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METHODS

Separation of Enantiomeric Triacylglycerols by Chiral-Phase HPLC

Tomáš Řezanka · Karel Sigler

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Abstract Liquid chromatography–atmospheric pressure chemical ionization mass spectrometry (LC–MS/APCI) with a chiral phase was used for separation of triacylglycerols (TAG) obtained either by organic synthesis or isolated from different algal species. We present chromatographic characteristics and tandem mass spectra of enantiomers and positional isomers (regioisomers) of C16, C18 and C20 polyunsaturated fatty acids. The retention time was found to depend on the structure of the given TAG, increasing with increasing number of double bonds and decreasing with increasing number of the carbons in TAG, with the exception of dieicosapentaenoyl-palmitoylglycerols.

Keywords Triacylglycerols · Atmospheric pressure chemical ionization mass spectrometry · Chiral LC · Alga and yeast

Abbreviations

APCI	Atmospheric pressure chemical ionization
CID	Collision induced dissociation tandem mass
MS/MS	spectrometry
DB	Double bonds
DMAP	4-Dimethylaminopyridine
EDCI	1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide
ESI	Electrospray ionization
FA	Fatty acid(s)
FAME	Fatty acid methyl ester(s)
LC–MS/APCI	Liquid chromatography–mass spectrometry/atmospheric pressure chemical ionization
NARP-LC	Non aqueous reversed phase-liquid chromatography
TAG	Triacylglycerol(s)
A	Arachidonic acid, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, 20:4ω6
E	Eicosapentaenoic acid, 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid, 20:5ω3
P	Palmitic acid, hexadecanoic acid, 16:0
Pl	7*Z*,10*Z*-Hexadecadienoic acid, 16:2ω6
Pm	4*Z*,7*Z*,10*Z*,13*Z*-Hexadecatetraenoic acid, 16:4ω3
Pn	7*Z*,10*Z*,13*Z*-Hexadecatrienoic acid, 16:3ω3
Po	Palmitoleic acid, 9*Z*-hexadecenoic acid, 9*Z*-16:1
Se	Stearidonic acid, 6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid, 18:4ω3

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T. Řezanka (✉) · K. Sigler
Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague, Czech Republic
e-mail: rezanka@biomed.cas.cz

Introduction

Natural triacylglycerols (TAG) are found in living organisms always as mixtures of different types of molecules, in many cases as stereoisomers, i.e., regioisomers or enantiomers. Natural mixtures of TAG, whether of plant or animal origin, always include tens, hundreds or even thousands of molecular species depending on the number of bound fatty acids (see Table 1).

The rapid development of liquid chromatography in recent years, especially in combination with mass

Table 1 The number of possible TAG [33]

Description	Number of possible TAG	Number of possible TAG for $y = 35$
Without isomers	$x = (y^3 + 3y^2 + 2y)/6$	7,770
Without optical isomers	$x = (y^3 + y^2)/2$	22,050
All isomers	$x = y^3$	42,875

x is the number of TAG, y is the number of FA in TAG

spectrometers with the soft ionization techniques of atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) [1] and related methods has facilitated the separation and identification of regioisomeric TAG [2]. The knowledge of the spatial configuration of TAG, i.e., esterification of individual fatty acids on the backbone of prochiral glycerol, is of key importance for elucidation of physiological and pathological changes in living cells. Many nutritional properties including oxidative stability, absorption and subsequent metabolism in the digestive system are known to be affected by the position of fatty acids (FA) in TAG [3]. Mammalian pancreatic lipase cleaves fatty acids in positions 1 and 3 on the glycerol backbone faster than those in position 2. TAG having the secondary hydroxyl esterified by a polyunsaturated fatty acid (PUFA), i.e., unsaturated fatty acid with 3–6 double bonds (DB), are therefore nutritionally more beneficial. These, so-called structured TAG can be produced either by a directed enzymatic esterification (transesterification) [4] or by directed biosynthesis by oleaginous microorganisms [5].

Separation of regioisomeric TAG has been described many times, beginning with the pioneering study [6] and including the review [7] describing their separations from tens of different oils and fats by non-aqueous reversed phase-liquid chromatography (NARP-LC) [8] and silver-LC [9]. The separation of regioisomeric TAG does therefore present no problems for chemists versed in this field. The same holds for identification of regioisomers that has also been in principle solved (see, e.g., the identification of 762 regioisomeric TAG from krill oil [10], though without any quantification of individual molecular species). However, one should be aware that even this large number of identified TAG is a mere fraction (about 1.38 %) of the theoretical number of TAG, see Table 1. The identification of regioisomeric TAG makes use of the well-known fact that regioisomers have different ratios of intensity of $[M+H–R_iCOOH]^+$ ions, i.e., the ratios of ions of type $[R_1R_1']^+/[R_1R_2]^+ + [R_1'R_2]^+$, so that, e.g., the formation of the 1,2-isomer of the $[R_1R_1']^+$ ion requires less energy than that involved in generating the analogous 1,3-ion from the TAG [11].

