Lipids (2015) 50:811–820
DOI 10.1007/s11745-015-4042-8

METHODS

Separation and Identification of Odd Chain Triacylglycerols of the Protozoan *Khawkinea quartana* and the Mold *Mortierella alpina* Using LC–MS

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Received: 5 March 2015 / Accepted: 17 June 2015 / Published online: 30 June 2015

Abstract Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC–MS/APCI) with reversed- and chiral phases was used for separation of triacylglycerols (TAG) from protozoan and mold. This study describes the separation and identification of odd numbered chains of regioisomers and enantiomers of triacylglycerols from different natural sources, i.e., the protozoan *Khawkinea quartana* and the mold *Mortierella alpina*. Using the above-mentioned separation methods and the synthesis of appropriate standards of TAG, we identified regioisomers and enantiomers of both even and odd numbered TAG. The biosynthesis of odd numbered TAG was found to be strictly stereospecific and to depend on the production microorganism, one enantiomer predominating in the protozoan and the other in the mold. It was proved that even numbered TAG are synthesized in a higher optical purity, which can be explained by a higher affinity of acyltransferases to the respective substrate, i.e., to even chain PUFA.

Keywords Protozoan · *Khawkinea quartana* · Mold · *Mortierella alpina* · Structured triacylglycerols · Chiral-LC/APCI-MS

Electronic supplementary material The online version of this article (doi:10.1007/s11745-015-4042-8) contains supplementary material, which is available to authorized users.

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Abbreviations

ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
CCF	Culture Collection of Fungi, Faculty of Science, Charles University, Prague
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)
iPrOH	2-Propanol
L	Linoleic acid
LC–MS/APCI	Liquid chromatography-mass spectrometry/atmospheric pressure chemical ionization
Ln	Linolenic acid
M	Myristic acid
Ma	Margaric acid
MEA	Malt extract agar
Mo	Margaroleic acid
MS^2	Tandem mass spectrometry
NARP-LC	Non aqueous reversed phase-liquid chromatography
NLS	Neutral loss scan
O	Oleic acid
ORD	Optical rotatory dispersion
P	Palmitic acid
PUFA	Polyunsaturated fatty acid(s)
S	Stearic acid
SAG	Culture Collection of Algae at the University of Gottingen, Germany
TAG	Triacylglycerol(s)
TIC	Total ion current

Introduction

Odd numbered fatty acids (odd FA) with straight chain are widespread in nature, but their quantitative content mostly does not exceed 1 % of the total fatty acids. They are found from bacteria to mammals, including humans [1–3]. In fact, in-depth analysis of each natural oil and fat can be said to identify odd FA, but their content is mostly in the order of several thousandths of the total FA amount. There are of course exceptions; while the analysis of odd FA is very well developed and documented, with a few exceptions the analysis of native TAG containing odd FA has not been published. We tried to obtain odd TAG (i.e., TAG having at least one odd FA in the molecule) from both natural sources and using organic synthesis, and analyzed these TAG by the nonaqueous reversed-phase high performance liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (NARP-HPLC/APCI-MS) and by chiral HPLC in the case of certain enantiomers.

The milk of mammals as well as milk products (cheese, yogurt, etc.) contains odd FA. These arise either by the biosynthesis performed by symbiotic bacteria living in the digestive tract but also by the *de novo* biosynthesis in the mammary gland [4]. Analysis of butterfat containing margaric (Ma) or margaroleic (Mo) acids using normal phase-HPLC/ESI–MS has been published, with hundreds of TAG being identified, among them dozens of odd TAG, e.g., OMaM, OMMa, PMoP, PPoMa, OPMa, OMaP, SMoP, etc. [5].

Holcapek *et al*. [6] analyzed eleven vegetable oils by NARP-HPLC/MS-APCI and found nearly 100 TAG. In each of the oils were identified odd TAG containing at least one odd FA, i.e., Ma or Mo, the concentrations of which in the TAG did not exceed 1 % of the total TAG. No TAG having 2 or 3 odd FA in the molecule were found.

Odd TAG containing Ma and Mo, e.g., LLMa, OOMo, OLMa, etc. were also identified in wild and cultivar Tunisian peanut kernels [7].

Conversely, both acids, Ma and Mo, plus pentadecanoic acid were significantly represented in studies dealing with odd chain TAG in the bacterium *Rhodococcus* [8, 9]. The authors even identified a TAG (MoMoO) having two odd FA in the molecule; this TAG is therefore even-numbered and is present in an amount of up to 2.8 % of the total TAG.

Quite a different situation is with TAG having odd FA with 2 or more double bonds in the molecule, i.e., polyunsaturated odd fatty acids (odd PUFA). We did not succeed in finding a single article dealing with the chromatography of these TAG and only a few publications describing odd FA with two or more double bonds in TAG.

