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Enantiomeric Separation of Triacylglycerols Consisting of Three Different Fatty Acyls and Their Chiral Chromatographic Elution Behavior

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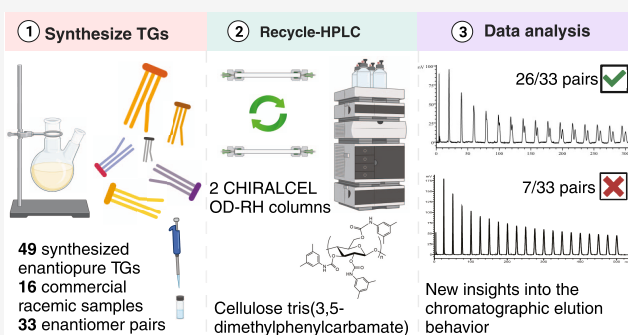
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ABSTRACT: Chromatographic separation of triacylglycerol (TG) enantiomers is a highly challenging task of analytical chemistry because of the similar physicochemical properties. The analysis of chiral TGs is crucial for improving the knowledge of lipid biochemistry and for understanding the nutritional properties of fats and oils. Thus, this study aimed to systematically investigate the chiral resolution of TGs consisting of three different fatty acyls (FAs). Thirty-three asymmetric TG enantiopairs, including 49 synthesized enantiopure TGs and racemic TGs, were analyzed with a recycling chiral HPLC system. Twenty-six enantiopairs were successfully separated. Overall, having both unsaturated and saturated FAs in the outer positions or a difference in carbon chain length between two saturated FAs at the outer positions favored the separation of enantiomers. The retention time at separation correlated negatively with the *sn*-3 carbon number of the early eluting enantiomer and positively with the carbon number difference between *sn*-1 and *sn*-3. When the samples were studied in separate groups based on unsaturation and regioisomers, both the acyl carbon number and the degree of unsaturation of FAs in all three positions influenced the separation and elution behavior of chiral TGs, indicating an active role of both intermolecular interactions and steric hindrances. This is the first systematic study of the chiral separation of TGs consisting of three different FAs using a large number of enantiopairs. The novel findings on the behavior of TG enantiomers in a chiral environment provide important guidance and reference for a stereospecific study of the chemistry and biochemistry of natural lipids.

① Synthesize TGs ② Recycle-HPLC ③ Data analysis

49 synthesized enantiopure TGs
16 commercial racemic samples
33 enantiomer pairs

2 CHIRALCEL OD-RH columns

Cellulose tris(3,5-dimethylphenylcarbamate)

26/33 pairs ✓
7/33 pairs ✗

New insights into the chromatographic elution behavior

■ INTRODUCTION

The chromatographic separation of triacylglycerol (TG) regioisomers and enantiomers has long remained a challenge despite the increasing interest in chiral chromatographic analysis of TGs.[1−3] Already in 1958, Mattson and Lutton[4] claimed that the natural distribution of fatty acyls (FAs) on the glycerol backbone is not random, a fact that is generally accepted. The TG molecule becomes chiral if different FAs are esterified to the outer positions (*sn*-1 and *sn*-3) of glycerol. Thus, TGs consisting of three different FAs (ABC-TGs) are chiral. Enantiomers differ in their three-dimensional configuration at the *sn*-2 stereogenic center[5] and thus typically show different biochemical behaviors[6,7] and physiological properties[8] despite identical chemical composition. For example, the melting behavior of a mixture of TG enantiomers is different from pure enantiomers,[9] and the crystallization behavior of asymmetric TGs affects the physical properties of fat-based products[10].

Although most common TGs in edible oils and fats are composed of one (such as O_O_O, L_L_L) or two kinds of FAs (such as O_O_P, O_P_P, P_L_L, P_L_P, and O_O_S), ABC-TGs also often represent characteristic compounds of some oils,[11] including P_Po_L, Po_L_S, P_Po_O, and P_O_A in olive oil[12,13] or P_O_M in palm oil.[14,15] In some oils, the abundance of ABC-TGs is remarkable, for example, lard contains P_O_S, P_L_O, and P_L_S as major TG species, with ABC-TGs accounting for more than 37% of the total TGs.[16] Further interesting examples are human milk containing O_P_L up to 20% and ABC-TGs up to 50%[17,18] and cocoa butter containing 38% of P_O_S.[19]
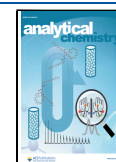
ABC-TGs can form six isomers, which are extremely hard to simultaneously separate chromatographically.[3] For the identification and separation of TGs, several chromatographic methods have been studied. Nagai et al.[3] evaluated the isomeric separation of ABC-TGs P_O_S, P_O_L, and S_O_L using the column CHIRALPAK IF-3 and acetonitrile as mobile phase. Although the isomers were not completely separated, they managed to differentiate six isomers of S_O_L and P_S_O and predict their regioisomeric elution order according to the ratio of diacylglycerol fragment ions analyzed by an ESI-MS/MS system. Lisa and Holčapek[20] analyzed several ABC-TGs including P_O_L, Ln_O_A, O_L_S, S_Ln_O, S_Ln_L, and L_O_Ln using two chiral Lux Cellulose-1 columns and hexane and hexane-2-propanol as mobile phases. All other enantiomers were at least partially separated except Ln_O(sn-2)_A and P_O(sn-2)_L that constituted both saturated FA (SFA) and di- or triunsaturated (U) FA at the outer positions. By varying the degree of the silver modification of the stationary phase of a cation exchange column, better control of the retention mechanism was achieved by Santoro et al.[21] Their method had the advantage of a shorter analysis time within which to separate P_O(sn-2)_S and P_S(sn-2)_O regioisomer pairs. Using a 2D HPLC system consisting of nonaqueous reversed-phase and silver ion HPLC, Zhao et al.[22,23] were able to separate some regioisomers of P_L(sn-2)_Ln, L_O(sn-2)_Ln, and S_L(sn-2)_O.