Natural polysaccharides such as cyclodextrin and cellulose are optically active substances owing to their D-(+) glucose units. However, cellulose or cyclodextrin alone are not good chiral selectors due to their low selectivity and are therefore used in the form of derivatives. Their selectivity can be improved by using, e.g., tris-(3,5-dimethyl-phenyl-carbamate) covalently bound to cyclodextrin or cellulose. Owing to the different conformation of cyclodextrin (cyclic conformation) and cellulose (linear conformation) the two chiral stationary phases equipped with the same chiral selector exhibit different enantioselectivities [12], modified cyclodextrin showing a higher chiral selectivity than cellulose [5, 13–15].

In this paper we describe the identification of regioisomers and enantiomers of TAG, both synthetic and natural (found in different strains of a snow alga), containing enantiomers and diastereoisomers of C16 and C20 polyunsaturated fatty acids. For the purpose we used chiral phase LC–MS with APCI. Chiral separation makes it possible to determine both qualitative and semiquantitative proportions of regioisomers and enantiomers of TAG containing C16, C18, and C20 PUFA. The method is suitable for a direct determination of TAG profiles after a genetic manipulation or a change in cultivation conditions of microorganisms.

Materials and Methods

Standards and Instrumentation

Acetonitrile, 2-propanol, THF, hexane, dichloromethane, 4-dimethyl-aminopyridine (DMAP), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDCI), 2,3-isopropylidene-*sn*-glycerol and 1,2-dieicosapentaenoyl-*sn*-glycero-3-phosphocholine were purchased from Sigma–Aldrich (Prague, Czech Republic). 1,2-Dieicosapentaenoyl-3-palmitoyl-*rac*-glycerol (EEP), 1,3-dieicosapentaenoyl-2-palmitoyl-*rac*-glycerol (EPE), 1,2-dipalmitoyl-3-eicosapentaenoyl-*rac*-glycerol (PPE), and 1,3-dipalmitoyl-2-eicosapentaenoyl-*rac*-glycerol (PEP) were obtained previously [5]. 1,2-Diarachidonoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA, and (9*Z*)-hexadecenoic, 7(*Z*),10(*Z*)-hexadecadienoic, 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acids, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine from Larodan (Malmö, Sweden). The 4(Z),7(Z),10(Z),13(Z)-hexadecatetraenoic acid was formerly supplied by Angene International Limited (Hong Kong, People's Republic of China). The synthesis of the following TAG-*sn*-PPA, *sn*-PAP, *sn*-APA and *sn*-AAP and *sn*-PPE, *sn*-PEP, *sn*-EPE and *sn*-EEP has been described earlier [5, 16].

Lipid Extraction and Isolation of TAG

The extraction procedure was based on the method of Bligh and Dyer [17], except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases [18]. The organisms used included snow alga—*Chloromonas pichinchae* [19] and yeast—*Kluyveromyces polysporus* [13]. The alcohol–water mixture of the frozen cells was cooled, one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure [20].

Total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted neutral lipids with 40 mL of chloroform–methanol (7:3), and acidic lipids with 30 mL of chloroform–methanol-concentrated aqueous ammonia (70:30:2) containing 0.4 % (w/v) ammonium acetate. The eluate of neutral lipids was reduced in volume, mixed with 1 mL of hexane and the mixture was stirred for 15 min. The solution was filtered, hexane was evaporated and the oil samples were dissolved in an acetonitrile–2-propanol–hexane mixture (1:1:1, v/v/v), which was injected on the HPLC column [19].

Stereospecific synthesis of enantiomers [21, 22], enzymatic synthesis of TAG, preparation of mixed FA composition TAG and FAME analysis [23, 24] are described in Supplements.

TAG Analysis by RP-HLPC/MS-APCI

LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and two columns Hichrom HIR-PB—250A 250 × 2.1 mm ID, 5 µm particle size, in series. This setup was used as a high efficiency column—approximately 26,000 plates/250 mm, with a flow rate of 1 mL/min and an injection volume of 10 µL, and column temperature of 25 °C. The TAG were separated using a solvent gradient program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 5 to 110 min of ACN/iPrOH 30:70 (vol/vol); held for 10 min. The composition was returned to the initial conditions over 10 min. The performance was measured by triheptadecenoin as an internal standard under the conditions given above.

The detector was an AB Sciex API 4000 mass spectrometer (AB Sciex, Concord, Ontario, Canada) using APCI mass spectra. The ionization mode was positive, using the following settings: source temperature 420 °C, nebulizer gas (GS1) 5, nebulizer current 3 mA, curtain gas 10, collision gas and high declustering potential 100 V. The optimal collision energy was dependent on the type of experiment and was set to +10 V (full scan mode) or +30 V (product ion mode). In all full scan runs, the spectra were obtained from *m/z* 200–1,100. To identify the exact composition of high *m/z* value TAG species, an enhanced product ion spectrum (optimal CE +35 V) was made of all ions with an *m/z* value over 500 Da.

