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Enantiomeric separation of triacylglycerols containing very long chain fatty acids☆

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ABSTRACT

Enantiomers of triacylglycerols (TAGs) containing any combination of very long chain fatty acids (VLCFAs) and/or very long chain polyunsaturated fatty acids (VLCPUFAs) with diolein, dilinolein and didocosahexaenoin were synthesized. Gradient non-aqueous reversed-phase high-performance liquid chromatography/high resolution atmospheric pressure chemical ionization-tandem mass spectrometry (NARP-HPLC/HRMS^2-APCI) and chiral liquid chromatography were used for the separation and identification of molecular species of these TAGs. Further, NARP-LC and chiral LC were used to separate natural mixtures of TAGs obtained from four natural sources, i.e. ximenia oil (*Ximenia americana*), green alga (*Botryococcus braunii*), brewerís yeast (*Saccharomyces pastorianus*) and a dinoflagellate (*Amphidinium carterae*). The ratio of regioisomers and enantiomers in individual samples was determined and a hypothesis has been confirmed on the biosynthetic pathway of natural TAGs, which is based on the preferential representation of VLCFAs and VLCPUFAs in the *sn*-1 position of the glycerol backbone.

1. Introduction

Vegetable oils and animal fats consist predominantly of triacylglycerols (TAGs) which are esters of three fatty acids (FAs) with glycerol. Natural TAGs are present in living organisms as mixtures of different types of molecules, in many cases as stereoisomers, i.e. regioisomers or enantiomers. Natural TAG mixtures, whether of plant or animal origin, always contain tens, hundreds or even thousands of molecular species depending on the number of bound fatty acids (see Table S1). Milk fat [1] and oil from marine organisms [2] are considered to be the most complex mixtures of TAGs. The number of identified molecular species of TAGs reaches hundreds, e.g. 762 regioisomeric TAGs from krill oil [3] and 568 for human milk [1].

Since four enzymes are primarily involved in TAG biosynthesis, three of which are acyltransferases [4] which specifically esterify each of the glycerol hydroxyls, the resulting combinations are not random. So, for example, *sn*-16:0/18:1/16:0 and *sn*-16:0/16:0/18:1 (16:0 is palmitic and 18:1 oleic acid) are present in sunflower oil at a ratio of 100: 0, whereas in pork fat they occur in a ratio of 8:92 [5]. Physical properties of regioisomers such as *sn*-18:0/18:2/18:1 and *sn*-18:0/18:1/18:2 (where 18:0 is stearic and 18:2 is linoleic acid) vary considerably. Appropriate melting points of, for example, α crystalline forms are ∼−4 °C and ∼−13 °C [6] and the difference of 9 ° is due simply to a change in the positions of two FAs, i.e. is caused by changing the acids esterified to primary and secondary hydroxyl. Molecular structure of TAG is therefore of great importance in terms of nutritional, biochemical and technological aspects.

The number of carbon atoms in the acyl chains of natural TAGs is usually 16, 18, 20 or 22. This results from their biosynthesis, where the FAs in plants and animals are biosynthesized from acetyl-CoA (malonyl-CoA) and therefore contain even-numbered chains. Fatty acids can be classified, e.g., by carbon chain length and number of double bonds. In FAs called long-chain fatty acids (LCFAs), chain lengths range from 12 to 22 carbon atoms, of which LCFAs with 16 and 18 carbon atoms are the most common types of FAs found in nature. FAs that are longer than C22 [7] are called very long chain fatty acids (VLCFAs) and are less widespread than LCFAs. VLCFAs with chain lengths above 26 carbon atoms are often called ultra-long chain FA (ULCFA) [8] and are found from fungal spores to specialized mammalian tissues (the brain, retina, etc.). In the plant kingdom, they range from algae to higher (flowering) plants. The

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chain lengths are sometimes defined differently; see, for example, the definition by Poulos et al. [9] which defines VLCFAs as 24–28C fatty acids and ULCFAs as fatty acids having a chain of 30–38 carbon atoms. If they contain 2 or more double bonds, they are called polyunsaturated FAs, and in combination with differently long chains they are abbreviated as PUFAs, VLCPUFAs, etc. Unfortunately, with very few exceptions (sponges), their representation in organisms is very low, usually below 1% of all FAs.

One of the most common methods for separating natural TAGs is non-aqueous reverse phase liquid chromatography (NARP-LC). It was used to separate hundreds of TAGs [10–12], which were then identified by electrospray ionization mass spectrometry (ESI–MS) and/or atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). Stereospecific analysis of individual molecular species of TAGs is relatively difficult. Using APCI, the regioisomers of TAGs can be distinguished by the lower relative occurrence of the $[M+H\text{-}R_2COOH]^+$ fragment ion which results in a neutral loss of FA from the *sn-2* position [13–15]. If the TAG is asymmetric, i.e., if the two primary hydroxyl groups are esterified by different FAs, then the TAGs may be chiral, i.e. they may form two enantiomers, e.g., di-stearoolein (*sn*-18:0/18:1/18:1 and *sn*-18:1/18:1/18:0).

The separation of enantiomers of TAGs is most often carried out on a stationary phase based on cellulose tris(3,5-dimethylphenylcarbamate) or cellulose tris(3-chloro-4-methylphenylcarbamate) [16]. In the case of enantiomers, it is always necessary to obtain a standard of at least one of the two possible enantiomers for identification because the order of elution from the column is virtually impossible to predict. Only minor structural modifications of the stationary phase, e.g. substitution of methyl for Cl (cellulose tris(3,5-dimethylphenylcarbamate) versus cellulose tris(3-chloro-4-methylphenylcarbamate)), where methyl is a group of electron donating and the chlorine group is electron withdrawing and therefore the electron density on the phenyl group, has an effect on the elution of TAGs [17]. Examples of important chiral separations of TAGs have previously been published [18–22], and the type of columns, the mobile phase, the molecular weight range (in Da), including the origin of samples and references have been given, so here we mention only the most recent articles concerned with, e.g., the composition of regioisomers and enantiomers of TAGs in immature chicken egg yolk, mature chicken yolk, and chicken meat [23].

For the analysis of TAGs containing VLCFAs, the sources (organisms) already described were selected. In the case of higher, i.e. flowering plants, VLCFAs are present e.g. in the seeds of the genus *Tropaeolum* [24] or *Ximenia* [25–27]. *Ximenia americana* is a small sprawling tree of woodlands native to the tropics. The fruits are lemon-yellow or orange-red. In all cases, TAGs contain monoenoic FAs (erucic, i.e. 22:1ω-9, nervonic, i.e. 24:1ω-9, ximenic, i.e. 26:1ω-9 and 19-octacosenoic, i.e. 28:1ω-9 acids) as the major VLCFAs. For the next analysis, we used ximenia oil (*Ximenia americana*) [26].

Mitei et al. [25], who published the data on TAGs in ximenia oil obtained by direct inlet high resolution positive ESI, identified only 7 molecular species of TAGs. Since tandem MS was not used, the TAGs were characterized only by the carbon number: double bond(s) ratio, i.e. CN: DB, given as 64:3, 64:5, 62:2, etc. Based on their FAs analysis, however, it can be assumed that, for example, the 64:3 TAG contains two oleic (18:1ω-9) and one 19-octacosenoic (28:1ω-9) acids. Furthermore, the same authors, based on chemical shift (13C NMR) of ester carbon (about 170 ppm) found that saturated FAs were distributed only at the *sn*-1/3 position while *sn*-2 contains unsaturated FAs.

Further, yeasts, in particular the genus *Saccharomyces*, are the most widely used microorganisms in biotechnological production of beer and wine. Worldwide production of yeast biomass remaining after beer production is about half a million tons of dry matter. Already in 1973 Welch and Burlingame [28] described the presence of VLCFAs up to tetratriacontanoic acid in the yeast species *Saccharomyces cerevisiae*, but monounsaturated FA only up to 28:1 (octacosenoic acid) were identified. Other papers dealing with VLCFA in yeast and specifically *S. cerevisiae* have been published only sporadically [29,30]. After over 40 years, the yeast obtained from beer production by bottom fermentation was analyzed by reversed-phase LC–MS using positive APCI or ESI [31] and TAGs as well as phospholipids containing VLCFAs [32] were identified. In total, 22 TAGs containing at least one VLCFA [31] were identified.