Although chiral chromatography has been applied for TG enantiomer separation, evidence on the eluting rule of ABC-TGs is presently inconclusive. There is a lack of knowledge of the elution behavior of TGs of varying isomeric structures in the chiral system. Such knowledge is crucial for understanding the mechanisms of retention, elution, and separation of TG positional isomers. Hence, this study aimed to carry out systematic research on the elution and separation of ABC-TGs containing different types of fatty acyls and to study the influence of the structural characteristics and positional distribution of fatty acyls on the separation of TG isomers on chiral columns. A great challenge in the stereospecific analysis of TGs is the lack of commercial standards of enantiopure reference compounds.[24,25] To facilitate the study, 49 enantiopure ABC-TGs (Table S1) were synthesized; these together with 16 commercial regiopure racemic TGs (Table S2) formed 33 enantiopairs (ECN 32−52). The enantiomers were analyzed via chiral HPLC with a sample recycling system and a UV detector. This study systematically investigated the enantiomeric separation of the TG enantiopairs and examined the relation between the structure and elution behavior. To the best knowledge of the authors, this is the first study reporting on the systematic analysis of such a high number of enantiomers of ABC-TGs. The results of this study provide new insights into the chromatographic elution behavior of chiral TGs, improving the current understanding of the retention mechanism of TG isomers on chiral stationary phases and advanced regio- and stereospecific analysis of complex natural lipids.

■ EXPERIMENTAL SECTION

Nomenclature and Abbreviations. To describe the TG structure, stereospecific numbering (sn) was used to distinguish the position of FAs on the glycerol backbone following the LIPID MAPS guideline.[26] TGs are named A_B_C if only the composition of FAs is known, regardless of the FA positional distribution. When only the FA in the middle position is known, e.g., as B, the terminology that differentiates between possible configurations was applied. More specifically, the TG mixture that contains both enantiomers at a molar ratio of 1:1 is named rac-A_B(sn-2)_C, or otherwise TG A_B(sn-2)_C referring to nonracemic mixtures. A/B/C indicates TG with FAs A, B, and C esterified in positions sn-1, 2, and 3, respectively. The equivalent carbon number (ECN) is defined as the number of carbons in the FA residues minus twice the number of double bonds.[27] SFA indicates saturated fatty acid; UFA, unsaturated fatty acid; ECN, equivalent carbon number; C, acyl carbon number; ΔC, difference in acyl carbon number between different positions; and DB, double bond. Abbreviations for individual FAs are denoted as Bu = 4:0 (butyric acid), C = 10:0 (capric acid), La = 12:0 (lauric acid), M = 14:0 (myristic acid), P = 16:0 (palmitic acid), S = 18:0 (stearic acid), O = 18:1 (oleic acid), L = 18:2 (linoleic acid), Ln = 18:3(n-6) (gamma linolenic acid), A = 20:0 (arachidic acid), E = 20:5 (eicosapentaenoic acid), and D = 22:6 (docosahexaenoic acid).

Chemicals and Triacylglycerol Reference Compounds. Methanol and hexane were purchased from Sigma-Aldrich Corporation (St. Louis, MO), and 2-propanol was from VWR International (Radnor, PA). All solvents were HPLC grade.

Forty-nine ABC-TG enantiomers (ECN 32−52, Table S1) were synthesized at the University of Iceland. These compounds can be divided into four categories based on the pattern of saturation or unsaturation of the incorporated FAs: first, those that possess two different SFAs, one esterified in a terminal position sn-1/3 (S) and the other in the sn-2 position (S′), and one UFA located in the remaining terminal sn-1/3 position, category USS′-type, and second, TGs possessing UFA in the sn-2 position and two SFAs in the two sn-1/3 positions, category SUS′-type. Similarly, the third and fourth groups are UU′S and USU′-type possessing two different UFAs and one SFA.

