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Analysis of regioisomers of short-chain triacylglycerols by normal phase liquid chromatography–electrospray tandem mass spectrometry

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Abstract

The regiospecific distribution of short-chain fatty acids in triacylglycerols (TAGs) influences their physico-chemical, nutritional, and organoleptic properties. This study demonstrates the applicability of normal phase HPLC with positive electrospray ionization mass spectrometry for the identification and structural analysis of regioisomers of short-chain triacylglycerols in nine interesterified mixtures of monoacid triacylglycerols. The finding that only one type of triacylglycerol adduct ion was formed without fragmentation or with minimal fragmentation was critical for liquid chromatography electrospray mass spectrometric quantification and identification of molecular species of TAGs. In the product ion electrospray tandem mass spectrometry, the 4–11 times lower abundance of the ion formed by cleavage of fatty acid from the secondary position relative to that formed by cleavage from the primary positions provided solid basis for differentiation between regioisomers of mono- and dibutyrates. Although the respective differences were lower, mono- and dicaproate regioisomers could be differentiated despite their incomplete separation. The tandem mass spectra of the short-chain triacylglycerols containing three different acyl groups revealed that the regioisomer having the shortest acyl chain in the secondary position, eluted in the first peak. The mixture of the other two regioisomers, where the shortest acyl chain was located in the primary positions, eluted in the second peak. In one instance only did the tandem mass spectra allow to differentiate between the reverse isomers eluting in the second peak. An effective chromatographic resolution of butyrate triacylglycerols and the linear relationship between the molar and area ratios of analyte and standard, enabled the mass spectrometric quantification of their regioisomers.

Keywords: Short-chain triacylglycerols; Regioisomers; Normal phase liquid chromatography; Electrospray ionization; Tandem mass spectrometry

Abbreviations: TAG, triacylglycerol; FAME, fatty acid methyl ester; FA, fatty acid; GLC, gas–liquid chromatography; LC, liquid chromatography; HPLC, high performance liquid chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; FAB, fast atom bombardment; EI, electron impact ionization; NMR, nuclear magnetic resonance spectroscopy; ISTD, internal standard; MCF, molar correction factor; S.D., standard deviation; 4:0, butyroyl; 6:0, caproyl; 7:0, enanthoyl; 12:0, lauroyl; 14:0, myristoyl; 16:0, palmitoyl; 16:1, palmitoleoyl; 18:0, stearoyl; 18:1, oleoyl; A, B, C, acyl groups; S, short-chain acyl groups (4:0, 6:0 or 7:0); L, long-chain acyl groups ($C_{12–18}$); *rac*, racemic; *sn*, stereospecifically numbered; BLaS, BLaO, BMP, BMPo, CoMP, CoMPo, CoPS, CoPO, and BCoE, interesterification mixtures prepared from equimolar amounts of the following monoacid triacylglycerols; B, tributyrin; Co, tricaproin; E, trienanthin; La, trilaurin; M, trimyristin; P, tripalmitin; Po, tripalmitolein; S, tristearin; O, triolein

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1. Introduction

In the present study, we examined 33 molecular species of synthetic short-chain TAGs of random distribution of fatty acids to demonstrate that normal phase LC/MS/MS with positive ammonia ionization can provide a clear cut differentiation between regioisomers of short-chain TAGs. An adequate chromatographic separation and linearity of response enabled a mass spectrometric quantification of regioisomers of mono- and dibutyrates.

Short-chain TAGs are common components of ruminant milk fats. Thus, the proportion of TAGs with one short-chain (C_4–C_6) per TAG molecule in butterfat has been shown to exceed 33 mol% [1,2]. In addition to TAGs with one short-chain fatty acid, ruminant milk fats contain TAGs with two or three short-chain fatty acids [3–5] but in much smaller amounts. In natural ruminant milk fats, the fatty acids are non-randomly distributed among the three positions of the glycerol moiety, with the short-chain fatty acids confined exclusively to the *sn*-3-position [6–8]. The regiospecific distribution of short acyl groups in the TAGs influences their physico-chemical and organoleptic properties [9]. In the production of structured TAGs, such as cocoa butter equivalents, human milk substitutes or lipids with enhanced nutritional properties, the regiospecific distribution of fatty acids needs to be controlled in order to obtain and maintain the desired physiological and nutritional effects.

In the past, the positional distribution of fatty acids in fats has been determined by stereospecific and regiospecific analyses involving partial chemical and enzymatic degradation of the TAGs or subfractions of TAGs, isolation of the degradation products, preparation of derivatives, further chemical or enzymatic degradation and a GLC determination of the fatty acids in appropriate monoacylglycerol (MAG) or enantiomeric diacylglycerol (DAG) fraction [6–8,10,11]. These methods are indirect, time-consuming and laborious, and not well suited for rapid monitoring of production processes, quality control and detecting adulteration of high quality fats with low-grade substitutes. In a few instances, positional isomers of intact short-chain TAGs have been resolved by argentation-thin-layer chromatography (TLC) [11], GLC [2,4,12] and HPLC [12].

In several studies, the potential applicability of MS and combinations of MS with GLC (GC/MS) or high performance liquid chromatography (LC/MS) have been demonstrated to the determination of regioisomers of long-chain TAGs [13–28]. In EI MS, $[M-RCOO+14]^+$ was observed for fatty acids esterified in the primary position but not for those esterified in the secondary position [13]. In direct inlet CI LC/MS, the abundance of $[M-RCOO]^+$ cleaved from the primary position was shown to be four times that cleaved from the secondary position [14]. Duffin et al. [15] had studied fragmentation of TAGs by low energy CID of $[M+NH_4]^+$ ions produced by ESI in the presence of ammonium acetate. At 50 eV collision energy, the major fragment ions were formed by loss of any of the three fatty acyl groups, and thus a preferential loss of fatty acyl groups from either primary or secondary positions was not observed. Similarly, in the high energy CID of $[M+NH_4]^+$ ions generated by ESI no positional information was obtained [16]. A limited differentiation of positional distribution in long-chain TAGs was achieved in low energy CID fragmentation of ESI produced $[M+NH_4]^+$ ions [17,18]. During the time this paper was written, an improved differentiation of long-chain TAG regioisomers using ESI–MS^n of $[M+NH_4]^+$ ions and ion trap instrument was reported [19]. The high energy CID fragmentation of $[M+Na]^+$ and $[M+Li]^+$ ions of long-chain TAGs produced by ESI have been used in unambiguous determination of regiospecific distribution of acyl groups in the molecular species of long-chain TAGs [16,20]. In the negative chemical ionization tandem mass spectra (NICIMS/MS), the $[M-H–RCOO-100]^-$ ions was observed to be produced primarily by loss of fatty acids from the primary positions and applied for regioisomer determination of medium- and long-chain TAGs in a variety of fats and oils [21–23]. In direct inlet APCI, MS and in APCI in combination with silver ion HPLC, lower abundance of $[M-RCOO]^+$ was found for fatty acids esterified in the secondary position than for those in the primary positions [24–27]. Sodiated $[M+Na]^+$ precursor ions produced by FAB and product ion mass spectra obtained by high energy CID have been used in the regiospecific analysis of short-chain TAGs [28].

High temperature GLC with positive [4] and negative [29] methane chemical ionization, and with positive electron impact ionization [29] and capillary supercritical fluid chromatography (SFC) with positive APCI/MS [30] have been applied to analysis of milk fat TAGs that naturally contain short-chain fatty acids, and no regioisomerism was found, as should have been anticipated because of exclusive association of the short-chain acids with the *sn*-3-position of native butterfat [6–8]. Regioisomerism was also not observed among the short-chain TAGs of enzymatically [31] and chemically [32] rearranged butterfat, when examined by positive ion EI GC/MS and LC/MS, respectively. These TAGs should have contained regioisomers of short-chain TAGs, and the inability to observe them must be attributed to the complexity of the short-chain TAG mixtures, or inappropriate analytical conditions. Also, the absence of standards prevented an unequivocal demonstration of the resolution of any regioisomers of short-chain TAGs. Likewise, Spanos et al. [33] failed to obtain positional information by applying isobutane positive chemical ionization and desorption tandem mass spectrometry to analysis of milk fat TAGs. Recently, Mottram and Evershed characterized milk fat TAGs of silica gel thin layer and gel permeation chromatography fractions by RP–HPLC–APCI/MS [34].

The objective of the present study was to investigate short-chain TAGs of interesterified mixtures of three monoacid TAGs using normal phase HPLC and positive ESI/MS

under experimental conditions favoring production of only one kind of adduct ions with minimal or no fragmentation. Our objectives were to study the identification of regioisomers of short-chain TAGs from tandem spectra of ammonium adducts and the quantification of regioisomers on the basis of extracted ammonium adduct ion data.