The green alga of the genus *Botryococcus* is known to be used as a potential source of biofuel because, unlike other microorganisms, it produces long-chain hydrocarbons and, in addition, produces them extracellularly. It is understandable that if alkadienes and alkatrienes with odd carbon numbers – 23–31 – or botryococcenes containing 30–37 carbon atoms are produced, the alga must also produce VLCFAs, which are their biosynthetic precursors. Lipid analysis by LC–MS confirmed that this alga contain TAGs with saturated and monounsaturated VLCFAs [33].

The last microorganism to be analyzed was *Amphidinium carterae* from the phylum Dinoflagellata (Dinophyta or Dinozoa). These unicellular flagellates live in freshwater, brackish or marine environments. They are often mixotrophic organisms, and their cells are covered by a complex structure formed by membranes and flattened vesicles. A bloom of certain dinoflagellates can result in a visible coloration of the water colloquially known as red tide, which can cause shellfish poisoning. Dinoflagellates produce characteristic lipids, especially VLCPUFAs [34–38], but the contents and structures of TAGs and phospholipids have also been described [38,39].

One of the most serious problems in analyzing regioisomers and enantiomers is the very poor availability of commercially available TAGs. The first step is almost always the necessity to prepare the appropriate standards by organic synthesis. We describe here a method for separating and identifying regioisomers and enantiomers of TAGs that enable identification of molecular species containing VLCFAs and VLCPUFAs, respectively.

2. Experimental

2.1. Standards and instrumentation

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1/18:1-PC) and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2/18:2-PC), 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphocholine (22:6/22:6-PC), 6(*Z*),9(*Z*),12(*Z*),15(*Z*),18(*Z*),21(*Z*)-tetracosahexaenoic, 15(*Z*)-tetracosenoic, 17(*Z*)-hexacosenoic, tetracosanoic, and hexacosanoic acids were purchased from Larodan (Malmo, Sweden). Ximenia oil was purchased from Augustus Oils Ltd. (Hampshire, England). All other chemicals were purchased from Sigma-Aldrich (Prague, CR).

2.2. Cultivation of microorganisms

For analyses of TAGs from microorganisms, the following organisms were used: green alga (*Botryococcus braunii)* [40], brewerís yeast (*Saccharomyces pastorianus*) [31] and dinoflagellate (*Amphidinium carterae*) [33] which have been previously cultivated in the laboratory under controlled conditions.

2.3. Enzymatic synthesis of TAGs

1,2-diacyl-*sn*-glycero-3-phosphocholines (18:1/18:1-PC, 18:2/18:2-PC and/or 22:6/22:6-PC) were treated with phospholipase C (Type I from *Clostridium perfringens* (*C. welchii*)) as described by Christie [41]. In brief, phospholipase C (3 mg) in 0.5 M tris(hydroxymethyl)methylamine (tris) buffer (pH 7.5; 2 mL),

2×10^{-3} M in calcium chloride, was added to phosphatidylcholine (~15 mg) in diethyl ether (2 mL). The mixture was shaken at room temperature for 3 h and extracted three times with diethyl ether (4 mL portions). The ether layer was dried over anhydrous sodium sulfate before being evaporated in a stream of nitrogen at ambient temperature. Pure 1,2-diacyl-*sn*-glycerol was obtained by preparative TLC on a layer of silica gel G impregnated with boric acid (10%, w/w), with hexane-diethyl ether (50:50, v/v) as solvent system. The appropriate band was located under UV light and was eluted from the adsorbent with diethyl ether.

2.4. Preparation of TAGs having different FAs

The esterification of glycerol derivatives with acids was described previously [42,43]. Briefly, a solution of 1,2-diacyl-*sn*-glycerol (0.1 mmol) and VLCFAs (0.011 mmol) as a free acid in dichloromethane (1.0 mL) was added to 4-dimethylaminopyridine (DMAP) (6.1 mg, 50 μmol) and *N,Ní*-dicyclohexylcarbodiimide (DCC) (18.6 mg, 0.12 mmol) in dichloromethane (1.0 mL) under an inert atmosphere (argon). The resulting solution was stirred at room temperature for 15 h. The reaction was stopped by passing the reaction mixture in ether/dichloromethane (10:90) through a short column packed with silica gel. Solvent removal in vacuo afforded the pure product as yellowish oil.

2.5. Preparation and analysis of fatty acid methyl esters (FAMEs)

The total TAGs (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A fatty acid fraction obtained from the saponification was partitioned between alkali solution (pH 9) and diethyl-ether to remove basic and neutral components. The aqueous phase containing fatty acids was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using BF_3/MeOH (14% solution of BF_3 from Sigma-Aldrich). Gas chromatography-mass spectrometry of FAMEs was done on a GC–MS system consisting of Varian 450-GC (Varian BV, Middelburg, The Netherlands), Varian 240-MS ion trap detector with electron ionization (EI), and CombiPal autosampler (Chromtech – Analytical Instruments, Idstein, Germany) equipped with split/splitless injector. A DB-5MS column was used for separation (60 m, 0.25 mm ID, 0.25 μm film thickness). The temperature program started at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 160 °C at a rate of 25 °C min^{-1}. The second temperature ramp was up to 280 °C at a rate of 2.5 °C min^{-1}, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 3 scans s^{-1} under electron ionization at 70 eV, mass range *m/z* 50–500. FAMEs were identified according to their mass spectra [44,45] and using a mixture of chemical standards obtained from Sigma-Aldrich.

2.6. Lipid extraction and isolation of TAGs

Briefly, the biomasses of different microorganisms were separately extracted according to Bligh and Dyer [46] and extracts of total lipids were applied to Sep-Pak Cartridge Vac 35 cc (Waters, Prague, Czech Republic; with 10 g of aminopropylsilica-based polar bonded phase), and neutral lipids were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (7:3), the solvents were 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.