The synthesis of the TGs belonging to the first group was brought about by a six-step chemoenzymatic route described by Gudmundsson et al. starting from enantiopure solketal (1,2-isopropylidene-sn-glycerol) with either (S)- or (R)-solketal as a chiral precursor.[28] The method is based on the use of a benzyl ether protective group, a highly regioselective immobilized *Candida antarctica* lipase (CAL-B), and finally an EDCI (1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide) coupling agent. Some of the SUS′-type TGs were synthesized by a similar chemoenzymatic approach also described by Gudmundsson et al.[28] and Kristinsson and Haraldsson[29] this time involving two *Candida antarctica* lipase steps. The synthetic strategy was based on the easy removal of the benzyl protective moiety by catalytic hydrogenolysis, which leaves the SFAs unaffected. This was different when UFAs were present because they are prone to undergo hydrogenation under deprotection conditions. The remaining SUS′-type TGs and the TGs belonging to the double-unsaturated UU′S and USU′ TG categories were prepared by a similar six-step chemo-enzymatic method that had been modified to accommodate an increased number of UFAs and the presence of a UFA during the deprotection. That required switching to a different p-methoxybenzyl protecting group that undergoes cleavage under mild oxidative conditions by use of DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) under which conditions monounsaturated FAs were observed to easily survive.[30] All of the intermediates and final TG products were obtained

in high chemical and enantiomeric purity. ^{1}H and ^{13}C NMR and IR spectroscopy and satisfactory high-resolution accurate mass spectrometry analyses were used for full characterization of molecular structures, and the measurement of the specific optical rotation and chiral recycling HPLC was used to establish the enantiomeric purity of the TGs.

Sixteen commercial racemic TG samples (ECN 42−52, Table S2) were purchased from Larodan (Solna, Sweden) containing SUS′-, SS′U-, UU′S-, and USU′-type structures and were of at least 98% purity. Among all of the samples, enantiomer pairs were mixed in unequal ratios, or a racemic mixture was spiked with one enantiomer to determine the elution order. All samples were diluted to isopropanol/hexane 4:1 with a final concentration of 1 mg/mL, and the injection volume was 15 μL. In some cases, where the response from the UV detector was insufficient, the concentration was increased to 2 mg/mL. The four samples, each containing regioisomer triplets, were prepared by mixing synthesized enantiopure TGs (Table 1) to test the applicability of the same method for regioisomer separation.

Table 1. Regioisomeric Triplets Formed by Enantiopure Compounds

| sample name	composition	proportion %
mixture 1	M/La/O	20
	La/M/O	30
	M/O/La	50
mixture 2	P/M/O	20
	M/P/O	30
	P/O/M	50
mixture 3	P/C/O	20
	P/O/C	30
	C/P/O	50
mixture 4	La/P/L	20
	P/L/La	30
	P/La/L	50

Chromatographic Conditions. The racemic TG samples and mixture TG samples were analyzed with our group's previously published method[31] using two chiral columns (CHIRALCEL OD-RH, 150 × 4.6 mm, 5 μm, Chiral Technologies Europe, Illkirch, France), methanol as mobile phase, and a sample recycling system. Both automatic valve-switching (by the LCsolution program, Shimadzu Corporation, Kyoto, Japan) and manual switching methods were used. To numerically determine the separation, the peak-to-valley (p/v) ratio for each enantiopair was calculated. When the p/v ratio was above 1, the peaks were considered separated, and the retention time (t_R) of the first eluting enantiomer is presented as the t_R at separation. The separation factors (α's) were calculated at the end of the chromatographic analysis. To evaluate the system performance, theoretical plate numbers (N's) were calculated by using the retention time and peak width of pure compounds of the first eluting enantiomer of each enantiopair after the first columns pass without recycling. All chromatographic parameters were calculated by the LCsolution software.

Statistical Analysis. The Pearson correlation analysis was performed to study the correlation among the carbon chain length, number of double bonds, carbon number difference between the chain lengths at different positions, t_R, ECN, and t_R at separation by the IBM SPSS Statistics 25.0 software

(IBM, Armonk, NY, USA) with two-tailed significance. The significance of this study was set at the 0.05 and 0.01 levels. All data were checked for normal distribution.