2. Experimental

2.1. Materials

Standards: Monoacid TAG standards of 99% purity were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, Switzerland), and 1-monomyristin, 1-monopalmitin, and 1-mono-olein from Larodan Fine Chemicals (Malmo, Sweden).

Solvents: All solvents were purchased from Rathburn, UK and were of HPLC grade.

2.2. Synthesis of short-chain TAGs

2.2.1. Chemically catalyzed interesterification

Nine mixtures of randomized TAGs were prepared by chemical interesterification of equimolar amounts of three monoacid TAGs at a time to yield the following randomized mixtures: BLaS = tributyrin + trilaurin + tristearin; BLaO = tributyrin + trilaurin + triolein; BMP = tributyrin + trimyristin + tripalmitin; BMPo = tributyrin + trimyristin + tripalmitolein; CoMP = tricaproin + trimyristin+tripalmitin; CoMPo = tricaproin+trimyristin+ tripalmitolein; CoPS = tricaproin + tripalmitin + tristearin; CoPO = tricaproin + tripalmitin + triolein; BCoE = tributyrin + tricaproin + trienanthin.

The interesterifications were performed using sodium methoxide as catalyst (1%) at 85–90 °C (1 h). The TAGs were isolated from the first eight reaction mixtures by TLC [2]. The TAGs from interesterification mixture BCoE were isolated using a 5.4 cm × 1.5 cm flash chromatography grade silica gel column by elution with 40 ml portions of dichloromethane/hexane 60:40 (v/v); dichloromethane/hexane 65:35 (v/v); and dichloromethane/hexane 85:15 (v/v). The eluents were combined. All interesterification mixtures were analyzed for randomness by GLC on polarizable 65% phenylmethylsilicone capillary columns as described by Kemppinen and Kalo [2]. In addition, three mixtures of randomized TAGs were prepared by chemical interesterification of equimolar amounts of two monoacid TAGs at a time to yield the following randomized mixtures: BM = tributyrin + trimyristin; BP = tributyrin + tripalmitin; BO = tributyrin + triolein. The interesterification was performed as above and the mono- and di-short-chain TAGs were isolated by flash chromatography as described above, except that the elution was performed with dichloromethane/hexane 65:35 (v/v) and dichloromethane/hexane 85:15 (v/v), respectively.

2.2.2. Synthesis of 1-long acyl chain-2,3-dibutyroyl-rac-glycerols

TAGs containing one long-chain (L) and two butyroyl residues were prepared by acylation of 1-monomyristin, 1-monopalmitin, and 1-monoolein as described by Kodali et al. [35]. The 1-monoacylglycerols were dissolved in tetrachloromethane and reacted with butyric acid in the presence of 4-dimethylaminopyridine by slow addition of dicyclohexylcarbodiimide in tetrachloromethane at ambient temperature. The 1-L-2,3-dibutyroyl-*rac*-glycerols were isolated from the reaction mixtures by flash chromatography as described above using dichloromethane/hexane 85:15 (v/v) as the eluent.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR spectra of the regioisomeric mixtures of di-short-chain TAGs prepared by interesterification and by synthesis from long-chain-1-monoacylglycerols dissolved in $CDCl_3$ were recorded. Varian Unity 600 (600 MHz) spectrometer (Palo Alto, CA), spectral width 10,000 Hz, was used and the signals were referenced to internal tetramethylsilane (TMS) (0.00) as previously described [12].

2.4. Liquid-chromatography–electrospray ionization mass spectrometry (LC/ESI/MS)

2.4.1. High performance liquid chromatography (HPLC)

Interesterified mixtures of standard TAGs were analyzed by normal phase HPLC using two Phenomenex Luna 3 silica columns (100 mm × 2.0 mm) and a guard column (4 mm × 2.0 mm), in series. For elution of TAGs, a multistep binary gradient of hexane (A) and hexane–methyl-*tert*-butyl ether–acetic acid (B) and flow rate of 0.1 ml/min was applied (Table 1).

2.4.2. HPLC with electrospray ionization mass spectrometry (LC/ESI/MS)

An ion-trap Bruker Esquire LC/ESI/MS (Bruker Daltonic, Bremen, Germany) was operated in positive

Table 1
Multistage binary gradient system in the NP-HPLC

Time (min)	% B (v/v)	Flow (ml/min)
0	0	0.1
2	15	0.1
16	15	0.1
22	18	0.1
30	18	0.1
31	90	0.1
49	90	0.1
50	99	0.5
64	99	0.5
65	0	0.5
75	0	0.5

Solvent A: hexane; solvent B: hexane/methyl-*tert*-butyl ether/acetic acid 60:40:1 (v/v).

electrospray ionization (ESI) mode. Capillary voltage was 3000 V, capillary exit offset 60 V, skimmer potential 20 V, and trap drive value of 55. Conventional ESI mass spectra were recorded using a scan range of 50–1000 *m/z* and summation of 15 spectra. Nebulizer (nitrogen) pressure was 40 psi, drying gas (nitrogen) flow 8 l/min and drying temperature 300 °C. Auto-MS/MS spectra for two most intense ions eluting concurrently were recorded using helium (99.996%) as the collision gas. The reagent solvent, chloroform/methanol/ammonia water (25%) 20:10:3 (v/v), was pumped with a flow rate of 6.0 ml/min via a 1:100 split device to effluent flow.

2.4.3. Linear calibration

Linear calibration was used in the determination of MCF. The conventional ESI mass spectra were recorded for three analyte/internal standard (trinonanoin) ratios for each of the nine interesterified standard mixtures. The ion chromatograms of ammonium adducts of short-chain TAGs and ISTD were extracted from total ion current data and integrated. The molar analyte/internal standard ratios were calculated from GLC data [2].

3. Results

3.1. NMR data of interesterified TAG mixtures, mixtures of regioisomers, and synthesized regioisomers

The ratios of primary and secondary butyrate methyl signals 1.96:1.0 and 1.77:1.0 measured by ^{1}H NMR for interesterified mixtures of tributyrin, trilaurin, and tristearin and tributyrin, tristearin, and triolein [12] are consistent with the random distribution of butyrate in TAGs of interesterified mixtures. The ratio of the most intense butyroyl methyl signals at the *sn*-1(3) (δ 0.957) to those at the *sn*-2 (δ 0.963) was 1.7:1, 2.1:1, 1.9:1 for regioisomeric mixtures of dibutyroylmyristoyl-, dibutyroylpalmitoyl-, dibutyroyloleoyl-glycerol, respectively, which are in agreement with theoretical random ratio 2:1 of isomers *rac*-L/4:0/4:0 and 4:0/L/4:0. The respective ratio of most intense methyl signals for 1-myristoyl-2,3-dibutyroyl-, 1-pamitoyl-2,3-dibutyroyl-, and 1-oleoyl-2,3-dibutyroyl-*rac*-glycerol was 1.1:1.0, 1.1:1.0, and 1.0:1.0, which are consistent with theoretical ratio of 1:1.

3.2. Ion chromatograms of ammonium adducts

Each of the eight equimolar mixtures of monoacid TAGs contained one TAG with three short-chain acyls (S/S/S) and two TAGs with three long-chain acyls (L/L/L, L′/L′/L′). During the interesterification reaction, a mixture of 10 molecular species in random ratio was formed: S/S/S, (L/S/S + S/L/S), (L′/S/S+S/L′/S), (L/L/S+L/S/L), (L′/L′/S+L′/S/L′), (L/L′/S + L′/L/S + L/S/L′), (L/L/L′ + L/L′/L), (L/L′/L′ + L′/L/L′) L/L/L, L′/L′/L′. The regioisomers are shown in the parenthesis. The ninth mixture (BCoE) contained the respective molecular species and regioisomers of TAG with the exception that only short acyl chains 4:0, 6:0, and 7:0 were present.

The extracted ion chromatograms were recorded for the ammonium adducts of TAGs containing one, two or three short acyl chains (Table 2). Fig. 1 shows the total ion chromatogram and extracted ion chromatograms of short-chain TAGs in the interesterified mixture of tributyrin, trilaurin, and tristearin and the mass spectra recorded at the peak maxima of extracted ion chromatograms. The electrospray mass spectra showed only $[M + NH_4]^+$ ions. Sodium adducts were not observed. Also fragment ions were not generally present, an exception being the very low intensity fragment ions in the spectra of ammonium adducts with molecular weight below 500 (Fig. 1B). In some mass spectra it was possible to see ammonium adducts of other closely eluting TAGs (Fig. 1D–F). Regioisomers of mono- and dibutyrates with different molecular weight and combination of fatty acids (4:0/4:0/L, 4:0/L/L, and 4:0/L/L′) were separated with baseline resolution or close to it (Figs. 1, 2A, 3 and 4A, Tables 3–5). Regioisomers of mono- and dicaproates were separated partially or eluted in the same peak (Figs. 2B and 4B, Tables 3–5).