It was shown that the marine opisthobranch *Scaphander lignarius* contained unusual PUFA such as several ω3 fatty acids and the ω7 acid—heneicosa-5,8,11,14-tetraenoic acid (21:4ω7) [10] and the activity of the isolated compounds against a range of human cancer cell lines (melanoma, colon carcinoma and breast carcinoma) was reported.

Several papers, e.g., Chang *et al*. [11, 12] address odd PUFA in thraustochytrids, heterotrophic protists inhabiting largely marine habitats. The genera *Schizochytrium* and *Thraustochytrium* were found to contain odd PUFA in an unusually high qualitative (from 19:1 to 19:4 and from 21:1 to 21:6) and quantitative amount (21:4ω7 constitutes 7.2 % of total FA) which is similar to that of the even-C20 and C22 PUFA.

The surface marine zooplankton contains tenths of percent of 21:5ω3, which is one of the most common odd chain PUFA [13]. A study of the fatty acid compositions and the positional distributions of fatty acids in TAG of four freshwater fish species (*Salmo trutta*, *Ictalurus punctatus*, *Ictalurus melas* and *Micropterus salmoides*) showed that, unlike the even PUFA, the 21:5 acid is not preferred in any of the three possible positions (*sn*-1 or *sn*-2 or *sn*-3) of the TAG [14].

The above data show that the number of sources of odd PUFA is very limited, basically to less common autotrophic microorganisms.

Another way to obtain odd chain PUFA from natural material is to use the ability of some fungi of the genera *Saprolegnia* or *Mortierella* to utilize odd precursors, e.g., pentadecane or pentadecanoic acid, as major carbon sources in the biosynthesis of PUFA [15, 16]. After the cultivation, the fungal mycelia were found to contain odd PUFA, especially 17:3ω5, 19:3ω5, 19:4ω5 and 19:5ω5.

The number of TAG molecular species rapidly increases with the number of fatty acids contained in the TAG. When considering both regioisomers and the enantiomers, if a TAG contains ten FA, then the number of theoretically possible TAG is 10^3, i.e., 1000 molecular species. One of the most common methods for separating TAG is NARP-LC and the identification is performed using APCI or ESI. On the basis of the relative abundances of ions $[M + H–R_2COOH]^+$ arising by neutral loss of FA from *sn*-2 position one can identify, after separation, regioisomers [17] but not enantiomers. These must first be separated by chiral LC and identified on the basis of compliance with the retention time of at least one standard. Most commonly used for chiral LC is a polysaccharide-based chiral stationary phase, e.g., cellulose-tris-3,5-dimethylphenylcarbamate [18, 19].

In this paper we describe the identification of regioisomers and enantiomers of odd-chain triacylglycerols, i.e., TAG having in the molecule at least one PUFA containing an odd number of carbon atoms, i.e., 15,17,19 and 21 carbon atoms, which were obtained by culturing the colorless

biflagellate *Khawkinea quartana* (Euglenophyceae, Euglenophyta) as well as the mold *Mortierella alpina* (Mortierellaceae, Glomeromycetes) growing on hydrocarbons (pentadecane and heptadecane). NARP-LC and chiral-LC was used for separation and tandem mass spectrometry (MS^2) for identification. The combination of LC–MS thus allowed the separation and identification of odd TAG. Although these TAG are present in nature in minor amounts in terms of quantity, they are widespread. Furthermore, their proportion can be considerably increased by genetic manipulation or a change in microorganism cultivation conditions and in this case it is suitable to have a method of analysis such as that described in this article. To our knowledge, analysis odd TAG molecules having the odd PUFA in the molecule has not yet been published.

Materials and Methods

Chemical and Standards

Acetonitrile, 2-propanol, THF (tetrahydrofuran), hexane, dichloromethane, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), and phospholipase C from *Clostridium perfringens* (*C. welchii*) Type I, lyophilized powder, 10–50 units/mg protein were purchased from Sigma-Aldrich (Prague, CR). Trilinoleoylglycerol, trilinolenoylglycerol, dilinoleoyl-linolenoylglycerol (racemic mixture of *sn*-LLLn or *sn*-LnLL and LLnL), all-*Z*-10,13,16-nonadecatrienoic (19:3ω3), all-*Z*-6,9,12,15-heneicosatetraenoic (21:4ω6), and all-*Z*-6,9,12,15,18-heneicosapentaenoic (21:5ω3) acids were purchased from Larodan Fine Chemicals, Malmö, Sweden. 1,2-Dilinoleoyl-sn-glycero-3-phosphocholine was purchased from Avanti Polar Lipids, Inc., AL, USA. The all-*Z*-4,7,10,13-nonadecatetraenoic acid (19:4ω6) was formerly supplied by Angene International Limited (Hong Kong, People's Republic of China).