■ RESULTS AND DISCUSSION

Separation of Enantiomers. Thirty-three enantiopairs were analyzed in this study, and all the chromatograms are shown in Figure S1. Seven pairs of them were not separated within chromatographic analysis time <8 h under the applied conditions. The analysis time could not be extended as peak broadening limits the number of cycles. Twenty-six enantiopairs were clearly separated (Figure 1). As an example of
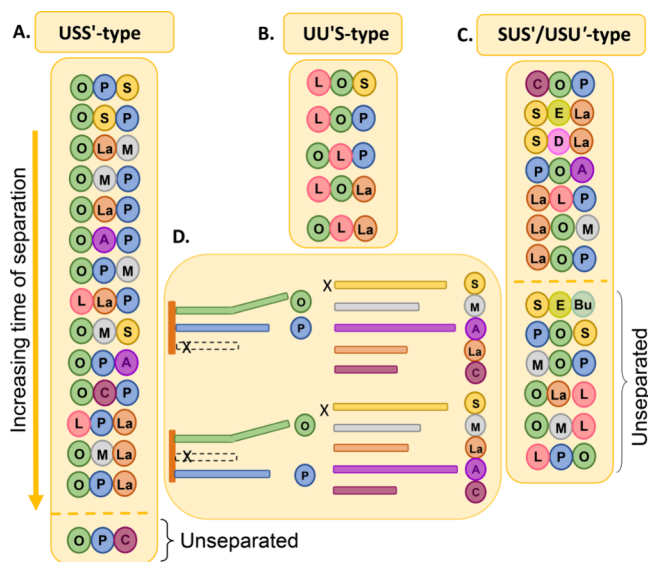
Figure 1. Separation results of USS′ (A), UU′S (B), and SUS′/USU′-type TGs (C). The sample sets of TG enantiopairs with both oleic acid and palmitic acid in either positions sn-1 and 2 or sn-1, and 3 and SFA in the third position (D).

baseline separation, the chromatogram of the O/La/P enantiomers is presented in Figure 3A. There was a visible shoulder after four column passes, and the two enantiomers were baseline separated after 15 column passes.

Under uniform conditions, structural factors, such as the acyl carbon chain length, number of double bonds, and ECN number, influence the separation efficiency of TGs. Table 2 provides the results of the Pearson correlation analysis of the separated enantiopairs. As known also from reversed-phase chromatography, the retention time and the ECN number are significantly positively correlated. This is consistent with the previous findings.[31] Only TGs with very long chains and highly unsaturated (DB > 6) FAs are exceptions.[32] The retention time affects the chromatographic behavior and the separation. For example, if the sample stays in too short a time within the stationary phase, it may not be separated as shown in the case of S_E(sn-2)_Bu (Figure S2A to AG). According to the same logic, the retention time at separation is negatively correlated to the retention time on a single column pass and the ECN number (Figure 2A). In other words, the larger the ECN number and the longer the retention time are, the shorter is the time needed for the separation of the enantiomers. In this data set, the retention time at separation was negatively correlated with the sn-3 carbon number (Figure 2B) and

Table 2. Pearson Correlation Analysis of the Separated Enantiopairs

factors	t'_R (min)[c]	t_R (min) at separation[d]
t_R (min) at separation	−0.399[a]	1
ECN	0.981[b]	−0.455[a]
sn-1 C	0.199	−0.253
sn-1 DB	0.043	−0.12
sn-2 C	0.137	0.17
sn-2 DB	−0.379	0.217
sn-3 C	0.737[b]	−0.541[b]
sn-3 DB	0.074	−0.25
ΔC *sn*-1 & 2[e]	−0.416[a]	0.089
ΔC *sn*-1 & 3	−0.519[b]	0.618[b]
ΔC *sn*-2 & 3	−0.368	0.265

[a]Correlation is significant at the 0.05 level (2-tailed). [b]Correlation is significant at the 0.01 level (2-tailed). [c]t'_R (min) = adjusted retention time. [d]Separation was determined when the p/v ratio ≥ 1. [e]The C represents the acyl carbon number; ΔC represents the acyl carbon number difference between different positions.
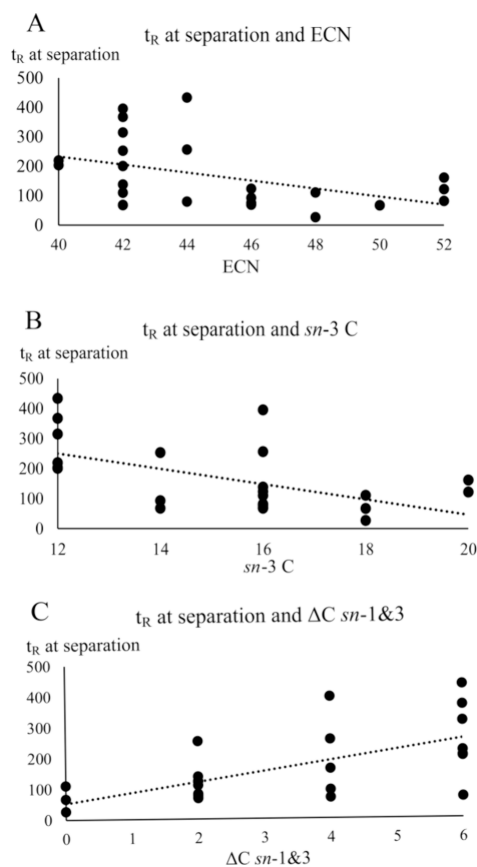
Figure 2. Scatter plots of significantly correlated parameters with t_R at separation. (A) The scatter plot of the correlation between the ECN and t_R at separation. (B) The scatter plot of the correlation between the *sn*-3 C (acyl carbon number) and t_R at separation. (C) The scatter plot of the ΔC *sn*-1 & 3 (acyl carbon number difference between *sn*-1 and *sn*-3) and t_R at separation. Separation was determined when the p/v ratio ≥ 1.

positively correlated with the acyl carbon number difference between the *sn*-1 and *sn*-3 FAs (Figure 2C).