3.3. Tandem electrospray mass spectra of ammonium adducts

3.3.1. Diacid TAGs containing one short (S) and two long (L) acyl chains

The extracted ion chromatograms in Figs. 1D–F and 2A show that regioisomers of monobutyrates (L/L/4:0 and L/4:0/L) were separated by normal phase HPLC to (or nearly to) the baseline. The regioisomers of monocaproates were separated only partially from each other (Fig. 2B). The product ion tandem mass spectra of ammonium adducts showed lower relative abundance of $[(M + NH_4)–NH_3–$short-chain acid$]^+$ for L/S/L than for L/L/S isomers. The relative abundance of $[(M + NH_4)–NH_3–$butyric acid$]^+$ was 13.4% for 1,3-distearoyl-2-butyroylglycerol and 41.6–47.7% for 1,2-distearoyl-3-butyroyl-*rac*-glycerol (Fig. 2A), and varied from 4.1 to 13.6% for the L/4:0/L and from 34.5 to 53.9% for the L/L/4:0 isomers studied. The relative abundance ratio $[(M + NH_4)–NH_3–4:0]^+/[(M + NH_4)–NH_3–L]^+$ varied in the range 0.0428–0.157 for the L/4:0/L isomers and was on the average 0.125, and with standard deviation (S.D.) 0.036, $n = 9$. The respective ratio for the L/L/4:0 isomers was in the range 0.527–1.127 and on the average 0.791 (S.D. = 0.171, $n = 12$). On the average the relative abundance ratio for the L/L/4:0 was 6.33 times higher than for the L/4:0/L. The relative abundance of $[(M+NH_4)–NH_3–$caproic acid$]^+$ was 17.2% for 1,3-distearoyl-2-caproylglycerol and 40.4–42.6% for 1,2-distearoyl-3-caproyl-*rac*-glycerol, and varied from 11.1 to 21.1% for the L/6:0/L and from 40.4 to 49.5% for the L/L/6:0 isomers studied (Table 3). The relative abundance ratio of $[(M + NH_4)–NH_3–6:0]^+/[(M + NH_4)–NH_3–L]^+$

Table 2
$[M + NH_4]^+$ ions observed in the HPLC–ESI–MS of short-chain TAG in the interesterified mixtures of standards

$[M + NH_4]^+$ (*m/z*)	Molecular species in di- and tri-short-chain TAG with two different acyls	Molecular species in short-chain TAG with three different acyls	Molecular species in mono-short-chain TAG
348	4:0–4:0–6:0		
362	4:0–4:0–7:0		
376	4:0–6:0–6:0		
390		4:0–6:0–7:0	
418	6:0–6:0–7:0		
432	6:0–7:0–7:0		
432	4:0–4:0–12:0		
460	4:0–4:0–14:0		
486	4:0–4:0–16:1		
488	4:0–4:0–16:0		
514	4:0–4:0–18:1		
516	4:0–4:0–18:0		
516	6:0–6:0–14:0		
542	6:0–6:0–16:1		
544	6:0–6:0–16:0		4:0–12:0–12:0
570	6:0–6:0–18:1		
572	6:0–6:0–18:0		
600			4:0–14:0–14:0
626		4:0–14:0–16:1	
626		4:0–12:0–18:1	
628		4:0–14:0–16:0	6:0–14:0–14:0
628		4:0–12:0–18:0	
654		6:0–14:0–16:1	
656		6:0–14:0–16:0	4:0–16:0–16:0
680			6:0–16:1–16:1
684			6:0–16:0–16:0
710		6:0–16:0–18:1	
712		6:0–16:0–18:0	4:0–18:0–18:0
740			6:0–18:0–18:0

varied between 0.125 and 0.267 for the L/6:0/L isomers, and was on the average 0.214 (S.D. = 0.064, $n = 4$). The respective ratio for the L/L/6:0 isomers was in the range 0.678–0.980 and on the average 0.766 (S.D. = 0.112, $n = 6$). On the average the relative abundance ratio for the L/L/6:0 was 3.58 times higher than for the L/6:0/L.

3.3.2. Triacid TAGs containing one short (S) and two long (L,L′) acyl chains

TAGs of this type possess three regioisomers: S/L/L′, L/S/L′ and L/L′/S as the racemates. It was already shown that the silica gel columns separated the butyrates of this type in two peaks (Figs. 1E and 3). The MS/MS spectra for the first peak of the four butyrates indicated that L/4:0/L′ isomers were eluted in the first peak. The relative abundance of $[(M + NH_4)–NH_3–\text{butyric acid}]^+$ was 11.4% for 1-stearoyl-2-butyroyl-3-lauroyl-*rac*-glycerol and varied from 11.4 to 19.4% for the L/4:0/L′ isomers studied (Table 4). The L/L′/4:0 and L′/L/4:0 were not chromatographically resolved. The MS/MS spectra clearly showed that the mixture of the latter regioisomers was eluted in the second peak. The relative abundance of $[(M + NH_4)–NH_3–\text{butyric acid}]^+$ for the mixture of 1-stearoyl-2-lauroyl-3-butyroyl-*rac*-glycerol and 1-lauroyl-2-stearoyl-3-butyroyl-*rac*-glycerol was 44.7%, and varied from 36.4 to 48.5% for the isomers having the butyric acid in the primary position. The relative abundance ratio of $[(M + NH_4)–NH_3–4:0]^+/[(M + NH_4)–NH_3–L]^+$ varied in the range 0.129–0.241 for the L/4:0/L′ isomers and was on the average 0.177 (S.D. = 0.047, $n = 4$). The respective ratio for the mixture of L/L′/4:0 and L′/L/4:0 isomers was between 0.572 and 0.942, and on the average 0.753 (S.D. = 0.159, $n = 4$). On the average, the relative abundance ratio for the mixture of L/L′/4:0 and L′/L/4:0 isomers was 4.25 times higher than for the L/4:0/L′ isomers. Although the regioisomers L/6:0/L′ and L/L′/6:0 were chromatographically poorly separated, the tandem mass spectra clearly indicated the elution of the former isomer with the leading edge and the latter with the tailing end of the peak for L/L′ = 16:0/14:0, 18:0/16:0, and 18:1/16:0. The relative abundance of $[(M+NH_4)–NH_3–\text{caproic acid}]^+$ varied from 32.9 to 51.5% and from 14.9 to 17.6% for the isomers having caproic acid in the primary and secondary positions, respectively. The relative abundance ratio of $[(M + NH_4)–NH_3–6:0]^+/[(M + NH_4)–NH_3–L]^+$ varied in the range 0.175–0.213 for the L/6:0/L′ isomers and was on the average 0.190 (S.D. = 0.020, $n = 3$). The respective ratio for the mixture of L/L′/6:0 and L′/L/6:0 isomers was in the range 0.490–1.062 and on the average 0.681 (S.D. = 0.268, $n = 4$). On the average the relative abundance ratio for mixture of L/L′/6:0 and L′/L/6:0 was 3.56 times higher than for the L/6:0/L′ isomers.