CID (collision-induced dissociation) mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas as a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 200–1,100 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3 % intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.2 software.

TAG Analysis by Silver Ion HPLC

The LC system used for separation in the silver ion mode was the same as that used in the reversed-phase mode. TAG (~1 µg/µL in methanol) were chromatographed on two silver-ion columns Chrom-Spher Lipids (250 × 4.6 mm, 5 µm, Varian, Palo Alto, CA, USA) connected in series. Mobile phase was a gradient from 100 % of A and 0 % of B at 0 min to 60 % of A and 40 % of during 120 min, where A is hexane–2-propanol–acetonitrile (99.8:0.1:0.1, v/v/v) and B is mixture of hexane–2-propanol–acetonitrile (96:2:2, v/v/v). The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 µL, respectively. Columns were conditioned by mobile phase A at flow rate of 0.5 mL/min for 1 hour before the every analysis. Other separation conditions used were as in the RP–LC, see [16].

TAG Analysis by Chiral LC/MS-APCI

The LC system used for separation in the chiral mode was the same as that used in the reversed-phase mode. TAG (~1 mg/mL in methanol) were chromatographed on two Astec cyclobond^{TM} I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin) chiral LC columns (5 µm, 25 cm × 4.6 mm) connected in series, from Sigma. Mobile phase was a gradient from 90 % of A and 10 % of B at 0 min to 50 % of A and 50 % of B during 180 min, and then isocratically for 30 min, where A is hexane and B is hexane-2-propanol (99:2, v/v) mixture [13]. The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 µL, respectively. Other separation conditions were as used in the RP–LC, see above.

Results and Discussion

In this article we report the chiral separation of synthetic and natural TAG which are not present in vegetable oils produced in large quantities, such as olive, sunflower, soybean, canola, linseed, etc. These oils contain the following dominant FA: palmitic acid and C18 FA, i.e., stearic, oleic, linoleic and linolenic acids. The latter two are considered essential FA for humans. Chiral chromatography of TAG containing these predominantly C18 acids has been described several times [15, 25–27]; the most detailed study was published by Lisa and Holcapek [14], who analyzed more than 120 TAG.

Important not only for human consumption are other PUFA found particularly in marine fish and mammals. These are final predators in the food chain that begins with phytoplankton and in which the C20 PUFA, i.e., arachidonic and eicosapentaenoic acids, are biosynthesized. Nagai et al. [27] described the striking differences between individual stereoisomers in marine fish (skipjack tuna, sardine) and mammals (Steller sea lion, harp seal), in which PUFA are in α-position of TAG, which is demonstrated by the fact that *sn*-PPE and *sn*-EPP are present while PEP is not.

Two methods were used for the synthesis of standards. The first one consisted in using phospholipase C to obtain, from commercially available phosphatidylcholines, one of the diacylglycerol enantiomers. This was then esterified to yield a corresponding TAG. This enabled us to obtain both regioisomers and enantiomers of TAG having one or two PUFA (eicosapentaenoic or arachidonic acids) and palmitic acid [5, 16].

The other method has already been described and successfully applied. The principle of the method consists in reacting the free acid with 2,3-isopropylidene-*sn*-glycerol, deprotecting and esterifying the remaining two free hydroxyls (at the *sn*-2 and *sn*-3) by the corresponding FA. The conditions of the synthesis are specifically described in Experimental. In fact, the only problem with any method of synthesis of optically active TAG is intramolecular and intermolecular transesterification. We tried to prevent the migration of the acyl(s) during synthesis by using low reaction temperature, i.e., 20 °C for removing protecting groups and a room temperature in the esterification of free hydroxyl groups of glycerol [19]. A chiral chromatogram of the reaction mixture with major *sn*-PmPnPn is shown in Fig. 1, tandem mass spectra are shown in Fig. 2, and the ratios of other synthesized regioisomers and enantiomers are presented in Table 2.

Lisa and Holcapek [14] successfully used a mobile phase which was a mixture based on hexane with acetonitrile and/or 2-propanol as modifiers. They found that TAG having more than 5 double bonds are separated when acetonitrile and 2-propanol, as well as mixtures of the two polar solvents with hexane are used as a mobile phase. Nagai et al. [20], using 100 % methanol and a column with cellulose tris (3,5-dimethyl-phenyl-carbamate) coated on silica gel, i.e., the same as that used by Lisa and Holcapek [14], failed to separate the key TAG, i.e., *sn*-PEE and *sn*-EEP. They succeeded only when using a Chiralcel OZ-3R (3-chloro-4-methyl-phenyl-carbamate), but the elution occurred in reverse order. The first to elute was *sn*-PEE followed by *sn*-EEP, i.e., a TAG having in position *sn*-1 saturated FA, not unsaturated FA as in the case of columns endcapped by 3,5-dimethyl-phenyl-carbamate stationary phase. This phenomenon, i.e., reversing the order of elution, is explained as being due to the fact that the two methyl groups have electron-donating characteristics, while the chlorine atom has electron-withdrawing characteristics [4]. Thanks to the use of the chiral phase, i.e.,