Based on the correlation results, we found that the degree of unsaturation of the FA in the sample influences the elution behavior. Therefore, the separation of enantiomers was examined according to the grouping described earlier. First, the TGs with one UFA and two SFAs belong to the category USS′-type TGs. Then, TGs that possess either two SFAs or two UFAs in the outer positions belong to categories SUS′- or USU′-type TGs, and finally, TGs with two UFAs and one SFA belong to UU′S-type TGs.

USS′-Type TGs. In total, 14 out of 15 enantiopairs of USS′-type TGs were separated (Table 3, Figure 1A). This was the largest group in this study. As can be noticed from Table 3, the FA in the *sn*-1 position of the first eluted enantiomer was in all cases unsaturated, which indicates that the TGs with UFA on the *sn*-1 position always elute before the corresponding enantiomers. This observation confirmed our previous findings on the elution order of USS′- and UU′S-type TG enantiomers.[31,32]

Instead of a resolution, the p/v ratio was applicable for all enantiopairs and also when the separation was weak. Another chromatographic parameter to follow separation was the separation factor (α), which expresses the relative retention of enantiomers but does not take the peak widths into account. The theoretical plate numbers (N's) were calculated to evaluate the overall performance of the system and using the retention time and peak width of the pure compound of the first eluting enantiomer of each enantiopair after the first column. With enantiopairs, the retention behavior of enantiomers affects the peak width. N is not the most optimal parameter to follow the efficiency of the recycling HPLC system, as "column length" varies depending on the number of cycles.

Contradictory to the findings of Lisa and Holčapek,[20] we found that FA in position *sn*-2 also had a significant influence on the chiral separation efficiency of TG enantiomers. Figure 1D compares the time needed for enantioseparation between the two unique sets of structured TGs both consisting of five pairs of enantiopure TGs. All these pairs had O and P as the FAs either in the positions *sn*-1/3 and *sn*-2 (the series of O_P(*sn*-2)_S, O_P(*sn*-2)_M, O_P(*sn*-2)_A, O_P(*sn*-2)_La, and O_P(*sn*-2)_C) (Figure S1F,Q,C,J,U) or in the positions *sn*-1 and *sn*-3 (the series of O_S(*sn*-2)_P, O_M(*sn*-2)_P, O_La(*sn*-2)_P, and O_C(*sn*-2)_P) (Figure S1G,P,H,D,V). As shown in Figure 1D, the FA at the middle position had a clear impact on the separation of enantiopairs: the smaller the difference in acyl carbon chain length between the *sn*-2 and *sn*-3 positions of the first eluting enantiomer is, the faster is the separation between the enantiomers. When the acyl chain length difference between *sn*-2 and *sn*-3 FAs reaches 6 as in the case of O_P(*sn*-2)_C, the enantiomers were not separated. When the carbon number difference was the same, the enantiomers that possessed a longer carbon chain at the *sn*-3 position than those at the *sn*-2 position were separated faster (Table 3). In addition, the enantiomeric separation time of O_P(*sn*-2)_S, O_P(*sn*-2)_M, O_P(*sn*-2)_A, O_P(*sn*-2)_La, and O_P(*sn*-2)_C increased compared with their regioisomers. Thus, the effect of *sn*-2 FAs on the separation efficiency can be even greater than that of the primary positions at least with the chromatographic conditions examined. Also, the acyl carbon number of SFA clearly affects the separation efficiency, but the number is not linearly correlated with the time needed for separation. However, the separation efficiency was similar in both sets of structured TGs; also, O_A(*sn*-2)_P (t_R min at separation 82.1 min, p/v ratio 1.24) is enantioseparated in practice before O_La(*sn*-2)_P (t_R at separation 80.1 min, p/v ratio 1.02) (Figure S1D,H).