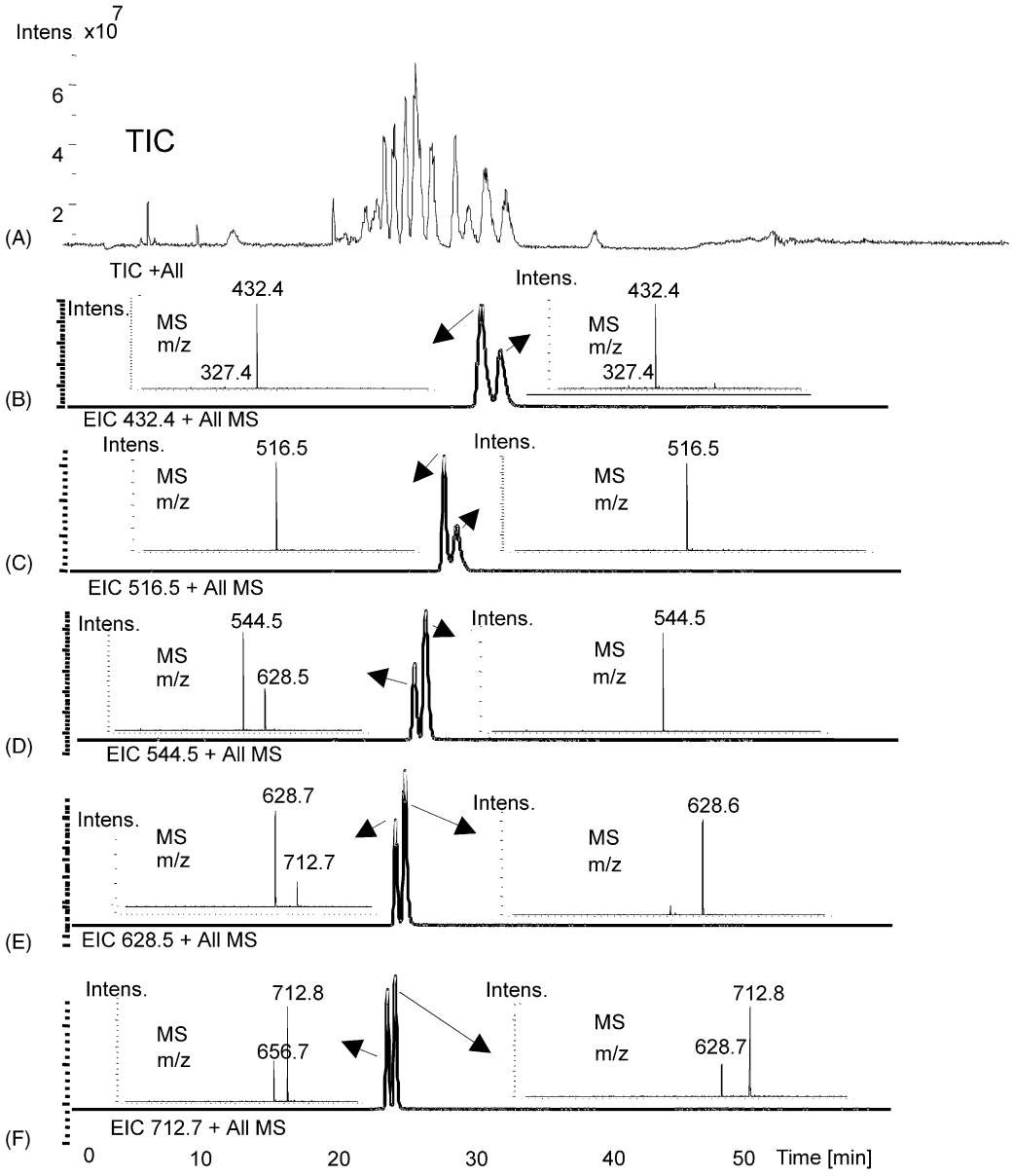

Fig. 1. Total ion chromatogram of interesterified mixture of tributyrin, trilaurin, and tristearin, extracted ion chromatograms of ammonium adducts of short-chain triacylglycerols, and conventional mass spectra recorded at the top of peaks. (A) Total ion chromatogram, (B) dibutyroyllauroylglycerol, (C) dibutyroylstearoylglycerol, (D) butyroyldilauroylglycerol, (E) butyroyllauroylstearoylglycerol, (F) butyroyldistearoylglycerol.

Among the above short-chain TAGs containing three different fatty chains may also be included the isomers of 4:0/6:0/7:0. The MS/MS spectra of these regioisomers revealed that 7:0/4:0/6:0 was eluted in the first peak, while 7:0/6:0/4:0 eluted in the leading edge and 6:0/7:0/4:0 in the tailing end of the second peak (Fig. 3). Thus, the reverse isomers 7:0/6:0/4:0 and 6:0/7:0/4 were partly separated within the same peak and could be clearly differentiated using tandem mass spectrometry. The relative abundance of [$(M + NH_4)$–NH_3–fatty acid 4:0, 6:0 and 7:0 in the *sn*-2-position] for these regioisomers was 4.5, 11.7, and 18.2%, respectively (Table 4). The data show that regioisomers L/S/L′ and L/L′/S (S being the shortest acyl chain) can be differentiated on the basis of tandem mass spectra. This indicates that the poor cleavage of fatty acid from the secondary position of the triacylglycerol molecule is independent of the chain length.

3.3.3. Diacid TAGs containing two or three short acyl chains

The regioisomers of the diacid TAGs containing two short and one long acyl chain are represented by S/L/S and L/S/S. By definition the short fatty chains are identical. All dibutyrate regioisomers with the long chains ranging from C_{14} to C_{24} were separated to (or close to) the baseline by normal phase HPLC (Figs. 1B, C and 4A, Table 5). In the product

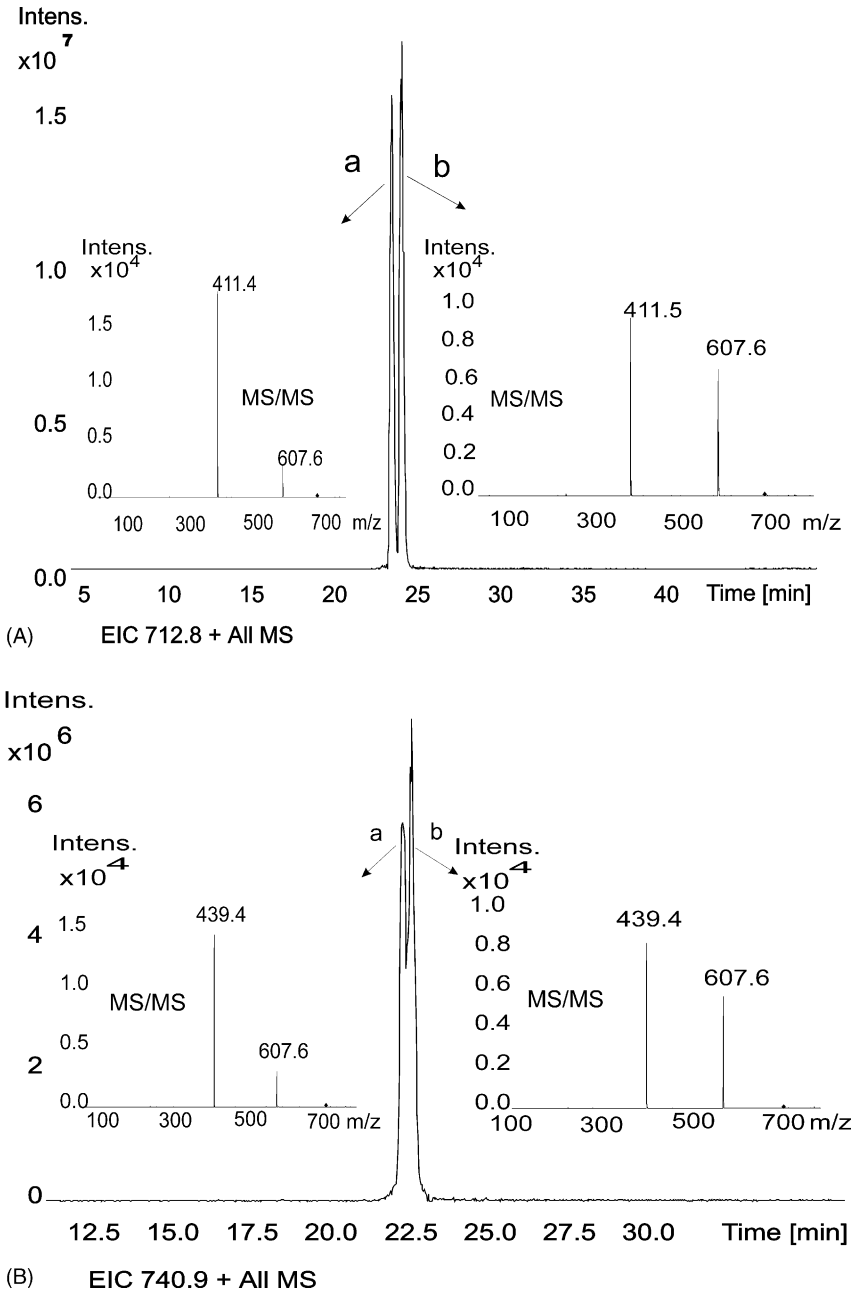

Fig. 2. Extracted ion chromatograms of mono-short-chain triacylglycerol ammonium adducts and tandem mass spectra of regioisomers. (A) Butyroyldistearoylglycerol: a, 1,3-distearoyl-2-butyroylglycerol; b, 1,2-distearoyl-3-butyroyl-*rac*-glycerol. (B) Caproyldistearoylglycerol: a, 1,3-distearoyl-2-caproylglycerol; b, 1,2-distearoyl-3-caproyl-*rac*-glycerol.

ion MS/MS spectra, the relative abundance of $[(M + NH_4)–NH_3–$long chain acid$]^+$ was much lower for S/L/S than for L/S/S isomer. The relative abundance of $[(M + NH_4)–NH_3–$long chain acid$]^+$ for 1,3-dibutyroyl-2-oleoylglycerol (Fig. 4A) was 7.3–7.8 and varied from 2.6 to 8.6% for 4:0/L/4:0 isomers with $C_{20–24}$ acyl carbons. The relative abundance of $[(M + NH_4)–NH_3–$long chain acid$]^+$ for 1-oleoyl-2,3-dibutyroyl-*rac*-glycerol was 38.4–45.7 and varied for L/4:0/4:0 isomers from 20.8 to 46.6%. The relative abundance ratio of $[(M + NH_4)–NH_3–L]^+/[(M + NH_4)–NH_3–4:0]^+$ varied between 0.0267 and 0.0941 for the 4:0/L/4:0 isomers and was on the average 0.0443 (S.D. = 0.022, $n = 20$). The respective ratio for the L/4:0/4:0 isomers varied in the range 0.262–0.873 and was on the average 0.516 (S.D. = 0.176, $n = 22$). On the average the relative abundance ratio for the L/4:0/4:0 isomers was 11.65 times higher than for the 4:0/L/4:0 isomers. The dicaproate and dienanthoate isomers separated partially, showed shouldering of the descending limb of the peak, or eluted in the same peak (Fig. 4B, Table 5). The abundance of $[(M + NH_4)–NH_3–$long chain acid$]^+$ for the 6:0/L/6:0 isomers with $C_{26–30}$ acyl carbons was in the range of 5.7–18.0% and that of L/6:0/6:0 isomers