2.7. TAGs analysis by RP-HPLC/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 HIRPB – 250A 250 × 2.1 mm ID,

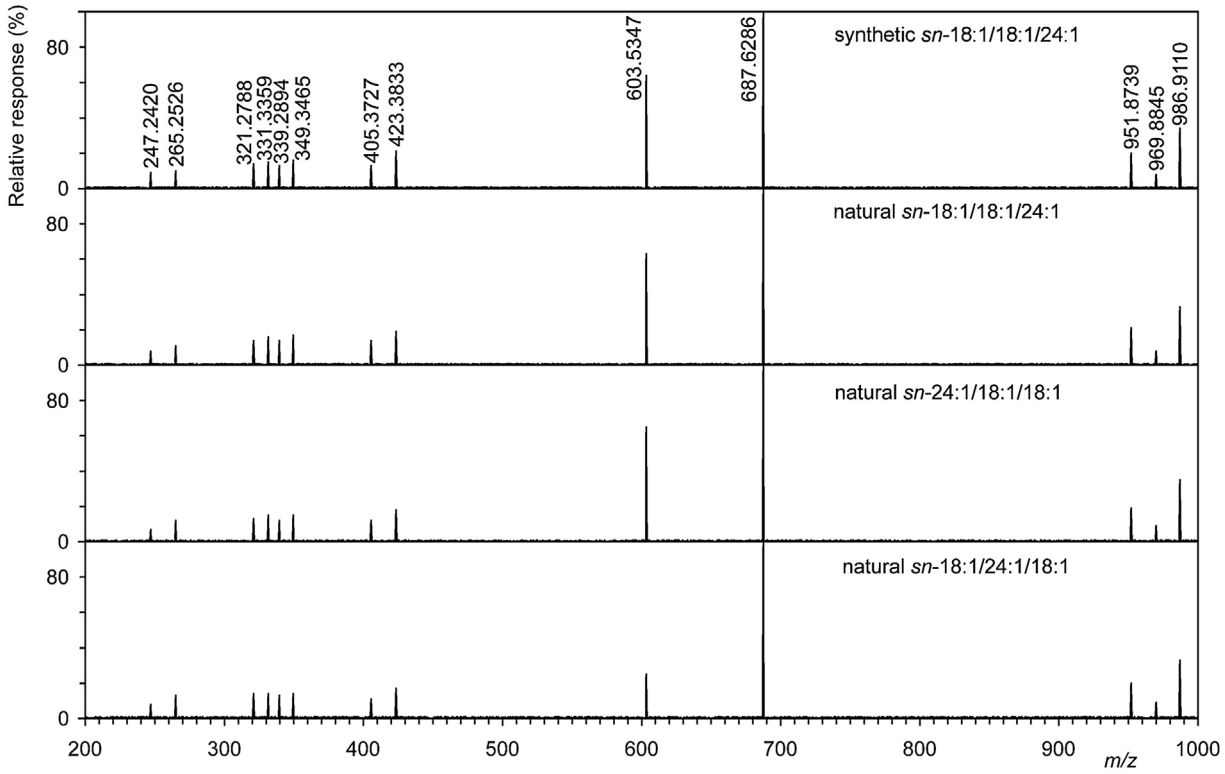

Fig. 1. Tandem mass spectra of the synthetic and natural regioisomer (*sn*-18:1/24:1/18:1) and enantiomers (*sn*-24:1/18:1/18:1 or *sn*-18:1/18:1/24:1). For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and Discussion.

5 μm particle size, in series. This setup was used as a high efficiency column – approximately 104,000 plates m^{-1}, with a flow rate of 1 mL min^{-1} and an injection volume of 10 μL, and column temperature of 25 °C. The TAGs 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 min to 250 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 high resolution hybrid mass spectrometer LTQ Orbitrap Velos (Thermo Scientific, FL, USA) was used. APCI–MS analysis was performed in the positive ion mode. MS spectra were obtained by the FT mode. MS spectra were acquired with target mass resolution of R = 30,000 at *m*/*z* 400 (lock mass 391.2842 Da (diisooctyl phtalate)). The ion spray voltage was set at +2500 V and the scan range of the instruments was set at *m*/*z* 200–1500. Spray current 4.0 μA, source temperature 350 °C, capillary temperature 300 °C. Nitrogen was used as a nebulizer gas set at 40 arbitrary units (sheath gas) and 10 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected by the high resolution FT mode. Further source conditions in the positive mode: vaporizer temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; tube lens voltage, 170 V.

2.8. TAGs 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. TAGs (~1 mg mL^{-1} in methanol) were chromatographed on two Astec cyclobond™ 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-Aldrich. 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 10 min, where A is hexane and B is hexane-2-propanol (99:2, v/v) mixture [18,19]. The flow rate, column temperature, and injection volume were 0.5 mL min^{-1}, 25 °C, and 10 μL, respectively. Other separation conditions were as used in the RP-LC, see above. The number of theoretical plates m^{-1} reached ~55 thousand (calculated using the synthetic standard (*sn*-18:1/18:1/24:1).

3. Results and discussion

3.1. Synthesis of TAG standards

There are basically three possibilities of obtaining the isomers of TAGs, including enantiomers. The first uses a randomization reaction of a mixture of two or three TAGs, each of which contains only one type of acyl, e.g. triolein and trinervonin. The resulting mixture is then a mixture of all regioisomers and enantiomers, i.e. 18:1/18:1/18:1, *sn*-18:1/18:1/24:1, *sn*-18:1/24:1/18:1, *sn*-24:1/18:1/18:1, *sn*-18:1/24:1/24:1, *sn*-24:1/18:1/24:1, *sn*-24:1/24:1/18:1, and 24:1/24:1/24:1. During randomization, which is basically a catalyzed inter- and intra-esterification, the starting fatty acyls, in the above-mentioned case oleoyl and nervonyl, are randomly distributed on the glycerol backbone. In the resulting mixture, however, the TAGs are represented equimolarly, without any preference of any regioisomers or enantiomers. Because the mass spectra of both enantiomers are identical, i.e., the mass spectrum of *sn*-24:1/18:1/18:1 is identical to the mass spectrum of *sn*-18:1/18:1/24:1, see Fig. 1, the method is unsuitable for identifying one of the enantiomers.

Another method, which is suitable for preparing a specific enantiomer, is a reaction using commercially available 1,2-isopropylidene-*sn*-glycerol and/or 2,3-isopropylidene-*sn*-glycerol, see Fig. S1. The free hydroxy group is esterified under mild conditions by the appropriate FA, the protecting group is removed by hydrolysis at low temperature and low pH and the two free hydroxy groups of the chiral monoacylglycerol are esterified with another acid to form the corresponding enantiomer. However, the method has several shortcomings [18,47]. Among the drawbacks is in particular the possibility of the fatty acyl migrating during the reaction. Optimum reaction conditions then give the desired enantiomer in a purity higher than 90% of the total molecular species of TAGs in 3 reaction stages, which reduces the yield of the respective TAG. This contrasts with randomization, where the yield of the reaction reaches almost 100%.

The third method employs commercially available chiral compounds, namely phosphatidylcholines. Fig. 2 shows the reaction

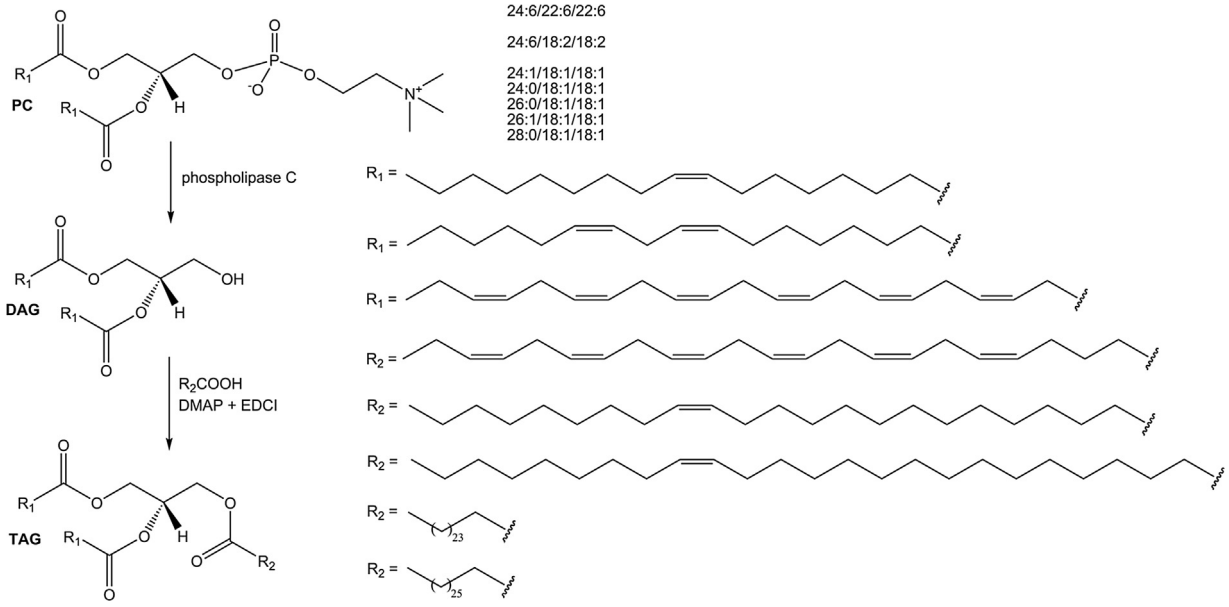

Fig. 2. Synthesis of enantiomers by stereospecific reactions.

Table 1
The ratio of enantiomers synthetized from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1/18:1-PC) and 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (18:2/18:2-PC), 1,2-didocosahexaenoyl-*sn*-glycero-3-phosphatidylcholine (22:6/22:6-PC); retention time (tR, min), and two diagnostic ions of type $[AA]^+$ and $[AB]^+$ including their abundances (%).