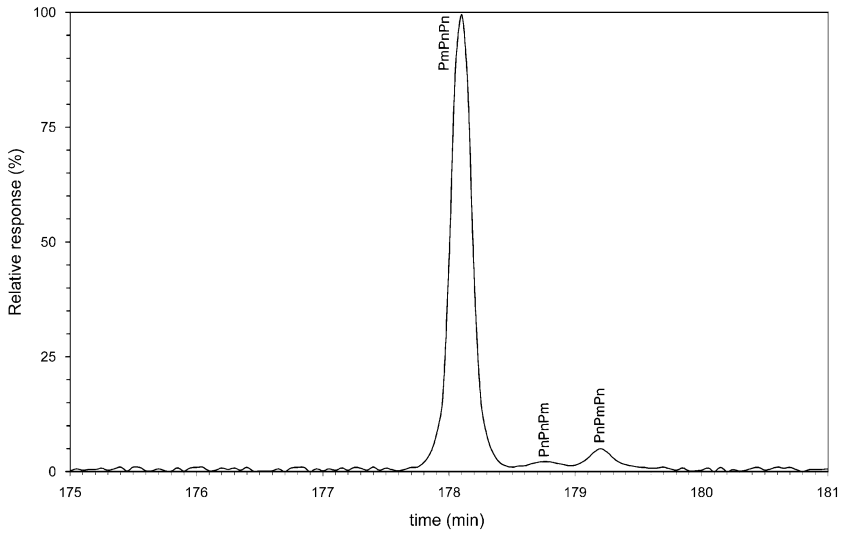

Fig. 1 Chiral LC of reaction mixture after synthesis of three isomers of dipalmitlinolenoyl-hexadecatetraenoyl-glycerol TAG

