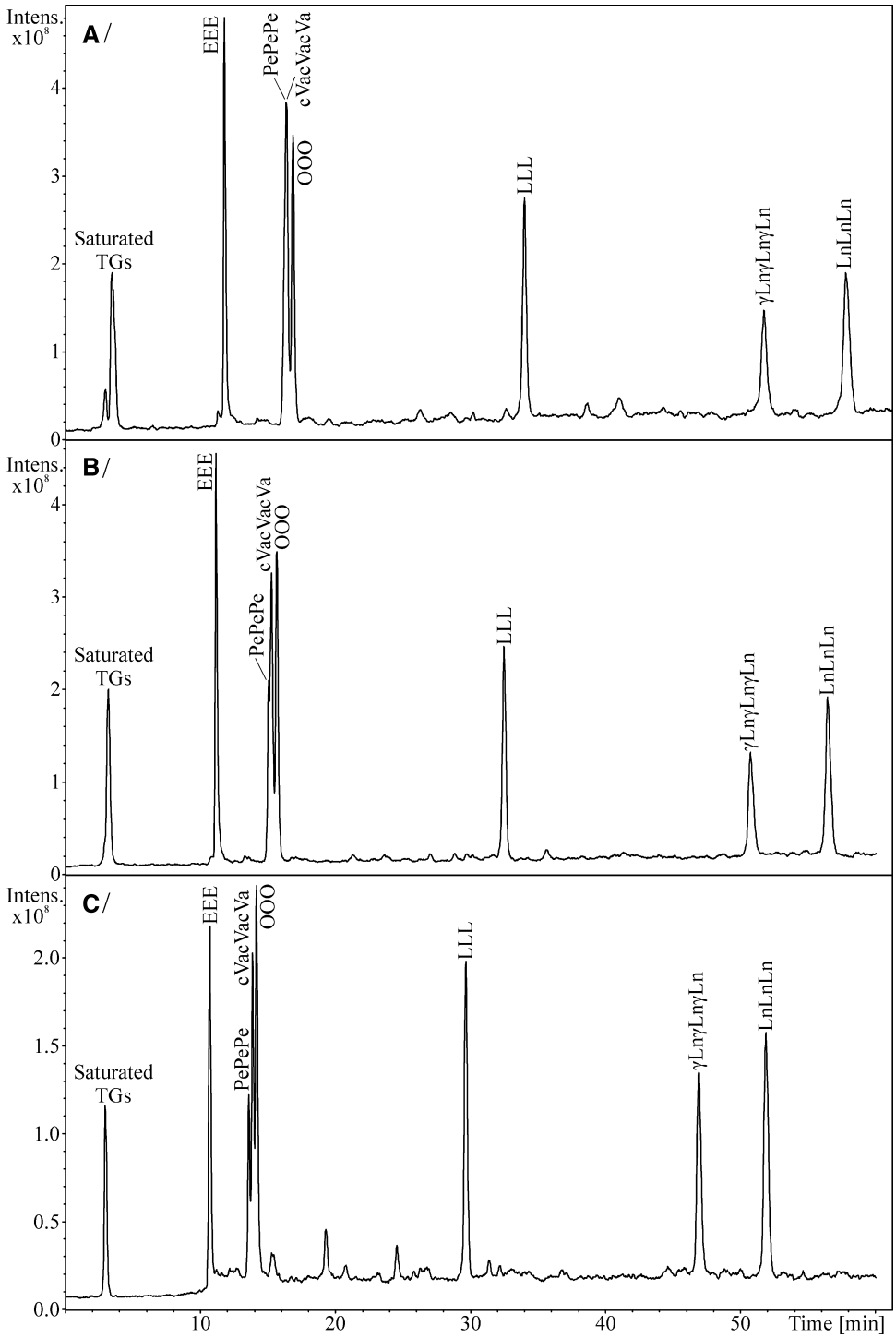

Figure 7. Effect of column temperature on the retention behavior of TGs with various fatty acyl chain lengths, degrees of unsaturation, DB configurations and positions (Mixture 1) in silver-ion HPLC–APCI-MS analysis using dichloromethane mobile phase: A/ 10, B/ 25, and C/ 40°C. HPLC conditions: ChromSpher Lipids column (250 × 4.6 mm, 5 μm, Agilent Technologies), flow rate 1 mL/min, gradient 0 min 100% A, 60 min 33% A + 67% B, where A is dichloromethane and B is dichloromethane/acetonitrile (90:10, v/v).