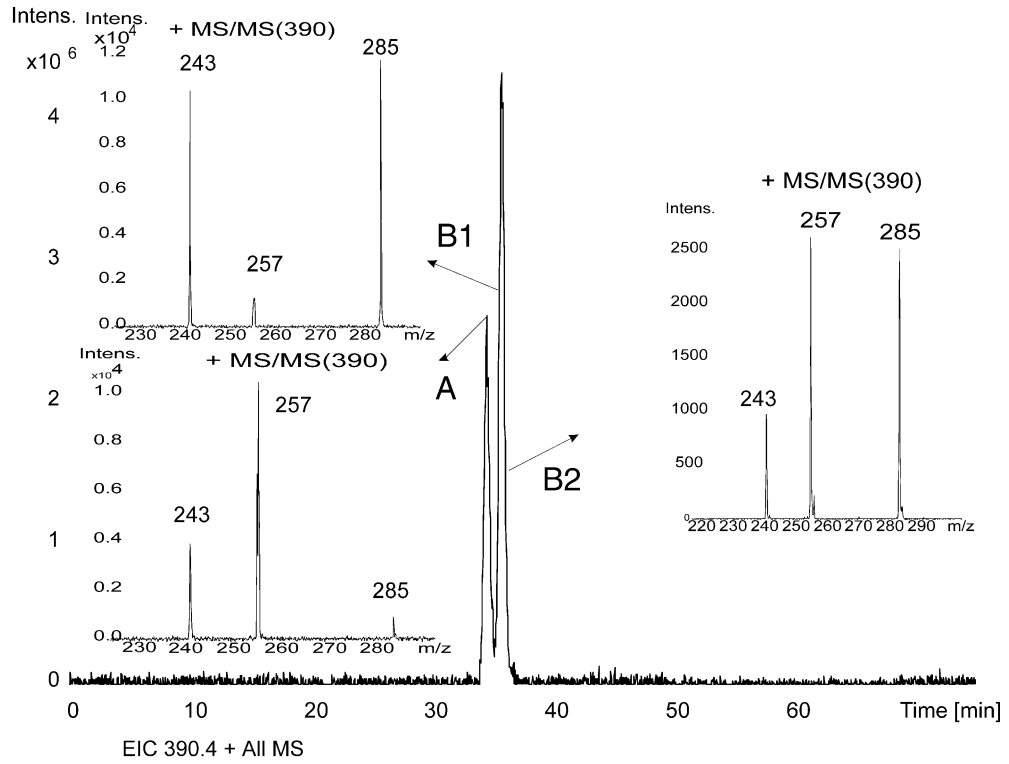

Fig. 3. Extracted ion chromatogram of butyroylcaproylenanthoylglycerol. (A) Tandem mass spectrum of peak A: 1-enanthoyl-2-butyroyl-3-caproyl-*rac*-glycerol. (B1) Tandem mass spectrum recorded at the leading edge of peak B: 1-enanthoyl-2-caproyl-3-butyroyl-*rac*-glycerol. (B2) Tandem mass spectrum recorded at the tailing end of peak B: 1-caproyl-2-enanthoyl-3-butyroyl-*rac*-glycerol.

in the range of 20.7–41.7%. The relative abundance ratio of $[(M + NH_4)–NH_3–L]^+/[(M + NH_4)–NH_3–6:0]^+$ was in the range 0.0604–0.220 for the 6:0/L/6:0 isomers and on the average 0.125 (S.D. = 0.051, $n = 16$). The respective ratio for the L/6:0/6:0 isomers varied between 0.216 and 0.715 and was on the average 0.457 (S.D. = 0.138, $n = 10$). On the average the relative abundance ratio for the L/6:0/6:0 isomers was 3.66 times higher than for the 6:0/L/6:0 isomers.

For the 6:0/L/6:0 and L/6:0/6:0 isomers separated partially, the respective ranges of relative abundance were 6.0–12.6 and 23.1−31.5%, respectively. The relative abundance ratios of $[(M + NH_4)–NH_3–L]^+/[(M + NH_4)–NH_3–6:0]^+$ varied between 0.0604 and 0.144 and for the 6:0/L/6:0 isomers and was on the average 0.109 (S.D. = 0.032, $n = 13$). The respective ratio for the L/6:0/6:0 isomers was in the range 0.216–0.637 and on the average 0.428 (S.D. = 0.111, $n = 9$). The average relative abundance ratio of isomers separated partially for the L/6:0/6:0 was 3.93 times higher than for 6:0/L/6:0 isomers.

The diacid TAGs with three short acyl chains, such as 6:0/4:0/6:0 was nearly completely separated from the 6:0/6:0/4:0 isomer. Also, the regioisomers of the triacid TAGs with three short acyl chains, such as the dibutyrates, could be clearly differentiated on the basis of their tandem mass spectra (Table 5). The regioisomers of the TAGs with three short acyl chains, such as dicaproates and dienanthoates could be distinguished on the basis of tandem mass spectra despite a partial chromatographic separation only.

3.4. Elution order of regioisomers

The regioisomers of the short-chain TAGs containing the shortest acyl chain in the secondary position were eluted first. The elution order of isomeric butyroylcaproyl enanthoyl glycerols was: 1-enanthoyl-2-butyroyl-3-caproyl-*rac*-glycerol < 1-enanthoyl-2-caproyl-3-butyroyl-*rac*-glycerol< 1-caproyl-2-enanthoyl-3-butyroyl-*rac*-glycerol. This is also the order of polarity of these regioisomers. The regioisomers of diacid TAGs containing two or three short-chain acids were eluted in a similar fashion. The TAGs with shortest acyl chain in the secondary position were eluted first followed by the mixture of the two regioisomers having the shortest acyl chains in the primary position.

3.5. Quantification

The theoretical random composition of molecular species was calculated for the eight interesterified mixtures from the composition (mol%) of the monoacid TAGs (starting materials). The experimental composition of molecular species was calculated from GLC data [2] for the same mixtures. The average deviation in percent from the theoretical random value (S.D.) was 11.1 (7.1), 11.8 (6.1), 9.0 (6.2), 6.4 (4.2),