Table 3. Results of Analyzed USS′-Type Enantiomer Pairs

sample[a]	first eluted enantiomer			t'_R[b] (min)	ECN	t_R (min) at separation[c]	p/v ratio at t_R	N[d]	α[e]
	sn-1	sn-2	sn-3						
O_P(sn-2)_S	O (18:1)	P (16:0)	S (18:0)	32.8	50	67.0	1.082	6500	1.029
O_S(sn-2)_P	O (18:1)	S (18:0)	P (16:0)	32.8	50	67.7	1.028	5200	1.011
O_La(sn-2)_M	O (18:1)	La (12:0)	M (14:0)	16.8	42	68.1	1.061	2700	1.017
O_M(sn-2)_P	O (18:1)	M (14:0)	P (16:0)	22.9	46	69.4	1.007	3300	1.020
O_La(sn-2)_P	O (18:1)	La (12:0)	P (16:0)	19.6	44	80.1	1.02	3900	1.019
O_A(sn-2)_P	O (18:1)	A (20:0)	P (16:0)	40.4	52	82.1	1.24	4700	1.035
O_P(sn-2)_M	O (18:1)	P (16:0)	M (14:0)	23.0	46	92.9	1.227	3700	1.026
L_La(sn-2)_P	L (18:2)	La (12:0)	P (16:0)	18.1	42	110.3	1.057	3500	1.02
O_M(sn-2)_S	O (18:1)	M (14:0)	S (18:0)	27.7	48	110.5	1.052	3500	1.02
O_P(sn-2)_A	O (18:1)	P (16:0)	A (20:0)	40.2	52	122.2	1.016	3800	1.031
O_C(sn-2)_P	O (18:1)	C (10:0)	P (16:0)	17.0	42	138.2	1.059	3000	1.018
L_P(sn-2)_La	L (18:2)	P (16:0)	La (12:0)	18.0	42	200.8	1.033	3500	1.011
O_M(sn-2)_La	O (18:1)	M (14:0)	La (12:0)	16.7	42	367.3	1.005	3100	
O_P(sn-2)_La	O (18:1)	P (16:0)	La (12:0)	19.6	44	433.7	1.019	3900	1.009
O_P(sn-2)_C	O (18:1)	P (16:0)	C (10:0)	16.9	42	NS[f]		4000	

[a]All samples are spiked samples containing both enantiomers. [b]t'_R = adjusted retention time. [c]Separation was determined when the p/v ratio ≥ 1. [d]N = theoretical plate number determined after single column pass. [e]α = separation factor after the last column pass at the end of the analysis. [f]Not separated.

Table 4. Results of Analyzed SUS′- and USU′-Type Enantiomer Pairs

sample[a]	first eluted enantiomer			t'_R[b] (min)	ECN	t_R (min) at separation[c]	p/v ratio at t_R	N[d]	α[e]
	sn-1	sn-2	sn-3						
C_O(sn-2)_P	C (10:0)	O (18:1)	P (16:0)	16.9	42	68.9	1.053	5600	1.020
P_O(sn-2)_A	P (16:0)	O (18:1)	A (20:0)	39.8	52	161.6	1.17	4500	1.020
S_E(sn-2)_La	S (18:0)	E (20:5)	La (12:0)	13.3	40	203.4	1.009	4200	1.012
S_D(sn-2)_La	S (18:0)	D (22:6)	La (12:0)	14.6	40	219.5	1.078	3200	1.011
La_O(sn-2)_M	La (12:0)	O (18:1)	M (14:0)	16.7	42	286.9	0.997	3100	1.011
La_O(sn-2)_P	La (12:0)	O (18:1)	P (16:0)	19.7	44	256.7	1.006	4000	1.010
La_L(sn-2)_P	La (12:0)	L (18:2)	P (16:0)	15.3	42	395.7	1.074	4000	1.008
P_O(sn-2)_S	P (16:0)	O (18:1)	S (18:0)	34.7	50	NS[f]			
S_E(sn-2)_Bu	S (18:0)	E (20:5)	Bu (4:0)	8.0	32	NS		1200	
M_O(sn-2)_P	M (14:0)	O (18:1)	P (16:0)	21.5	46	NS		3000	
O_La(sn-2)_L	O (18:1)	La (12:0)	L (18:2)	19.2	42	NS			
O_M(sn-2)_L	O (18:1)	M (14:0)	L (18:2)	21.7	44	NS		3900	
L_P(sn-2)_O	L (18:2)	P (16:0)	O (18:1)	24.7	46	NS		4000	

[a]All samples are spiked samples containing both enantiomers. [b]t'_R = adjusted retention time. [c]Separation was determined when the p/v ratio ≥ 1. [d]N = theoretical plate number determined after single column pass. [e]α = separation factor after the last column pass at the end of the analysis. [f]Not separated.

Table 5. Results of Analyzed UU′S-Type Enantiomer Pairs

sample[a]	first eluted enantiomer			t'_R[b] (min)	ECN	t_R (min) at separation[c]	p/v ratio at t_R	N[d]	α[e]
	sn-1	sn-2	sn-3						
S_O(sn-2)_L	L (18:2)	O (18:1)	S (18:0)	26.3	48	27.0	1.195		1.023
P_O(sn-2)_L	L (18:2)	O (18:1)	P (16:0)	18.1	42	75.4	1.04	3700	1.021
O_L(sn-2)_P	O (18:1)	L (18:2)	P (16:0)	16.1	42	123.8	1.015	3800	1.016
La_O(sn-2)_L	L (18:2)	O (18:1)	La (12:0)	19.1	46	315.0	1.019	3700	1.008
O_L(sn-2)_La	O (18:1)	L (18:2)	La (12:0)	19.1	46	shoulder[f]		3700	

[a]All samples are spiked samples containing both enantiomers. [b]t'_R = adjusted retention time. [c]Separation was determined when the p/v ratio ≥ 1. [d]N = theoretical plate number determined after single column pass. [e]α = separation factor after the last column pass at the end of the analysis. [f]At the end of the run (t_R at 480 min), there was only a shoulder to indicate the separation.