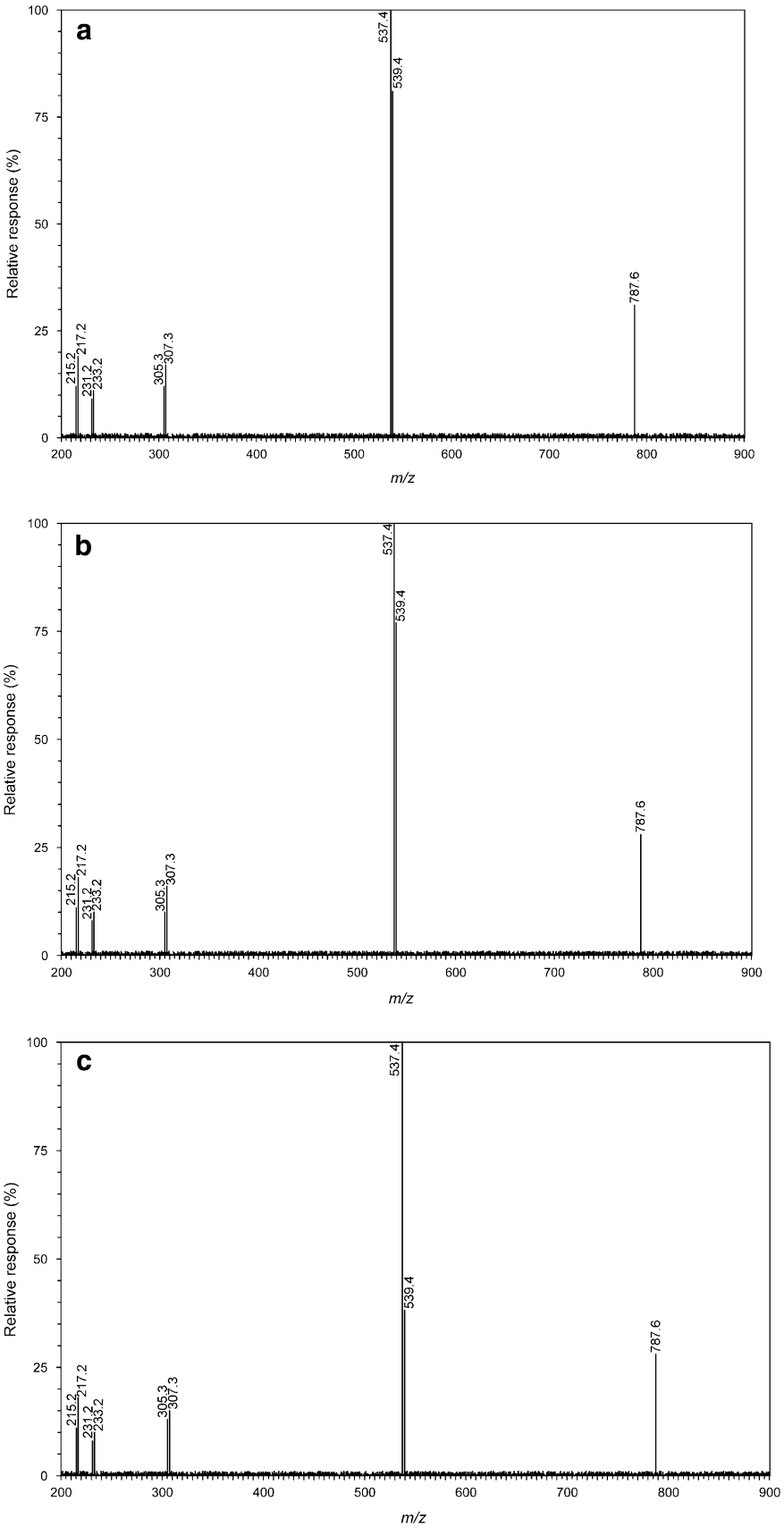

Fig. 2 **a** Tandem mass spectrum of 1-hexadecatetraenoyl-2,3-dihexadecatrienoyl-glycerol TAG (*sn*-PmPnPn). **b** Tandem mass spectrum of 1,2-dihexadecatrienoyl-3-hexadecatetraenoyl-glycerol TAG (*sn*-PnPnPm). **c** Tandem mass spectrum of 1,3-dihexadecatrienoyl-2-hexadecatetraenoyl-glycerol TAG (*sn*-PnPmPn)

Table 2 The ratio of enantiomers and diastereoisomers synthetized from appropriate free acids and 2,3-isopropylidene-*sn*-glycerol

TAG	***sn*-ABB**[a,b]	BAB	*sn*-BBA
sn-PPmPm	94	0	6
sn-PoPmPm	93	0	7
sn-PlPmPm	93	0	7
sn-PnPmPm	92	1	7
sn-PlPnPn	91	2	7
sn-PmPnPn	90	4	6
sn-PnPlPl	96	0	4

[a] In boldface—the molecular species we wanted to synthesize

[b] A, B, C denote different FA in different positions of the TAG. Thus *sn*-AAA is a TAG with identical FA (denoted A) in all three positions (e.g., 1,2,3-tripalmitoyl glycerol), ABB TAG has one FA (denoted A) in position 1 and a different FA (denoted B) in positions 2 and 3 (e.g., *sn*-APP = 1-arachidonoyl-2,3-dipalmitoyl-*sn*-glycerol, *sn*-PPA = 1,2-dipalmitoyl-3-arachidonoyl-*sn*-glycerol, *sn*-PAP = 1,3-dipalmitoyl-2-arachidonoyl-*sn*-glycerol), etc