Enantiomer	Optical purity of the major enantiomer (%)	Retention time (min)	*m/z* of the diagnostic ion type $[AA]^+$ (abundance)	*m/z* of the diagnostic ion type $[AB]^+$ (abundance)
18:1/18:1/24:0	93	104.60	603.5347 (66)	689.6444 (100)
18:1/18:1/24:1	92	115.08	603.5346 (66)	687.6289 (100)
18:1/18:1/26:0	94	102.38	603.5348 (66)	717.6759 (100)
18:1/18:1/26:1	92	113.14	603.5348 (66)	715.6604 (100)
18:2/18:2/24:0	91	123.72	599.5032 (66)	687.6286 (100)
18:2/18:2/24:1	90	130.95	599.5034 (66)	685.6133 (100)
18:2/18:2/26:0	92	122.16	599.5033 (66)	715.6603 (100)
18:2/18:2/26:1	91	129.73	599.5034 (66)	713.6447 (100)
22:6/22:6/24:6	88	163.71	695.5038 (66)	723.5351 (100)

scheme. In the first step, chiral 1,2-diacyl-*sn*-glycerol is prepared using phospholipase C (commercially available); the next step performed under moderate reaction conditions, i.e. at room temperature and in the presence of DMAP (the catalyst) and DCC (an agent binding the water produced in the reaction) yields one of two possible enantiomers in an chiral purity greater than 90% of the total molecular species of TAGs. A total of 9 enantiomers were prepared in this way. Table 1 gives the basic data of each enantiomer, in particular the retention time of the respective enantiomer, its chiral purity after the organic synthesis, and the abundance ratio of the diagnostic ions determining the stereochemistry of the respective TAG.

3.2. Analysis of TAGs from natural oils on reverse-phase column

Analysis of ximenia oil on the C18 reverse phase column (see Fig. 3 and Table S3) confirmed the presence of TAGs with VLCFAs. Subsequent analysis using two serially coupled columns containing a chiral phase (3,5-dimethyl-phenyl-carbamate-modified β-cyclodextrin), see Fig. 4 and Table 2, confirmed the presence of enantiomers of individual TAGs. Although according to our own studies as well as the analyses performed by other authors [25,27] the ximenia oil contains only 11 FAs (Table 3), the number of theoretically possible TAGs reaches 1331 molecular species, including both regioisomers and enantiomers.

The results of analysis on reverse phase column of TAGs containing VLCFAs from green alga (*Botryococcus braunii*) and brewer's yeast (*Saccharomyces pastorianus*) were very similar to TAGs from ximenia oil due to the similar content of VLCFAs. In analyzing TAGs containing at least one VLCFAs in the molecule, see Table S3, certain anomalies appeared in retention times (tR) of TAGs. This is best documented especially for TAGs that have large chain length variations in the molecule, see below.

Furthermore, one can hardly rely on the fact that it is possible to predict the order of elution from the column based on ECN values. We believe that the main cause is the spatial arrangement of very long chains in the TAGs molecule, which partly corresponds to the TAGs model, see Fig. S4. We can far more rely on the value of ACN: DB, since the decreasing number of DBs while maintaining ACN increases without any exception tR. In addition, some anomalies in tR were observed in some TAGs, e.g. 24:1/18:1/20:1, i.e. TAG having a small scattering in acyl lengths has tR of 117.7 min, whereas TAG with the same number of carbon atoms and double bonds (26:1/18:1/18:1) but with the difference eight methylenes between the shortest and the longest acyls, had a tR greater by 7.7 min. The same effect was seen in TAGs containing palmitic (16:0) or palmitoleic (16:1) acids in the molecule. This phenomenon is very well observed on a pair of TAGs, e.g. 24:0/16:1/16:0 having ECN-56 and TAG (30:1/18:2/18:2), where ECN equals 56, but tR differs by more than 20 min. In conclusion, to rely on identifying TAGs according

Table 2
Chiral LC/MS-APCI+ of the TAGs from the ximenia oil, retention time (tR, min) and relative quantities (%) of molecular species (including enantiomers) of TAGs. (**bold** – synthesized enantiomers).

Peak No.	*sn*-TAG	tR (min)	%
1	26:0/18:1/18:1	100.30	31.3
2	18:1/26:0/18:1	100.50	19.0
3	24:0/18:1/18:1	102.15	6.6
4	**18:1/18:1/26:0**	102.40	4.5
5	18:1/24:0/18:1	102.60	2.5
6	**18:1/18:1/24:0**	104.60	3.3
7	30:1/18:1/18:1	108.85	8.0
8	18:1/30:1/18:1	109.40	2.0
9	28:1/18:1/18:1	110.20	11.0
10	18:1/18:1/30:1	110.45	7.9
11	18:1/28:1/18:1	110.90	4.0
12	26:1/18:1/18:1	111.55	56.4
13	18:1/18:1/28:1	111.70	40.0
14	26:0/18:2/18:1	111.90	23.0
15	18:1/26:0/18:2	112.60	3.6
16	18:1/26:1/18:1	112.70	3.3
17	24:1/18:1/18:1	113.30	29.1
18	**18:1/18:1/26:1**	**113.45**	22.7
19	24:0/18:2/18:1	113.55	19.0
20	18:1/18:2/26:0	113.85	4.0
21	18:1/24:0/18:2	114.50	3.3
22	18:1/24:1/18:1	114.70	2.9
23	**18:1/18:1/24:1**	115.10	3.3
24	18:1/18:2/24:0	115.75	3.4
25	28:1/18:2/18:1	116.60	100.0
26	18:2/28:1/18:1	116.85	24.0
27	26:1/18:2/18:1	117.90	40.8
28	18:1/28:1/18:2	118.10	22.9
29	18:2/26:1/18:1	118.80	5.3
30	18:1/26:1/18:2	119.05	4.0
31	24:1/18:2/18:1	119.35	18.5
32	26:0/18:2/18:2	120.35	19.7
33	18:1/18:2/24:1	120.55	13.8
34	18:2/24:1/18:1	121.35	1.6
35	18:2/26:0/18:2	121.65	2.2
36	24:0/18:2/18:2	121.95	3.0
37	**18:2/18:2/26:0**	122.10	2.7
38	18:2/24:0/18:2	123.30	2.3
39	**18:2/18:2/24:0**	123.70	2.4
40	30:1/18:2/18:2	126.70	16.3
41	28:1/18:2/18:2	127.75	63.2
42	18:2/30:1/18:2	128.50	3.4
43	18:2/18:2/30:1	128.65	3.8
44	26:1/18:2/18:2	128.85	29.7
45	18:2/28:1/18:2	129.60	2.9
46	18:2/18:2/28:1	130.00	6.3
47	24:1/18:2/18:2	130.20	12.8
48	18:2/26:1/18:2	131.00	2.0
49	**18:2/18:2/26:1**	**132.00**	2.9
50	18:2/24:1/18:2	132.90	2.9
51	**18:2/18:2/24:1**	**134.40**	1.7