better chromatographic resolution of TG species compared to the hexane mobile phase, but does not enable the resolution of most TG regioisomers. Significant differences between the hexane and dichloromethane mobile phases are observed mainly in the retention order of TG species differing in positions of the DBs in the fatty acyl chains probably due to the different effect of hexane and dichloromethane on the stationary phase influencing the retention of TGs. Temperature affects the retention of TGs depending on the mobile phase composition. At elevated temperatures, TGs are retained more strongly in hexane and more weakly in the dichloromethane mobile phase. Temperature does not significantly influence

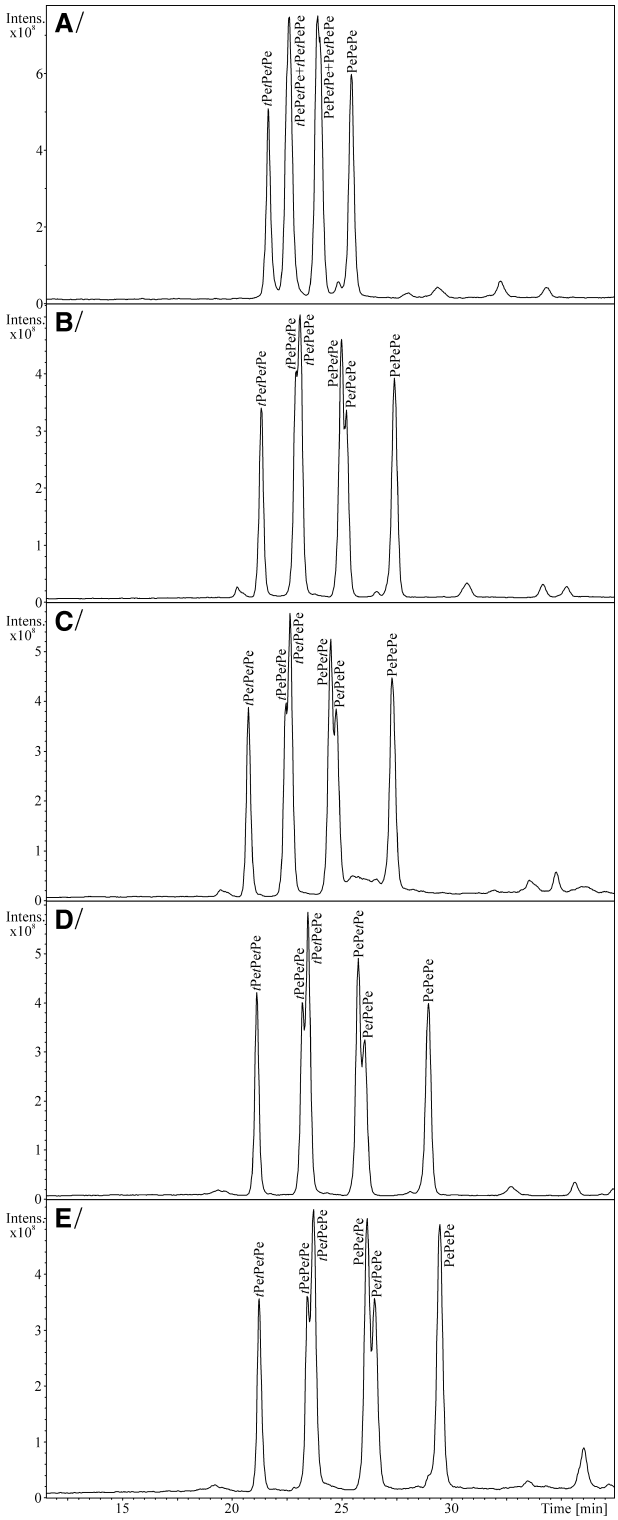

Figure 8. Effect of column temperature on the retention behavior of TGs (Mixture 7) prepared by the random esterification of glycerol with Pe and *t*Pe fatty acid standards according to Method 2 in silver-ion HPLC–APCI-MS analysis using the hexane mobile phase: A/ 10, B/ 20, C/ 25, D/ 30, and E/ 40°C. HPLC conditions are the same as those stated for Fig. 6.

the chromatographic resolution of TG regioisomers with the exception of *cis*/*trans* TG regioisomers using the hexane mobile phase. In general, the mobile phase composition affects the separation of TGs with silver-ion HPLC on a ChromSpher Lipid column more strongly than the temperature.

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