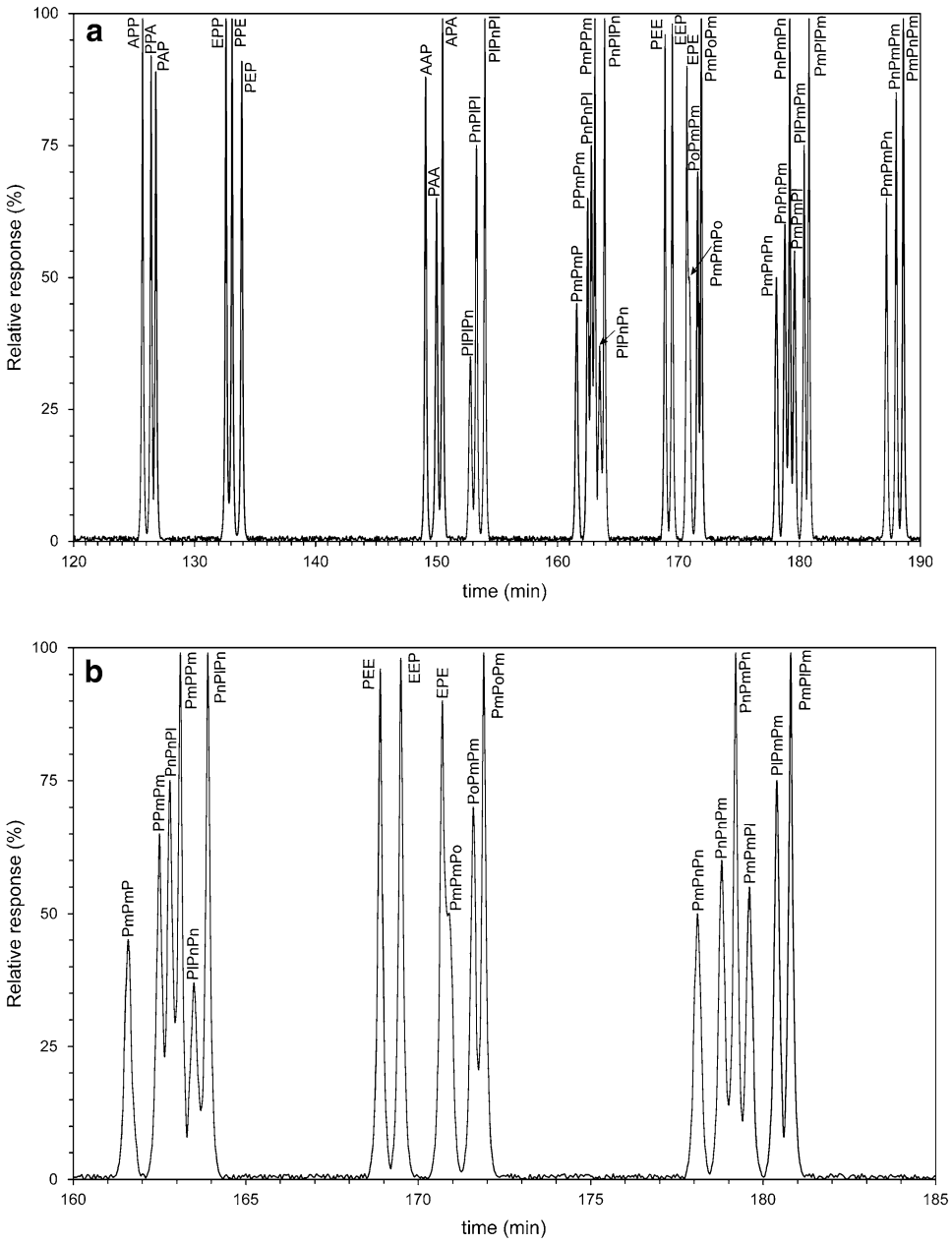

Fig. 3 **a** Chiral LC of natural and synthetic standards, see Table 3. The prefix "*sn*" was omitted in order to abbreviate the acronyms in the Figure. **b** Chiral LC of natural and synthetic standards, see Table 3. A chromatogram cutoff from Fig. 3a, expanded between 160 and 185 min

column with cellulose tris (3,5-dimethyl-phenyl-carbamate) coated on silica gel and a non-polar mobile phase (see above) we avoided the problem of the lack of separation of *sn*-EEP and *sn*-PEE. A small amount, of the order of percent units, of a polar or a polar aprotic solvent is particularly important for modulating retention time, chromatographic resolution and reproducibility [14]; the scatter of retention times for the last eluting peak (*sn*-PmPnPm) was ±1 % (~1.9 min).

Individual TAG were identified on the basis of the tandem mass spectra. We confirmed the known phenomenon that both regioisomers and enantiomers have identical spectra in terms of *m*/*z* values of individual ions but not in terms of their abundance. Symmetrical TAG, e.g., *sn*-EPE, obviously differs from the asymmetric TAG (i.e., the two enantiomers, *sn*-EEP versus *sn*-PEE) only in the intensity of diacylglycerol ions ($[EE]^+$ versus $[PE]^+$)—see the familiar rule that says that, based on the intensities of ions $[M+H\text{-}R_iCOOH]^+$, one can determine if the TAG is a 1,2-isomer (2,3-isomer) or 1,3-isomer derived from the original TAG [28, 29]. The individual enantiomers have almost identical mass spectra, see Fig. 2, so it cannot be determined from the mass spectra which enantiomer is present.

Figure 3a, b show a chromatogram of all standards, whether derived from a natural mixture [17] or TAG synthesized in this study. Table 3 gives further characteristics including the retention times, equivalent carbon number (ECN), where ECN is defined as: ECN = CN-n × DB, where CN is carbon number of the triacylglycerols, n is the number of double bonds (DB). Both Fig. 3 and Table 3 show that the retention time increases with increasing number of double bonds, with the exception of palmitoyl-dieicosapentaenoine, which will be discussed below. Conversely, retention time decreases with increasing chain length, which indicates that *sn*-AAP (having 8 double bonds and 56 carbon atoms) has a retention time 13.7 min shorter than *sn*-PnPnPl (also 8 double bonds, but 48 carbons). Overall, the contribution of one methylene group is seen to be about −1.5 min, whereas the contribution of one double bond is about +8 min. The unusual behavior of the three isomers of palmitoyl-dieicosapentaenoine, i.e., retention times much longer than these molecular species should have, can be explained by the combination of chain length and number of double bonds. Figure 1S show the models of two TAG (*sn*-PmPPm and *sn*-EPE). It is clearly seen that despite a similar structure they have completely different spatial arrangements.