Because the time needed for separation was not positively correlated with the acyl carbon number of SFA, it can be concluded that both attractive and repulsive molecular interactions[33] are involved in the enantioseparation of USS′-type TGs.

SUS′- and USU′-Type TGs. Most (7/10) of the SUS′-type TGs were separable (Table 4). For the separable SUS′ pairs, except those containing long-chain n-3 PUFAs, the carbon chain length of two SFAs influenced the elution order. The enantiomer with a shorter chain FA in the sn-1 position always elutes first. When TGs contain PUFA with five or more double

bonds, their chromatographic elution behavior no longer follows the ECN values.[25,32] This could also explain the behavior of S_E(sn-2)_Bu enantiopair, which was not separated despite the large difference in acyl carbon number between the sn-1 and sn-3 positions. The larger difference in the carbon number between the sn-1 and sn-3 FAs favored the separation of the enantiomers, a fact evident, e.g., when comparing the separation time of C_O(sn-2)_P with P_O(sn-2)_A as well as La_O(sn-2)_M with La_O(sn-2)_P. P_O(sn-2)_S and M_O(sn-2)_P were not enantioseparated under the conditions examined probably due to the small difference in the carbon chain length between the sn-1 and the sn-3 FAs. Increasing the number of double bonds in the middle position was seen to increase the time needed for enantiomeric separation, as seen in the comparison of La_O(sn-2)_P and La_L(sn-2)_P.

Enantiomers with UFAs in both the sn-1 and sn-3 positions were expected to be difficult to separate due to the highly symmetrical structure. Indeed, whereas enantioresolution was achieved for their UU′S-type counterparts (La_O(sn-2)_L and P_O(sn-2)_L), at least the three USU′-type TG enantiopairs (O_La(sn-2)_L, O_M(sn-2)_L, and L_P(sn-2)_O) were not separable under the chromatographic conditions used in this study (Table 5).

UU′S-Type TGs. The results of analyzed UU′S-type enantiopairs are shown in Table 5. Combining the results from the USS′-type and UU′S-type TGs, all 19 TGs that possess both UFA (C18:1 or C18:2) and SFA (C12:0-C20:0) in the sn-1 and sn-3 positions were successfully separated regardless of the type of FAs in the sn-2 position (U or S). The only exception was the enantiopair O−P(sn-2)−C.

By comparing the separation times of enantiopairs L_O(sn-2)_S, L_O(sn-2)_P, and L_O(sn-2)_La, the results showed that the longer the SFA carbon chain was, the faster the enantiomers were separated. The O_L(sn-2)_P and O_L(sn-2)_La enantiomers followed this rule as well. Within the same ECN, the more double bonds in the sn-1/3 position were, the faster was the enantioseparation, as noticed for L_O(sn-2)_P and O_L(sn-2)_P, and L_O(sn-2)_La and O_L(sn-2)_La. Consistent with the rule of USS′-type TGs, the enantiomer with UFA located in the sn-1 position always eluted first.[31]

The obtained results deviated from those reported by Lísa and Holčapek.[20] In their research, rac-P_O(sn-2)_L was not separated, but rac-O_P(sn-2)_L and rac-P_L(sn-2)_O were. They concluded that TGs with saturated and di- or triunsaturated FAs located in positions sn-1/3 were not separable by chiral columns (Lux Cellulose-1) using hexane and hexane-2-propanol as the mobile phase. The other example of this type of TG in our study, L_O(sn-2)_La, was also enantioseparated. Lísa and Holčapek's results were consistent with the findings of the studies by Řezanka and Sigler[34] and Chen et al.[35] According to Chen and others, rac-O_P(sn-2)_L and rac-P_L(sn-2)_O were separated with a chiral column packed with cellulose tris-3,5-dimethylphenyl-carbamate. They used mainly hexane as the mobile phase. On the other hand, rac-P_O(sn-2)_L was not separable with this method. Nagai et al.[3] tried to separate the TG enantiomers and positional isomers of P_O_L and P_S_O simultaneously with the CHIRALPAK IF-3 (amylose tris-3-chloro-4-methylphenyl-carbamate) column using acetonitrile as the mobile phase. They were able to separate the P_S_O partially into several peaks, but the P_O_L isomers were not resolved sufficiently. Together, these results indicate that the use of different stationary and mobile phases has a significant impact on whether certain TG isomers can be separated.