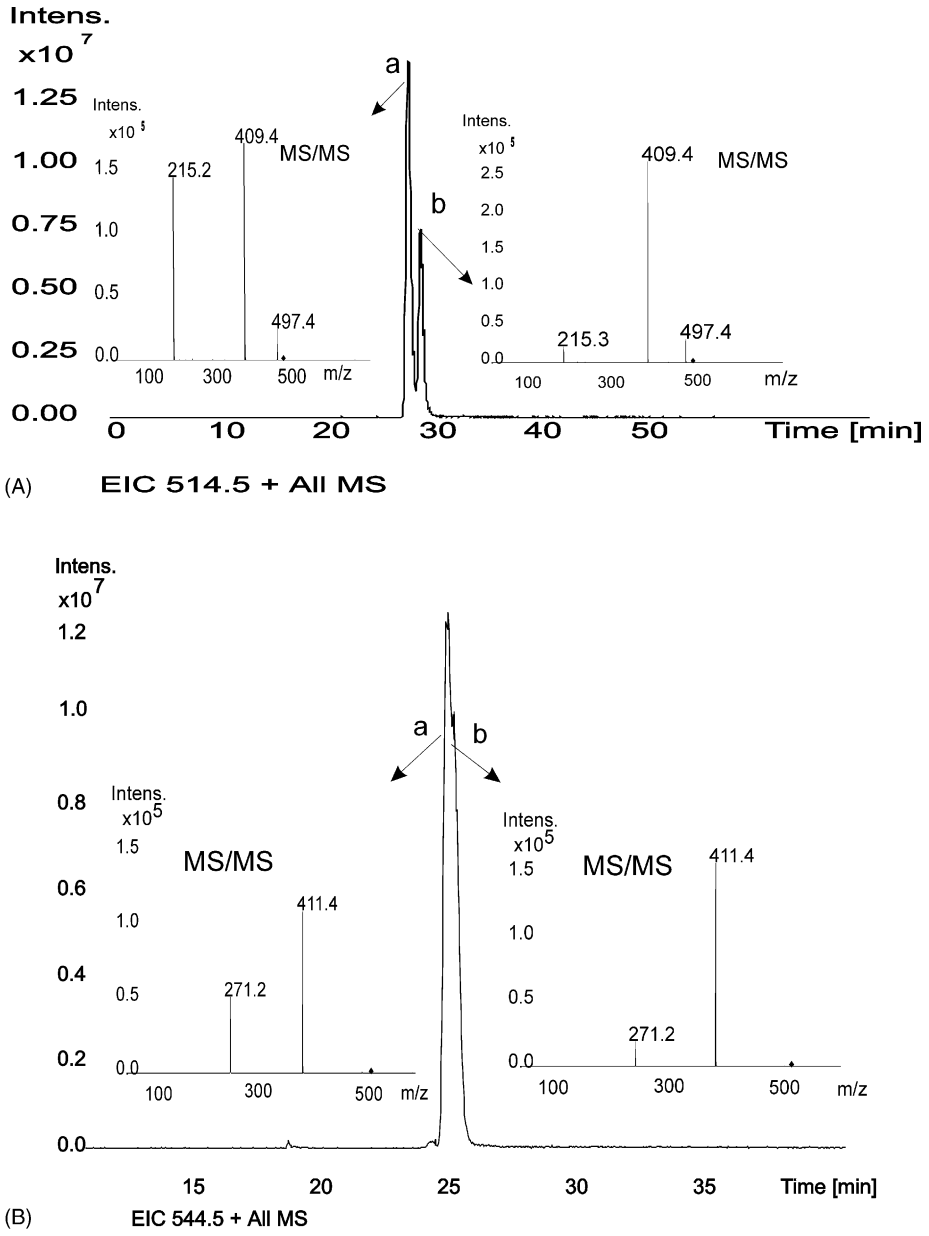

Fig. 4. Extracted ion chromatograms of di-short-chain triacylglycerol ammonium adducts and tandem mass spectra of regioisomers. (A) Dibutyroyloleoylglycerol: a, 1-oleoyl-2,3-dibutyroyl-*rac*-glycerol; b, 1,3-dibutyroyl-2-oleoylglycerol. (B) Dicaproylpalmitoylglycerol: a, 1-palmitoyl-2,3-dicaproyl-*rac*-glycerol; b, 1,3-dicaproyl-2-palmitoylglycerol.

5.5 (3.1), 6.5 (4.0), 7.9 (5.1), 6.5 (6.2) for the diacid TAGs in BLaS, BLaO, BMP, BMPo, CoMP, CoMPo, CoPS, and CoPO, respectively. In gas chromatograms separating the regioisomers very close to the baseline, the ratio of L/L/4:0 and L/4:0/L isomers on the average was 1.90 (S.D. = 0.075, $n = 6$), 1.95 (S.D. = 0.077, $n = 6$), 1.94 (S.D. = 0.043, $n = 9$). These data indicated that the distribution of fatty acids between the three positions of glycerol was in fact random. The $n(i)/n$(ISTD) ratio of each regioisomer was calculated from gas chromatographic data as follows. Molar amount of each molecular species was calculated from the areas of each analyte and internal standard and the amount of internal standard. Then the molar amount was divided in random ratio (2:1) to get molar amounts of each regioisomer. The plots $n(i)/n$(ISTD) versus area(i)/area(ISTD) in the ESI ion chromatograms for regioisomers of 4:0/14:0/14:0 (A and B), 4:0/14:0/16:1 (C and D), and 4:0/4:0/12:0 (E and F) are presented to demonstrate the linearity of response (Fig. 5). The regression analysis reveals linear dependence and different MCF for regioisomers. The MCFs were calculated for regioisomers of 20 diacid and triacid TAGs with one to three short acyl chains. The coefficients of determination, R^2 varied between 0.878 and 1.000 and were on the average 0.990 (S.D. = 0.021, $n = 40$). MCFs varied between 0.386 and 2.695. The minimum values were found for saturated TAGs 22:0 and 24:0, the smaller and bigger

Table 3
Tandem mass spectrometric data for identification of diacid triacylglycerols containing one short acyl chain

Interesterified mixture	CN:DB[a]	$[M + NH_4]^+$ (m/z)	Regioisomers[b]	RRT[c]	Regioisomer ratio[d]	Relative abundance[e] (%)
BLaS	28:0	544	*rac*-12:0–12:0–4:0	1.051	2.0:2.0:1.8	47.0; 48.1
			12:0–4:0–12:0	1.018		4.1; 10.5; 10.1
	40:0	712	*rac*-18:0–18:0–4:0	0.972	2.0:2.4:2.7	41.6; 47.7
			18:0–4:0–18:0	0.948		13.4
BLaO	28:0	544	*rac*-12:0–12:0–4:0	1.049		46.7; 43.0
			12:0–4:0–12:0	1.014		11.5; 13.6
	40:2	708	*rac*-18:1–18:1–4:0	0.984	2.0:2.3:2.3	34.5
			18:1–4:0–18:1	0.964		10.2
BMP	32:0	600	*rac*-14:0–14:0–4:0	1.018	2.0:1.9:1.7	43.1; 45.3
			14:0–4:0–14:0	0.988		13.6
	36:0	656	*rac*-16:0–16:0–4:0	0.993	2.0:2.4:2.6	53.0
			16:0–4:0–16:0	0.968		12.4
BMPo	36:2	652	*rac*-16:1–16:1–4:0	1.017	2.0:1.8:2.1	36.5; 37.8
			16:1–4:0–16:1	0.992		n.d.[f]
CoMP	34:0	628	*rac*-14:0–14:0–6:0	0.971[g]		43.9; 49.5
			14:0–6:0–14:0	0.971[g]		11.1
	38:0	684	*rac*-16:0–16:0–6:0	0.952		40.4
			16:0–6:0–16:0	0.941		20.3
CoMPo	34:0	628	*rac*-14:0–14:0–6:0	0.974		42.4
			14:0–6:0–14:0	0.960		21.1
CoPS	42:0	740	*rac*-18:0–18:0–6:0	0.925		40.4; 42.6
			18:0–6:0–18:0	0.910		17.2

[a] The number of acyl group carbons:the number of double bonds.
[b] Regioisomers in the reverse order of elution.
[c] Relative retention time.
[d] The ratio of the corrected areas of the former regioisomer to the latter in the highest, medium and lowest analyte/internal standard ratio of interesterified mixture.
[e] Relative abundance (%) of the $[(M + NH_4)–NH_3–\text{short-chain acid}]^+$. Normalized: $\sum[(M + NH_4)–NH_3–\text{fatty acid}_i]^+ = 100\%$.
[f] Not determined.
[g] Regioisomers separated partially.

TAGs had higher values. The unsaturated TAGs had higher MCFs than respective saturated TAGs. The MCFs of regioisomers differed by 2.0–45.4%. The MCFs of unsaturated regioisomers differed less than respective saturated regioisomers. The regioisomer ratios were determined for three analyte/internal standard ratio of five interesterified standard mixtures (BCoE, BLaS, BLaO, BMP, BMPo) by multiplying the areas with respective MCF and calculating the ratio of corrected areas (Tables 3–5). The regioisomer ratios of L/L/4:0 and L/4:0/L, L/L′/4:0 + L′/L/4:0 and L/4:0/L′, and L/4:0/4:0 and 4:0/L/4:0, for the highest analyte concentration were in general 2.0:1.0, which is in accordance with the NMR data presented in the Section 3.1. The ratios for lower analyte concentration differed to some extent from that ratio.

4. Discussion

The present study demonstrates that the lower abundance of fragment ions produced by the loss of a fatty acid from the secondary versus primary position can be utilized to differentiate between regioisomers of short-chain TAGs.