According to Lisa and Holcapek [14], the fatty acyl in *sn*-2 position does not affect the chiral separation, but this is true only for TAG with FA having 0–2 double bonds on the primary hydroxyl. If the FA has 3 or more double bonds, then the retention times of the enantiomers are reversed so that the enantiomer having in the *sn*-3 position a fatty acyl having more double bonds has also a higher retention time than the corresponding enantiomer having the same FA in the *sn*-1 position. As an example, the study [14] gives two enantiomers, LnOO with RT 109.3 min and OOLn with a retention time of 112.5 min. Nagai et al. [27] found that the enantiomer having an unsaturated acyl in *sn*-

Table 3 Basic data of the TAG standards obtained by synthesis and isolated from natural sources (snow algae) (relative %)

RT	TAG	Carbons	DB	ECN	Abundance (%)
125.7	APP	52	4	44	99
126.4	PPA[b]	52	4	44	92
126.8	PAP	52	4	44	89
132.6	EPP	52	5	42	99
133.1	PPE[a]	52	5	42	99
133.9	PEP	52	5	42	91
149.1	AAP[b]	56	8	40	88
150.0	PAA	56	8	40	65
150.5	APA	56	8	40	99
152.8	PlPlPn[c,d]	48	7	34	35
153.3	PnPlPl[c]	48	7	34	75
154.0	PlPnPl[c]	48	7	34	99
161.6	PmPmP[c]	48	8	32	45
162.5	PPmPm[c,d]	48	8	32	65
162.8	PnPnPl[c]	48	8	32	75
163.1	PmPPm[c]	48	8	32	99
163.5	PlPnPn[c,d]	48	8	32	37
163.9	PnPlPn[c]	48	8	32	99
168.9	PEE	56	10	36	96
169.5	EEP[a]	56	10	36	98
170.7	EPE	56	10	36	90
170.9	PmPmPo[c]	48	9	30	50
171.6	PoPmPm[c,d]	48	9	30	70
171.9	PmPoPm[c]	48	9	30	99
178.1	PmPnPn[c,d]	48	10	28	50
178.8	PnPnPm[c]	48	10	28	60
179.2	PnPmPn[c]	48	10	28	99
179.6	PmPmPl[c]	48	10	28	55
180.4	PlPmPm[c,d]	48	10	28	75
180.8	PmPlPm[c]	48	10	28	99
187.2	PmPmPn[c]	48	11	26	65
188.0	PnPmPm[c,d]	48	11	26	85
188.6	PmPnPm[c]	48	11	26	99

[a] Obtained by two-step synthesis from diacyl-*sn*-glycero-3-phosphocholine, its hydrolysis by phospholipase C and reaction of diacylglycerol with free acid under catalysis with DMAP and EDCI [16]

[b] Ditto, but according to Ref. [5]

[c] Molecular species whose separations were achieved for the first time in this study

[d] Synthetized from appropriate free acids and 2,3-isopropylidene-*sn*-glycerol, see Table 2

1 position is eluted earlier than the other enantiomer, which is demonstrated on the example of *sn*-EPP with a retention time of 105.0 min and *sn*-PPE with a retention time 108.5 min. Our findings, see Fig. 3 and Table 3, are actually the intersection of results of the two articles. This can again be demonstrated in our results, where *sn*-EPP had a retention time of 132.6 while *sn*-PPE has 133.1 min. It is surprising that in this case the composition of the mobile phase does not matter (normal- or reversed-mode), the most important factors being only the type of stationary phase and the structure of the corresponding enantiomer. Although it would seem that the retention times in chiral chromatography of the enantiomers of TAG can be predicted, the opposite is true. From this observation it follows that at least one of the two enantiomers must be obtained either from commercial sources or by organic synthesis to ensure correct identification. Unfortunately with TAG one cannot not rely on semi-preparation and the measurement of optical activity of the TAG so obtained, because they have $[\alpha]_D$ only negligibly different from 0; thus, e.g., 1-oleoyl-2,3-palmitoyl-*sn*-glycerol had $[\alpha]_D^{20} = 0.00$ (c 7.05, $CHCl_3$) [30].

This finding does not hold for regioisomers because they can be distinguished on the basis of the abundance of diacylglycerol ions, i.e., ions of the type ($[AA]^+$ versus $[AB]^+$).

We have previously used chiral LC/MS-APCI for exploring the biotechnological sources of two major PUFA, i.e., arachidonic [16] and eicosapentaenoic acids [5]. The snow alga *Chloromonas pichinchae* was collected in nature and further analyzed by argentation and NARP-LC/MS [19]. Figure 4 shows chiral LC fraction of the fraction having 11 double bonds after Ag-LC [17]. Unfortunately for some TAG (marked with crosses) we could not identify whether they were R- or S-enantiomers, either because of a poor separation of the enantiomers, overlapping peaks (see e.g., PnPmSe or LnSePm) (Se is stearidonic acid, see also Abbreviations) or because of the lack of standards, which is particularly reflected in minority TAG. Identification of some individual regioisomers, i.e., TAG having fatty acyls in *sn*-2 and *sn*-1 (*sn*-3) positions presented no problem. To achieve a complete separation, it would be ideal to use on-line two dimensional LC, wherein in the first dimension the TAG would be separated using either NARP-LC or Ag-LC and in the second dimension by chiral-LC. With on-line chromatography the second dimension has to be accomplished in a very short time. In case of off-line 2D LC, the times do not play a very important part. Unfortunately, this combination has not yet been published. For example Dugo et al. [31], described on-line NARP-LC and Ag-LC. The mass spectra of enantiomers are demonstrated with the regioisomers of dipalmitoyl-eicosapentaenoin (Figs. 2S–4S).