Separation of Regioisomeric TG Mixtures. To further apply recycling HPLC, 4 regioisomeric mixed TG samples (Table 1) were analyzed by the same method. The chromatograms of four TG mixtures and individual enantiomers are shown in Figure S2. After more than 20 column passes (total analysis time of more than 400 min), all peaks showed some asymmetry. Using this chiral column combined with the recycling method, the mixed TGs can be separated theoretically as long as the number of cycles is large enough. But in practice, peak broadening and long analysis time restrict the operation. To identify the separated peaks, the individual TG enantiomer was analyzed under the same conditions. Mixture 1 was difficult to separate, and only a little asymmetry was detected after 400 min. Mixture 2 showed asymmetry after 200 min, and two shoulders could be distinguished after 350 min. Based on the retention time of individual TG enantiomers, the first shoulder is P/O/M, and the second shoulder is P/M/O. Mixture 3 was separated into two peaks with different widths, and the second peak had a shoulder that tended to separate into a third one. Based on the retention time of individual TG enantiomers, the first peak is C/P/O, and the second peak is the mixture of P/C/O and P/O/C. Because the shoulder of the second peak was on the right, it could be P/C/O. Mixture 4 (Figure 3B) was separated into
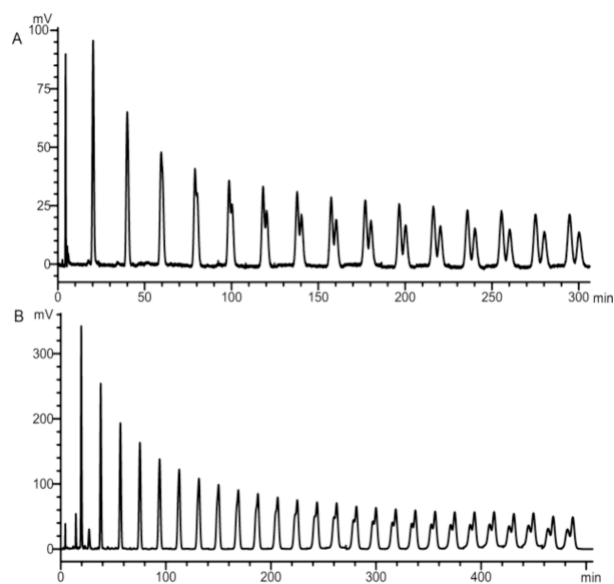
Figure 3. UV chromatogram of the enantiopair O/La/P:P/La/O (60:0%) (A) and the regioisomeric mixture TG 4 (P/La/L, La/P/L, and P/L/La) (B). Chromatographic conditions of recycle HPLC-UV: CHIRALCEL OD-RH (150 × 4.6 mm, 5 μm) columns; mobile phase: methanol.

two peaks after 400 min. Based on the retention time of individual TG enantiomers, the first peak is the P/L/La. The second one is the mixture of La/P/L and P/La/L. This was consistent with the previous research[36] that TGs with two SFAs located in position sn-1,2 or position sn-2,3 are expected to have longer retention times because the interaction of the stationary phase with double bonds, even the stationary phase and the solvent combination were not the same with current research.

Overall, regioisomers tend to have a very similar elution behavior, and they are difficult to separate with the column-solvent combination studied. Thus, mass spectrometric detection is preferred in TG regioisomer analysis.[37,38]

CONCLUSIONS

This is the first systematic study of the enantiomeric separation and eluting behavior of a large number of ABC-TGs using recycling chiral chromatography. Thirty-three enantiopairs were studied including 49 asymmetrically synthesized enantiopure TGs and 16 regiopure racemic TGs. Twenty-six out of 33 enantiopairs were separated, and the chiral chromatographic resolution and elution behavior were systematically studied.

In addition to the basic correlation between the ECN number of TGs and the adjusted retention time, novel correlations were also revealed. The t_R at separation is correlated not only with the sn-3 carbon number but also with the carbon number difference between the primary FAs, which indicates that the length of the FA in the primary position is significant in the separation process. Even though the FA on the sn-2 position did not show a significant correlation with the t_R at separation when all the data were studied, it showed an apparent influence on the separation of USS′-type TGs. Overall, it was clear that both the length of the acyl carbons and the degree of unsaturation in all three positions were involved in chiral separation. Our results suggest that both intermolecular interactions and steric hindrances play roles in the separation process.

This method was not efficient for regioisomeric separation, where MS is widely used as an effective method due to the structural information received.[39] For the enantiomeric analysis of TG, chiral chromatographic separation is unavoidable. The new knowledge provided by this study on the chiral chromatographic elution behavior of ABC-TGs is valuable for studying TGs in natural fats and oils. However, for a thorough analysis of TG enantiomers in complex natural fats and oils such as human milk fat, preseparation is necessary to reduce the interference of isobaric TGs; therefore, 2D LC separation would be needed.[40]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c02513.

List of synthesized enantiopure TG reference compounds, list of commercial racemic standards, chromatograms of 33 pairs of enantiopure TGs analyzed in this study, and the part of chromatograms of TG isomers illustrating the separation of regioisomeric triplets (PDF)

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Notes

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