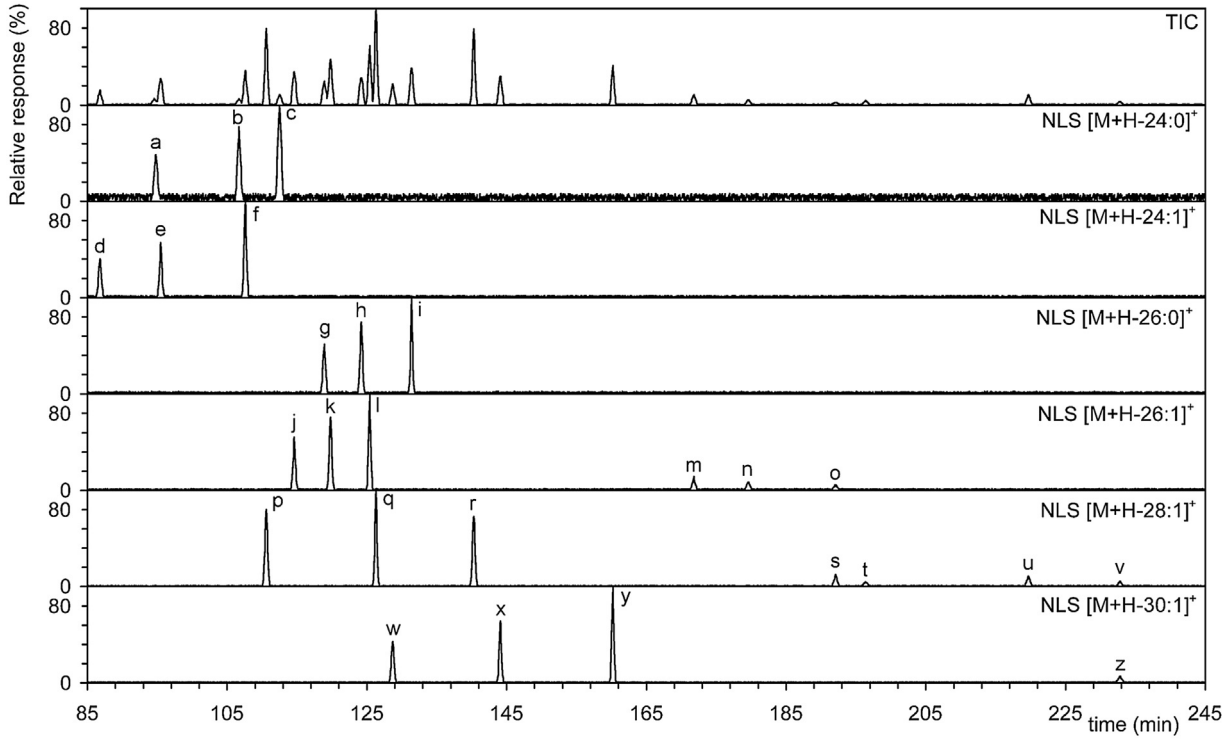

Fig. 3. Reversed phase liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (RP-LC/APCI-MS) chromatogram from 85 to 245 min of total ion current (TIC) of the TAGs from the ximenia oil, and five NLS – $[M+H-24:1]^+$, $[M+H-26:0]^+$, $[M+H-26:1]^+$, $[M+H-28:1]^+$, and $[M+H-30:1]^+$. Identification of the peaks, see Table S3.

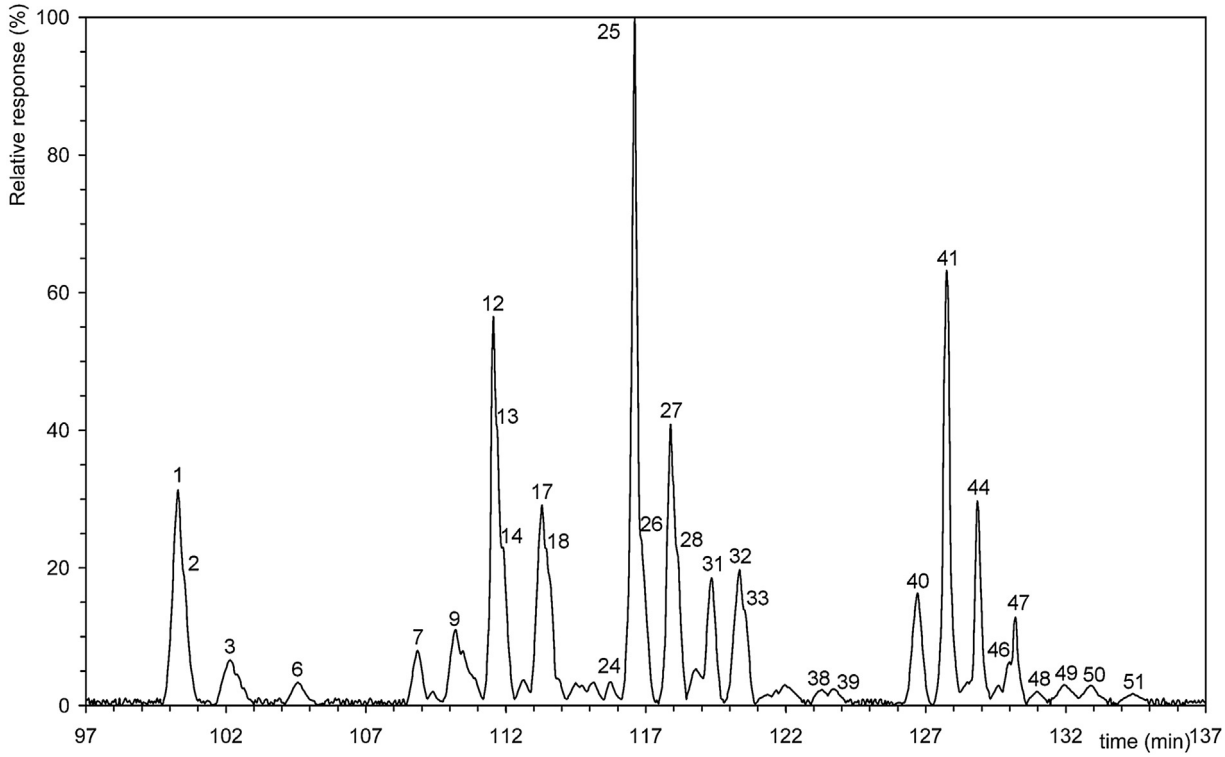

Fig. 4. The separation of enantiomers in natural mixtures of TAGs obtained from *Ximenia* oil. Identification of the peaks, see Table 2.

to the values commonly used, i.e. ACN or ECN is very unreliable, and basically the only method that is suitable for identification is LC–MS. Elution order is determined not only by the equivalent carbon number (ECN) (which is defined as ECN=CN – (2 x DB) where CN is the number of carbon atoms and DB is the number of double bonds), but also by the structure of the TAG. ECN can be calculated much more accurately when considering the location of double bonds. If n1, n2 and n3 are the numbers of double bonds in oleic

Table 3
Fatty acid composition (% of total FA), determined by GC–MS of FAME from ximenia oil (*Ximenia americana*).

Fatty acid	%
16:1	1.9
18:2	23.2
18:1	35.3
18:0	2.8
22:1	2.5
24:1	5.1
24:0	1.3
26:1	7.4
26:0	4.2
28:1	10.5
30:1	5.8

acid, linoleic acid and linolenic acid, then ECN can be calculated according to the formula: ECN=CN − d1n1 − d2n2 − d3n3. The coefficients d1 to d3 can be obtained using the TAG standards; the values of 2.60, 2.35, and 2.17, respectively, are given. The formula then looks like this: ECN=CN − (2.60n1) − (2.35n2) − (2.17n3)[48]. For understandable reasons discussed below, a comparison of tR from a sample of TAGs with tR of TAG standards cannot be used.

The *Amphidinium carterae* was also analyzed. The content of TAGs which contained VLCPUFAs was obviously minor but still measurable, see Table S1. The major TAGs, containing VLCPUFAs, unfortunately, in the order of tenths of a percent of total TAGs, was 22:6/28:8/28:8 while the TAG 28:7/28:8/28:8 containing the longest and the most unsaturated VPLPUFAs was represented in an amount only per mile of total TAGs. The TAGs that we identified, i.e. TAGs containing at least two C28 acids together with C14-C22 PUFAs, are the longest and most unsaturated TAGs isolated from a natural source. According to our current knowledge they have not yet been obtained by organic synthesis.

3.3. Chiral chromatography

Stereospecific analysis of TAGs on a chiral phase column comprises three consecutive steps. The first is to choose a suitable natural oil (fat) that contains VLCFAs or VLCPUFAs. The second step is the synthesis of relevant standards, and the third step is actual analysis. Although both VLCFAs and VLCPUFAs are fairly well spread in nature, the content of these acids in TAGs is often very low; usually well below 1% of total FAs. Another, non-negligible step in the analysis is the synthesis of the respective enantiomers; it should be noted that no companies, not even specialized ones selling lipid standards, sell the enantiomers of any TAG. For analysis, we used both the synthesized TAGs enantiomers (see above) and four natural mixtures of TAGs containing VLCFAs or VLCPUFAs.