Total ion current in the interval 200–900 Da was used for determining their percent proportions and diacylglycerol ions $[AA]^+$ and $[AB]^+$ for determining the contents of individual isomers. Unfortunately, in many cases the peaks were not separated and their abundance could not be determined because, for example, TAG of the *sn*-AAB and *sn*-BAA types give the same fragments, i.e., AA and AB

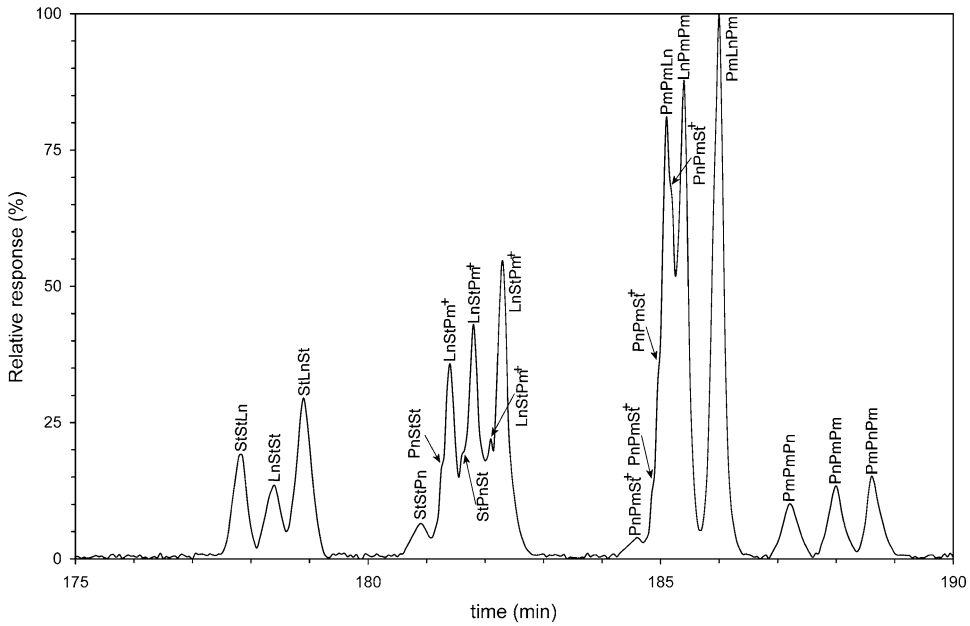

Fig. 4 Chiral LC of natural mixture of TAG, i.e., fraction having 11 double bonds after Ag-LC [17] obtained from snow alga. The content of FA (after transesterification to FAME) in this fraction was determined by GC–MS, i.e., Pn 3.8 %, Pm 47.2 %, Se 18.3 %, and Ln 30.7 %

ions. However, the identification of regioisomers, i.e., *sn*-ABA and *sn*-AAB (*sn*-BAA) was feasible because the emerging fragments have different abundances [28, 29].

Conclusion

The practical use of chiral LC with TAG is seen primarily in the analysis of structured TAG (STAG) important in the nutrition of both livestock and humans. Based on our previous experiments with targeted cultivation of algae [16] or by selecting suitable algae [32] one could prepare microbial oils with a predominance of one type of not only regioisomers but also enantiomers.

Synthesis of appropriate TAG or isolation of TAG from algae was used to obtain standards, especially C16 and C20 PUFA, i.e., acids given in Table 3, which were further separated and identified by chiral LC/MS-APCI. Their retention times were found to be in a direct relationship with the structure of given enantiomers and regioisomers of TAG, increasing with increasing number of double bond(s) and decreasing with increasing number of the carbon atoms in TAG, with the exception of dieicosapentaenoyl-palmitoyl-glycerols.

Unfortunately, the chiral analysis of TAG cannot be performed without the use of synthetic standards. For determination of the structure of individual enantiomers (but not regioisomers) it is necessary to chromatograph at least one of the two enantiomers, though in very similar structures one can infer the structure of the molecular species from retention time. Further, we chose as the most suitable method of synthesis the esterification of 2,3-isopropylidene-*sn*-glycerol based on chiral phospholipids, mainly because it gives excellent results in terms of stereospecificity.

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Conflict of interest The authors have declared that there is no conflict of interest.

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