The mass spectrometric regiospecific studies of TAGs, reviewed in the introduction, showed that most methods have been tested only to differentiate the regioisomers of long-chain TAGs. Some methods applicable to differentiate regioisomers of long-chain TAGs have failed when tested for short- or medium-chain TAGs. Kallio and Currie [21] have shown that in the negative ion chemical ionization tandem mass spectra (NICIMS/MS) the $[M - H–RCOO - 100]^-$ ion is produced primarily by loss of fatty acids from the primary positions. The abundances of $[M–H–RCOOH–100]^-$ product ions were not strongly dependent on collision energy or collision gas pressure, and the ratio was constant in all investigated reference compounds regardless of the fatty acid combinations. When the mixtures containing different proportions of reference TAGs of type *rac*-AAB and *sn*-ABA (A and B representing different fatty acyl groups) were analyzed, the product ion ratio $[M - H–B - 100]^-/[M - H–A - 100]^-$ was increased as the proportion of *rac*-AAB increased from 0 to 100%. This result enabled the identification and determination of the proportions of the regioisomers of the TAGs and the proportions of the regioisomers comprising the molecular weight species. This method has been applied to the analysis of molecular weight distribution of regioisomers

Table 4
Tandem mass spectrometric data for identification of triacid triacylglycerols

Mixture	CN:DB[a]	$[M + NH_4]^+$ (m/z)	Regioisomers[b]	RRT[c]	Regioisomer ratio[d]	Relative abundance[e] (%)
BCoE	17:0	390	*rac*-6:0–7:0–4:0	1.316[f]	2.0:2.0:2.1	44.4/18.2/37.4
			rac-7:0–6:0–4:0	1.316[f]		39.3/11.7/49.0
			rac-7:0–4:0–6:0	1.270		22.6/4.5/72.8
BLaS	34:0	628	(*rac*-12:0–18:0–4:0 + *rac*-18:0–12:0–4:0)	1.005	2.0:1.8:1.6	32.5/22.8/44.7
			rac-18:0–4:0–12:0	0.978		35.5/11.4/53.1
BLaO	34:1	626	(*rac*-18:1–12:0–4:0 + *rac*-12:0–18:1–4:0)	1.013	2.0:2.0:1.9	33.1/26.1/40.8
			rac-18:1–4:0–12:0	0.986		45.1/15.2/39.7
BMP	34:0	628	(*rac*-14:0–16:0–4:0 + *rac*-16:0–14:0–4:0)	1.004	2.0:1.9:1.9	29.5/22.0/48.5
			rac-16:0–4:0–14:0	0.979		36.2/13.8/50.0
BMPo	34:1	626	(*rac*-16:1–14:0–4:0 + *rac*-14:0–16:1–4:0)	1.015	2.0:2.0:2.0	37.7/25.9/36.4
			rac-16:1–4:0–14:0	0.989		46.5/19.4/34.1
CoMP	36:0	656	(*rac*-16:0–14:0–6:0 + *rac*-14:0–16:0–6:0)	0.961[g]		30.1/29.4/40.5
			rac-16:0–6:0–14:0	0.961[g]		40.3/15.3/44.4
CoMPo	36:1	654	(*rac*-16:1–14:0–6:0 + *rac*-14:0–16:1–6:0)	0.969		35.2/32.0/32.9
			rac-16:1–6:0–14:0			
CoPS	40:0	712	(*rac*-16:0–18:0–6:0 + *rac*-18:0–16:0–6:0)	0.945		20.9/27.5/51.5
			rac-18:0–6:0–16:0	0.934		45.9/14.9/39.2
CoPO	40:1	710	(*rac*-18:1–16:0–6:0 + *rac*-16:0–18:1–6:0)	0.951[b]		37.6/29.3/33.0
			rac-18:1–6:0–16:0	0.951[b]		46.0/17.6/36.4

[a] The number of acyl group carbons:the number of double bonds.
[b] Regioisomers in the reverse order of elution.
[c] Relative retention time.
[d] The ratio of the corrected areas of the former regioisomer to the latter in the highest, medium and lowest analyte/internal standard ratio of interesterified mixture.
[e] Relative abundance (%) of the $[(M+NH_4)–NH_3–\text{fatty acid}]^+$ in the order shown in the formula. Normalized: $\sum[(M+NH_4)–NH_3–\text{fatty acid}_i]^+ = 100\%$.
[f] Eluted in the same peak.
[g] Shoulder in the front of peak.

of medium- and long-chain TAGs from a variety of fats and oils. Kurvinen et al. [23] recently reviewed these studies and proposed a computer program to facilitate the application of this method to regiospecific analysis of natural fats and oils. Kurvinen et al. [22] applied this method to synthetic TAGs containing long-chain fatty acids in combination with octanoic acid and observed that the presence of the medium-chain fatty acid may cause difficulties in the quantification of regioisomers. In case of 8:0/18:2/8:0 and 8:0/8:0/18:2 TAGs, the ratio of $[M - H - 18{:}2 - 100]^-$ and $[M - H - 8{:}0 - 100]^-$ was increased by a factor of 18, when 8:0/18:2/8:0 was compared to 18:2/8:0/8:0. A linear relationship was obtained between the ion intensities and the molar proportion of 18:2/8:0/8:0 in the mixture. When the same analysis of the ratio of $[M - H - 8 : 0 - 100]^-$ and $[M - H - 18 : 2 - 100]^-$ ions was performed with 18:2/8:0/18:2 and 8:0/18:2/18:2 TAGs, a different result was obtained. Unexpectedly the ratio of intensities of these ions increased only by a factor of 5 when 18:2/8:0/18:2 and 8:0/18:2/18:2 were compared. This result indicated that the fragmentation was less specific in case of the TAGs with a single 8:0 residue than in case of TAGs with two 8:0 residues. The ion ratio was constant until the proportion of 8:0/18:2/18:2 in the mixture reached about 50% after which the ion ratio increased with increasing proportion of the 8:0/18:2/18:2 isomer. This finding made the exact quantification of this type of regioisomers in an unknown mixture impossible, although the predominant isomer may still be estimated, provided its proportion exceeded 50%.

Myher et al. [14] used reversed phase HPLC in combination with direct inlet positive chemical ionization to analyze the long-chain TAGs of randomized corn and peanut oils and showed that the abundance of $[M - H–RCOOH]^+$ cleaved from the primary positions was four times that cleaved from the secondary position.

Mottram and Evershed demonstrated the applicability of APCI/MS in the structural analysis of long-chain TAGs [25]. The APCI/MS show low intensity protonated molecular ion $[M + H]^+$, abundant "diglyceride" ions $[M - RCOO]^+$, and low intensity acyl ions. The abundance of $[M - RCOO]^+$ formed by cleavage of fatty acid from the energetically less favorable secondary position was lower than those formed

Table 5
Tandem mass spectrometric data for identification of diacid triacylglycerols containing two or three short acyl chains

Mixture	CN:DB[a]	$[M + NH_4]^+$ (*m*/*z*)	Regioisomers[b]	RRT[c]	Regioisomer ratio[d]	Relative abundance[e]
BCoE	14:0	348	*rac*-6:0–4:0–4:0	1.431	2.1:1.9:1.5	59.2
			4:0–6:0–4:0	1.466		0; 21.0
	15:0	362	*rac*-7:0–4:0–4:0	1.390	2.1:2.1:1.6	48.6; 38.4
			4:0–7:0–4:0	1.430		6.1
	16:0	376	*rac*-6:0–6:0–4:0	1.346	2.0:2.0:1.7	55.6; 58.2; 52.3
			6:0–4:0–6:0	1.315		95.4; 97.9
	19:0	418	*rac*-7:0–6:0–6:0	1.176[f]		27.5; 40.5
			6:0–7:0–6:0	1.176[f]		21.6; 20.9
	20:0	432	7:0–6:0–7:0	1.141[f]		86.4
			rac-7:0–7:0–6:0	1.141[f]		58.8; 55.3
BLaS	20:0	432	*rac*-12:0–4:0–4:0	1.206	2.0:2.2:2.3	35.9; 35.1; 33.7
			4:0–12:0–4:0	1.263		2.6; 2.6; 2.8
	26:0	516	*rac*-18:0–4:0–4:0	1.119	2.0:2.4:2.6	26.1; 26.0; 27.3
			4:0–18:0–4:0	1.157		3.8; 3.6
BLaO	20:0	432	*rac*-12:0–4:0–4:0	1.204	2.3:2.5:2.8	34.8; 35.6; 38.6
			4:0–12:0–4:0	1.260		3.2; 4.2; 4.7
	26:1	514	*rac*-18:1–4:0–4:0	1.133	2.0:2.1:2.1	41.9; 45.7; 38.4
			4:0–18:1–4:0	1.185		7.3; 7.8; 7.8
BMP	22:0	460	*rac*-14:0–4:0–4:0	1.178	2.0:2.2:2.6	29.4; 30.5; 25.0
			4:0–14:0–4:0	1.237		3.2; 2.7; 3.4
	24:0	488	*rac*-16:0–4:0–4:0	1.143	2.0:2.1:2.3	26.8; 28.4; 20.8
			4:0–16:0–4:0	1.199		2.7; 2.6; 3.4
BMPo	22:0	460	*rac*-14:0–4:0–4:0	1.174	2.4:2.6:3.0	29.2; 29.8
			4:0–14:0–4:0	1.235		3.3; 3.7
	24:1	486	*rac*-16:1–4:0–4:0	1.188	2.0:2.1:2.4	45.7; 46.6
			4:0–16:1–4:0	1.240		8.6
CoMP	26:0	516	*rac*-14:0–6:0–6:0	1.040[g]		30.7; 23.1
			6:0–14:0–6:0	1.050[g]		6.5; 11.3
	28:0	544	*rac*-16:0–6:0–6:0	1.023[f]		32.5
			6:0–16:0–6:0	1.023[f]		8.7; 6.0
CoMPo	26:0	516	*rac*-14:0–6:0–6:0	1.037[g]		31.5
			6:0–14:0–6:0	1.050[g]		10.7; 12.2
	28:1	542	*rac*-16:1–6:0–6:0	1.043[h]		41.7
			6:0–16:1–6:0	1.043[h]		18.0; 17.6; 16.6
CoPS	28:0	544	*rac*-16:0–6:0–6:0	1.026[f]		31.9
			6:0–16:0–6:0	1.026[f]		5.7; 9.1
	30:0	572	*rac*-18:0–6:0–6:0	1.010[f]		20.7
			6:0–18:0–6:0	1.010[f]		7.1; 12.3
CoPO	28.0	544	*rac*-16:0–6:0–6:0	1.023[g]		30.8; 26.5
			6:0–16:0–6:0	1.035[g]		8.7; 12.6
	30:1	570	*rac*-18:1–6:0–6:0	1.021[f]		38.9
			6:0–18:1–6:0	1.021[f]		12.5