TAGs analysis was performed using the atmospheric pressure chemical ionization mass spectrometry (APCI-MS) and molecular weights were determined based on both adducts of NH_4 molecules and ion fragments in mass spectra using tandem MS (MS^2). All regioisomers and enantiomers provide the same APCI mass spectra, differing only in the case regioisomers the intensity of ions of the neutral loss type of $R_xCOOH + NH_3$ from $[M + NH_4]^+$. This fact is based on the phenomenon that has been described many times, viz. that the neutral loss of fatty acid from the *sn*-2 position is less advantageous (more energy-consuming) than the loss of the primary TAG hydroxyls [13,14]. In the case of regioisomers, see also Fig. 1 and Table S2, i.e. *sn*-24:1/18:1/18:1, it can be seen that ions of the $[18:1/18:1]^+$ type and those of the $[24:1/18:1]^+$ type have different abundances depending on the initial TAG. In the case of the asymmetric TAG, i.e. two enantiomers (*sn*-24:1/18:1/18:1 or *sn*-18:1/18:1/24:1), the ratio is 66:100, whereas for the symmetric TAG *sn*-18:1/24:1/18:1 the ratio of $[18:1/18:1]^+$ versus $[24:1/18:1]^+$ ion is 25:100. For the enantiomers, i.e. *sn*-24:1/18:1/18:1 versus *sn*-18:1/18:1/24:1, it cannot be distinguished by tandem MS which enantiomer is involved [18–20,33].

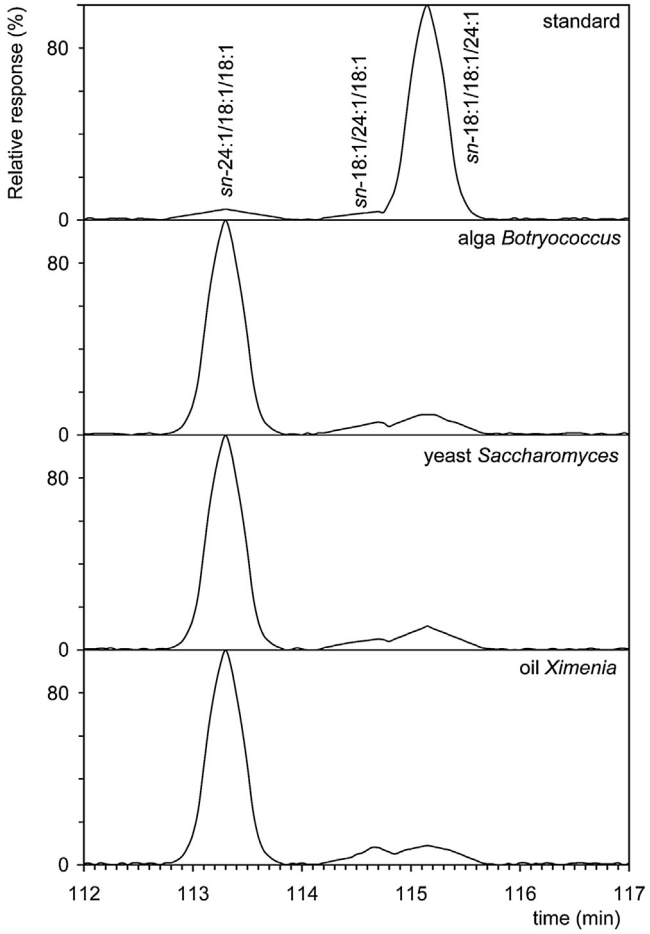

Fig. 5. VLCFAs preferences at the *sn*-1 position (nervonic acid) at the glycerol backbone. Three natural samples, i.e. alga, yeast and ximenia oil, including synthetic standard were analyzed on chiral column.

3.4. Retention characteristics of TAGs on a chiral column

A TAGs-separating column filled with 3,5-dimethyl-phenylcarbamate-modified β-cyclodextrin was used for chiral analysis as described previously [18–20,22,32]. Separation was carried out in the normal phase by gradient elution with the hexane-2-propanol mixture. The analysis has been optimized as previously described. Two chiral columns had to be connected in series for the successful separation of both the synthetically prepared standards, see above, and mainly for the separation of natural mixtures. Unfortunately, due to this connection, the retention time of TAGs was prolonged for between 2 and 3 h, see also [47].

In accordance with previously published data on TAGs containing acyls with an even number of carbon atoms, i.e. C16 to C20 fatty acids, the retention time increases with increasing polarity of the TAGs. Increasing polarity is caused by two factors, the first being an increasing number of double bonds in the molecule, and the other is the length of the hydrocarbon chain. Briefly, cerotyl-diolein (26:0/18:1/18:1) has a lower retention time than lignoceryl-diolein (24:0/18:1/18:1), see Table 2. The cerotyl-diolein chain is two CH_2 groups longer than lignoceryl-diolein. On the other hand, nervonyl diolein (24:1/18:1/18:1) has a higher retention time than lignoceryl-diolein due to the fact that nervonyl diolein contains three double bonds, in contrast to lignoceryl-diolein which contains only two.

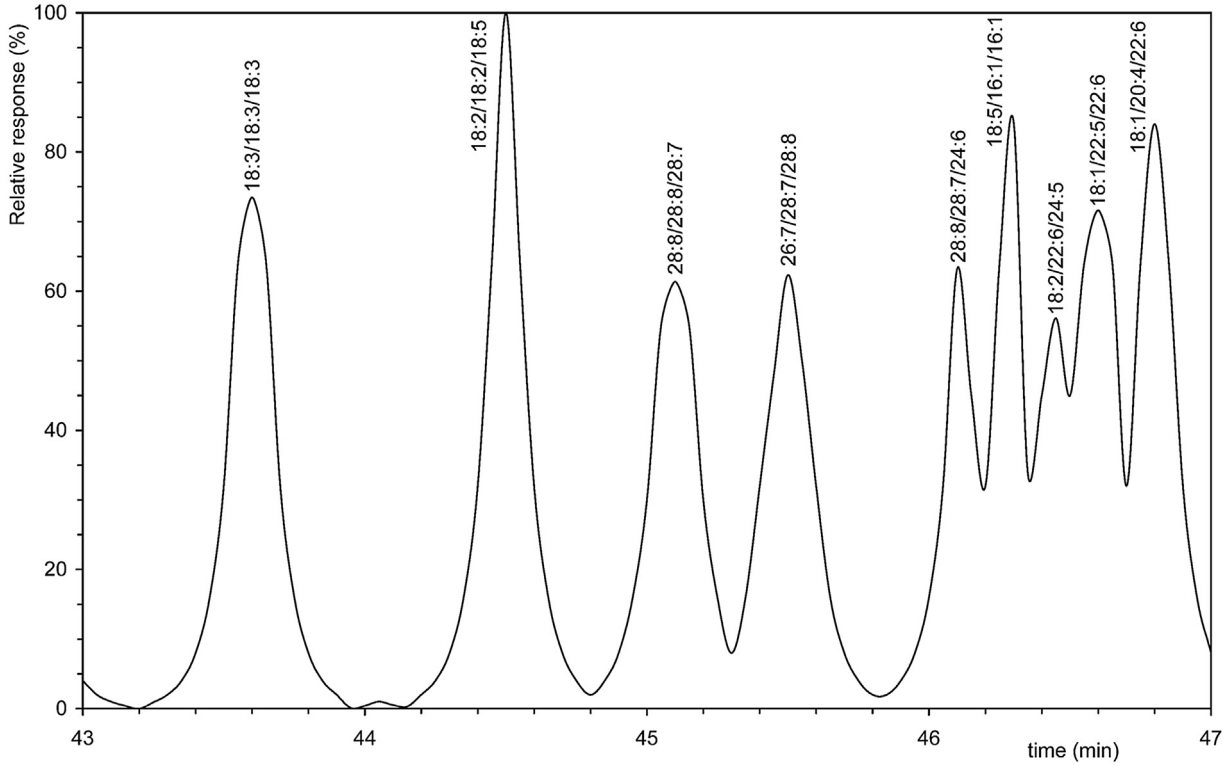

Fig. 6. Reversed phase liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (RP-LC/APCI-MS) chromatogram of total ion current (TIC) of the TAGs from the dinoflagellate *Amphidinium carterae*.

Predictions of retention times and thus molecular species of TAGs including enantiomers can be made based on the values shown in Figs. S2 and S3 . In the case of dioleins, the retention time decreases with increasing chain length. For instance, the sum of the acyl lengths of ximenyl-diolein is higher than that of nervonyl-diolein, and the retention time difference corresponding to the two methylene groups (-CH_2—CH_2-) is ~1.6 min. For more details see Table 2 and Fig. S2. A similar situation arises with the other enantiomers and also symmetric TAGs (regioisomers). Conversely, a change in acyl unsaturation, i.e. the replacement of lignoceric acid (24:0) by nervonic acid (24:1) that increases the number of double bonds by one bond, results in an ~11 min increase in retention time. Similar values, were found in acyls-dilinoleins, see Fig. S3.

The importance of using standards was clearly documented in the analysis of TAGs differing in the number of double bonds. While in the case of nervonyl-dioleins the order of elution was enantiomer – symmetric TAG – enantiomer (*sn*-24:1/18:1/18:1, *sn*-18:1/24:1/18:1, *sn*-18:1/18:1/24:1), the order of elution in the case of nervonyl-dilinoleins (*sn*-24:1/18:2/18:2, *sn*-18:2/18:2/24:1, *sn*-18:2/24:1/18:2,) was enantiomer – enantiomer – symmetric TAG. The same swapping of the retention times has been pointed out by Lisa and Holcapek [47], who found in stearyl-dioleins the elution order *sn*-18:0/18:1/18:1 < *sn*-18:1/18:0/18:1 < *sn*-18:1/18: 1/18:0, whereas the order for stearyl –dilinoleins was *sn*-18:0/18:2/18:2 < *sn*-18:2/18:2/18:0 < *sn*-18:2/18:0/18:2. The unusual chromatographic behavior of some molecular species of TAGs is probably due to a combination of different interactions of the analyte (spatial arrangement) with the chiral stationary phase, in which essentially two opposing mechanisms come into play – interaction with the non-aqueous reversed phase and interaction with the normal phase additionally enhanced by chiral interactions.

All enantiomers were at least partially separated, see Fig. 4. Unfortunately, in many cases the separation was not sufficient despite the serial connection of two columns, although the number of theoretical plates m^{-1} reached ~ 55 thousand (calculated using the synthetic standard (*sn*-18:1/18:1/24:1). However, on the basis of the synthesis of 9 enantiomers, see Table 1, all three organisms, i.e. small tree *Ximenia americana*, green alga *Botryococcus braunii* and brewer's yeast *Saccharomyces pastorianus* biosynthesize TAGs in which VLCFAs are preferably esterified at the *sn*-1 position of the glycerol backbone. This is the first direct evidence that eukaryotic organisms prefer this position in the biosynthesis (Fig. 5).

So far, only analyses of TAG mixtures have been performed, mostly using lipases, see, e.g. Christie [41]. Using pancreatic lipase it has been found that *Tropaeolum speciosum* seed oil lacks erucic, nervonic and ximenic acids in *sn*-2, so these acids must be present in the *sn*-1 or *sn*-3. To our knowledge, a method of directly determining the position of VLCFAs on a glycerol backbone, e.g. by means of a series of reactions (partial hydrolysis using Grignard reagents, conversion to phosphatidylcholines, phospholipase A2 hydrolysis, separation of products, i.e. monoacyl-, diacylphosphatidylcholines and free fatty acids, and determination of fatty acid methyl esters after derivatization by GC–MS) in natural fats and oils containing VLCFAs has not yet been performed.

Because the composition of dinoflagellate fatty acids and TAG is completely different from all three of the above-mentioned organisms, another enantiomer (*sn*-22:6/22:6/24:6) was synthesized which is structurally much more similar to TAGs in the previously found dinoflagellates. One of the biggest problems that need to be addressed before our analysis is the inability to purchase another VLCPUFAs than 24:6ω-3. Although Mansour et al. [34] published an article on the preparation of VLCPUFA from dinoflagellates, these acids cannot be commercially obtained. Therefore, a single TAG enantiomer was synthesized, viz. *sn*-22:6/22:6/24:6. It should also be noted that TAGs containing saturated and monoenoic VLCFAs were not identified in dinoflagellates. Although analysis of the dinoflagellate TAG by NARP-LC/MS-APCI$^+$ on a reverse-phase col-

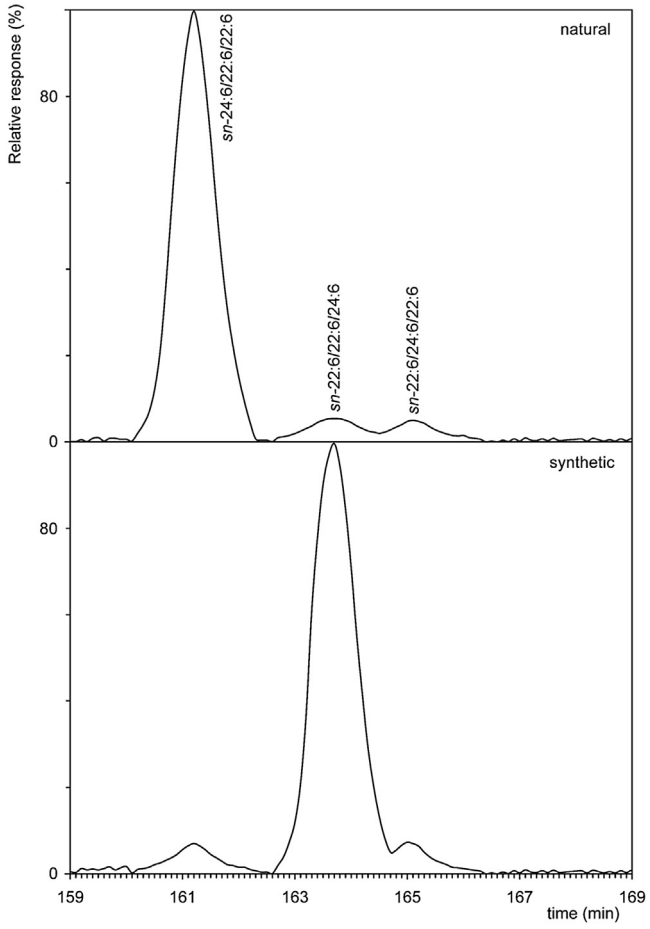

Fig. 7. Chiral separation of two enantiomers (*sn*-24:6/22:6/22:6 or *sn*-22:6/22:6/24:6) and a regioisomer (*sn*-22:6/24:6/22:6), both synthetically prepared and natural (from a dinoflagellate).

umn was performed, see Fig. 6, and Rezanka et al. [33], analysis of the same sample on a chiral column was completely unsuccessful. Therefore, a method that was time and material demanding was chosen to manually collect the respective molecular species of TAGs eluted from the reverse phase and their further separation on the chiral column. Three TAGs were obtained; see Figs. 7–9 which illustrates the separations of individual enantiomers and regioisomers.

The two enantiomers and the regioisomer of the synthetically prepared TAG (*sn*-24:6/22:6/22:6 or *sn*-22:6/22:6/24:6 and *sn*-22:6/24:6/22:6) were separated without problems (Fig. 7) and identified by tandem MS. Again, the tandem mass spectra of the two enantiomers are identical, regioisomer 22:6/24:6/22:6 differs only by the abundance of ions at *m*/*z* 723.5352 (neutral loss of *sn*-1/3 RCOOH + NH_3 from $[M + NH_4]^+$) and ion at *m*/*z* 695.5034 (neutral loss of *sn*-2 RCOOH + NH_3 from $[M + NH_4]^+$).