[a] The number of acyl group carbons:the number of double bonds.
[b] Regioisomers in the order of elution.
[c] Relative retention time.
[d] The ratio of the corrected areas of the former regioisomer to the latter in the highest, medium and lowest analyte/internal standard ratio of interesterified mixture.
[e] Abundance (%) of the $[(M + NH_4)–NH_3–\text{long-chain acid}]^+$. Normalized: $\sum[(M + NH_4)–NH_3–\text{fatty acid}_i]^+ = 100\%$.
[f] Shoulder in the tail of peak.
[g] Regioisomers separated partially.
[h] Both regioisomers eluted in the same peak.

by cleavage of fatty acid from the primary positions. For the ABA and AAB type long-chain TAGs they showed that the relative abundance ratios $[AA]^+/[AB]^+$ was 0.95–1.07 for AAB isomers and 0.20–0.29 for ABA isomers, and thus 4.75–3.7 times higher for AAB isomers. When analyzing milk fat TAGs of silica gel thin layer and gel permeation chromatography fractions using RP–HPLC–APCI/MS, Mottram and Evershed [34] presented recently abundandantly data for identified molecular species of milk fat TAGs. In addition, they stated that, in general, butyric acid was not esterified in the secondary position and showed tandem MS of two monobutyrates.

In the pioneer ESI/MS work of Duffin et al. [15] as well as in later studies of Cheng et al. [16], Dorschel [18], and Marzilli et al. [19], ammonium acetate and in the work of Hvattum [17] ammonium formate, was used for formation

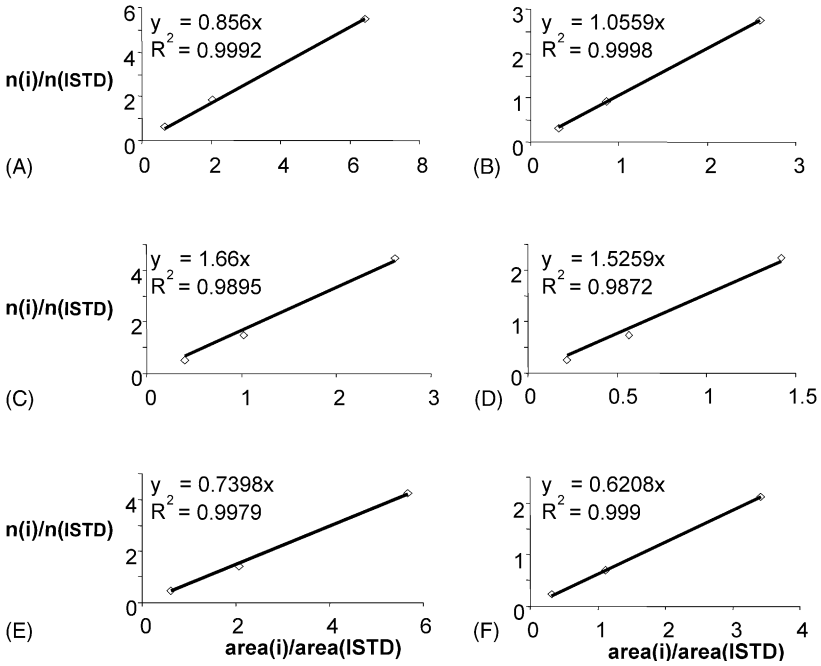

Fig. 5. Determination of molar correction factors. Plots of $n(i)/n$(ISTD) vs. area(i)/area(ISTD). (A) 1,2-Dimyristoyl-3-butyroyl-*rac*-glycerol; (B) 1,3-dimyristoyl-2-butyroylglycerol; (C) 1-palmitoleoyl-2-myristoyl-3-butyroyl-*rac*-glycerol; (D) 1-palmitoleoyl-2-butyroyl-3-myristoyl-*rac*-glycerol; (E) 1-lauroyl-2,3-dibutyroyl-*rac*-glycerol; (F) 1,3-dibutyroyl-2-lauroylglycerol.

of ammonium adducts. Duffin et al. [15] and Cheng et al. [16] could not observe preferential loss of fatty acid from the primary positions of ammonium adducts and thus were unable to differentiate regioisomers. Hvattum [17] and Dorschel [18] showed that loss of the fatty acid from the secondary position of long-chain TAGs was to some extent less favorable than from the primary positions. Contrary to these results, Marzilli et al. [19] were able to differentiate distinctly the regioisomers of long-chain TAGs. The relative abundance ratios $[AB]^+/[AA]^+$ varied between 5.2 and 5.9 for ABA isomers and between 1.1 and 2.0 for AAB isomers. The abundance ratio of the ion formed by cleavage of acyl from the primary position was 2.9–4.7 times that formed by cleavage of acyl from the secondary position.

In the present study, chloroform–methanol–ammonia water mixture was added post column to the effluent flow for formation of ammonium adducts. The abundance of ions formed by cleavage of butyrate from the primary positions was on the average 6.3 times that formed by cleavage from the secondary position for diacid monobutyrates and 4.3 times for triacid monobutyrates. The abundance of ion formed by cleavage of long-chain acyl from the primary positions of diacid dibutyrates was on the average 11.6 times that formed by cleavage from the secondary position. The differences of abundances were greater for the monobutyrates than monocaproates, the regioisomers of which were fully resolved by normal phase HPLC. However, mono- and dicaproate isomers could also be differentiated despite their incomplete separation by normal phase HPLC. The abundance of ion formed by cleavage of caproate from the primary positions was on the average 3.6 times than that formed by cleavage from the secondary position for diacid and triacid monocaproates. The abundance of ion formed by cleavage of long-chain acyl from the primary positions of diacid dicaproates was on the average 3.6 times that cleaved from the secondary position. On the average, these ion ratios are similar or higher than those reported by Myher et al. [14] for direct inlet positive chemical ionization MS of long-chain TAGs, Mottram and Evershed [25] for APCI/MS of long-chain TAGs, and Marzilli et al. [19] for ESI/MS of long-chain TAGs.

Unlike in the present study, the high energy CID fragments of ESI and fast atom bombardment (FAB) produced $[M+Na]^+$ ions of long-chain TAGs, studied by Cheng et al. [16], revealed also information on the position of double bonds in the unsaturated moieties. The same information was obtained from the high energy CID fragments of FAB produced $[M+Na]^+$ ions of synthetic TAGs containing two short-chain fatty acids and of similar TAGs isolated from bovine udder in the investigation of Kim et al. [28]. Hsu and Turk [20] used low energy CID of $[M+Li]^+$ ions of long-chain TAGs produced by ESI and observed that the ion formed by neutral loss of acyl groups as the free fatty acid or as lithium salt was less abundant from the secondary position than from primary positions, enabling assignment of regiospecific positions of long acyl groups.

In the present study, the ability of silica gel HPLC columns to separate regioisomers of short-chain TAGs was critical for the demonstration of difference in fragmentation in the tandem mass spectra and for the determination of MCFs for

regioisomers of butyrate TAGs. For identification of regioisomers of caproate TAGs and reverse isomers 7:0/6:0/4:0 and 6:0/7:0/4:0 partial separation within the same chromatographic peak was sufficient. The resolution of L/4:0/4:0 and 4:0/L/4:0, L/L/4:0 and L/4:0/L, and L/L′/4:0 and L/4:0/L′ regioisomers to the baseline or close to the baseline allowed the calculation of MCFs for these types of regioisomers. A linear response was found for 20 pairs of regioisomers of butyrate TAGs. The MCFs varied widely according to chain-length and unsaturation. The MCFs of regioisomers also differed to varying extent. When the regioisomer ratio was determined using MCFs in calculation of corrected areas, the random ratio was obtained for pairs of regioisomers present in the highest concentration in the interesterified standard mixtures. The applicability of the method to the determination of regioisomer ratios in natural and modified fats depends on the ability of the column to separate regioisomers from each other and from acyl-chain isomers. The latter depends on the nature of fat and on the type of modification.

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