Fig. 8, which shows a separation of 26:7/22:6/22:6 TAG, documents the fact that a homolog having one double bond and two methylene groups more than a synthesized standard is again separated into three molecular species, i.e. two enantiomers (*sn*-26:7/22:6/22:6 and *sn*-22:6/22:6/26:7) and a symmetric TAG (*sn*-22:6/26:7/22:6). In both TAGs containing VLCPUFAs, an enantiomer having a VLCPUFA in the *sn*-1 position was found to be the major one. This result has been confirmed by the fact that natural TAGs, if they contain VLCPUFAs, are preferably bound to the glycerol backbone in the *sn*-1 position.

Unfortunately, the separation of the *sn*-28:8/28:8/28:7 and *sn*-28:7/28:8/28:8 enantiomers, see Fig. 9, has not been successful.

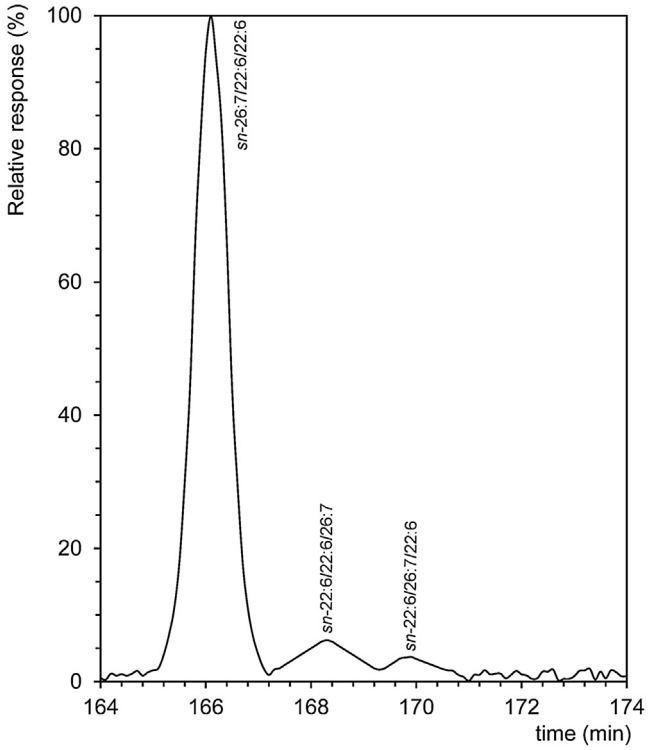

Fig. 8. Chiral separation of natural TAG, i.e. two enantiomers (*sn*-26:7/22:6/22:6 and *sn*-22:6/22:6/26:7) and a regioisomer (*sn*-22:6/26:7/22:6).

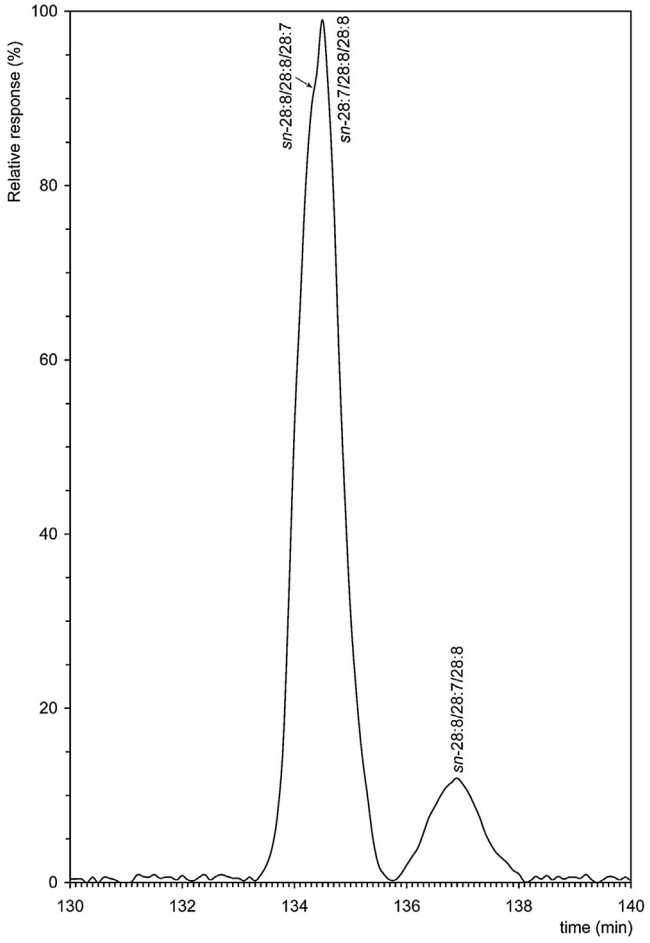

Fig. 9. Chiral separation of natural TAG, i.e. two enantiomers (*sn*-28:8/28:8/28:7 and *sn*-28:7/28:8/28:8) and a regioisomer (*sn*-28:8/28:7/28:8).

As can be seen from Fig. S4, where a model of this TAG is depicted, three polyunsaturated acyls with seven or eight cis (*E*) double bonds completely enclose the chiral center on the glycerol skeleton. With the use of two chiral columns connected in series, the enantiomers were not separated from each other and were merely separated from the regioisomer (*sn*-28:8/28:8/28:7 and *sn*-28:7/28:8/28:8 versus *sn*- 28:8/28:7/28:8).

4. Conclusion

We analyzed VLCPUFAs of total lipids of four organisms. Tandem MS, and simultaneously neutral loss scan and product ion scan were used to identify more than 150 molecular species of TAGs and it was strongly suggested that VLCPUFAs being always esterified at primary hydroxyl of glycerol backbone. In other words, VLCPUFAs are always at the *sn*-1(3) position in TAGs, as we found on reverse phase liquid chromatography. Based on these results, it can be assumed that all organisms examined from protozoa, yeasts and green algae to higher (flowering) plants biosynthesize such TAGs that contain VLCFAs and/or VLCPUFAs in *sn*-1 position. Based on the above analysis, mainly on positive high resolution tandem ESI, we identified dozens molecular species of TAGs containing VLCFAs and/or VLCPUFAs. The methods allowed us to expand the number of molecular species of triacylglycerols containing VLCPUFAs. These methods are of course not limited to dinoflagellates, but can in the future be used for other organisms including higher plants.

For example, a total of 23 TAGs were identified in *B. braunii* by chiral chromatography, slightly fewer than the 28 peaks determined by NARP-LC. Although the number of TAGs determined by chiral chromatography is lower, this method makes it possible to separate and identify some enantiomers, which is not possible with NARP-LC. The lower number of TAGs is mainly due to the fact that one TAG is separated into up to three molecular species (two enantiomers and a symmetric TAG, see, e.g., Fig. 5). One of the options, though very time and material demanding, is the separation of TAGs by NARP-LC, collection of individual peaks and their subsequent separation by chiral chromatography. This method was used in the dinoflagellate *Amphidinium carterae*. Of course, the best solution would be 2D LC using NARP-LC in the first dimension and chiral LC in the second. However, as far as we know, nobody has so far used 2D LC of this type, not even for the separation of standards.

The chromatographic results on the separation of the enantiomers of four natural mixtures of TAGs were supplemented by the synthesis of 9 enantiomers with an chiral purity greater than 90%, including an enantiomer having 18 double bonds. Furthermore, the hypothesis that individual enzymes in the biosynthetic pathway of natural TAGs, and especially acyl-CoA: *sn*-glycerol-3-phosphate acyltransferase (EC: 2.3.1.15) are specific for very long chain polyunsaturated acyls. This fact, at least to our current knowledge, has not yet been confirmed experimentally with TAGs. The results suggesting this fact have been published only with phospholipids, biosynthetic precursors of TAGs.

Conflict of interest disclosure

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.04.064.

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