Cultivation of Protozoan

The *Khawkinea quartana* 1204-20a was obtained from SAG, Germany (www.epsag.uni-goettingen.de). It was cultivated in 1-L glass bottles in ErbsE medium 55 days at RT with no agitation. Soil Water Medium with Pea (ErbsE medium) was prepared according to Pringsheim [20] as follows: garden pea soaked 12 h before (1/8 of the final volume) was placed in the bottom of the bottle, 2 cm of garden soil was added, filled up with distilled water till the bottle was ¾ full, autoclaved. Vitamins B1 (250 µg/L) and B12 (10 µg/L) were added. The biomass was harvested after 55 days, centrifuged at 4000 rpm for 3 min, frozen at −80 °C and lyophilized.

Cultivation of Molds

Three strains of the genus *Mortierella*, *M. alpina* CCF 2873, *M. humilis* CCF 1627, and *M. elongata* 2633 were purchased from the Culture Collection of Fungi (CCF) (Department of Botany, Faculty of Science, Charles University, Prague, Czech Republic). The strains were stored at 4 °C on a solid medium (malt extract agar, MEA), fresh subcultures on MEA were prepared by microfungal inoculation one week before their use in the experiment (7 d, MEA, 25 °C).

Mycelial biomass was prepared by cultivating the *Mortierella* strains under aerobic conditions in 100 mL of liquid nutrient medium containing 1 % yeast extract and 0.5 or 1 % glucose. The medium was sterilized at 121 °C for 20 min before inoculation and 1 mL of pentadecane or heptadecane was added after inoculation. Incubation carried out at 25 °C for 10 days on a rotary shaker (3.4 Hz). The biomass was harvested by centrifugation at 5000 rpm for 5 min, frozen at −80 °C and lyophilized.

Enzymatic Synthesis of 1,2-Dilinoleoyl-3-Odd Chain FA-*sn*-Glycerols

1,2-Dilinoleoyl-*sn*-glycero-3-phosphocholine was treated with phospholipase C (Type I from *C. perfringens* (*C. welchii*)) as described by Christie [21]. In brief, phospholipase C from *C. welchii* (3 mg) in 0.5 M tris(hydroxymethyl) methylamine (tris) buffer (pH 7.5; 2 mL), containing 2×10^{-3} M 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-dilinoleoyl-*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 [21].

The esterification of 1,2-dilinoleoyl-*sn*-glycerol with acids was described previously [22, 23]. Briefly, a solution of 1,2-dilinoleoyl-*sn*-glycerol (~6 µM) and all- *Z*-7,10,13-nonadecatrienoic acid (19:3ω6), 6,9,12,15-heneicosatetraenoic acid (21:4ω6), and 6,9,12,15,18-heneicosapentaenoic acid (21:5ω3) (~10 µM) as a free acid in dichloromethane (1.0 mL) was added to DMAP (6.1 mg, 5 µM) and EDCI (18.6 mg, 1.2 µM) 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 products as a yellowish oil. The yields of the target *sn*-LL19:3 (Fig. 1S), *sn*-LL19:4 (Fig. 2S), *sn*-LL20:4 (Fig. 3S), and *sn*-LL20:5 (Fig. 4S) were 3.8, 4.1, 3.9 and 3.7 mg, respectively.

Isolation of TAG

The extraction procedure was based on the method of Bligh and Dyer [24]. The alcohol-water mixture was cooled and one part chloroform was added and the lipids from the lyophilized cells of different cultivations of protozoan and molds 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.

First, total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35 cc (Waters; with 10 g of silica normal-phase), and TAG were subsequently eluted from the cartridge with 40 mL of chloroform–methanol (9:1, v/v). The eluate was evaporated and the oil samples were dissolved in an acetonitrile-2-propanol-hexane mixture (1:1:1, v/v/v), which was injected for HPLC analysis.

FAME Analysis

The total TAG (~5mg) 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 FA fraction was methylated using BF_3/MeOH [25, 26].

GC/MS of FAME was done on a Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization, and a CombiPal autosampler (CTC, USA). Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increased at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. The structures of FAME were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAME (Supelco, Prague).

Picolinyl Esters Analysis

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate FAME (~0.5 mg) in dry dichloromethane (1 mL) were added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated [27].

A FA picolinyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C/min to 180 °C and at 2 °C/min to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m*/*z* 70–650.

RP-HPLC/MS-APCI

LC–MS contained HP1090 series system (Agilent, USA) connected to an MS system Navigator (Finnigan MAT, USA). All conditions were the same [8, 9], except the linear gradient, wherein the elution was extended from 120 to 150 min. Detailed conditions are listed in the supplementary material.

TAG Analysis by Chiral LC/MS-APCI

The LC system used for separation in the chiral mode was the same as previously described [28]. Briefly, the Astec cyclobond™ I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin) chiral LC column was used. Other separation conditions were as used in the RP-LC, see "Electronic supplementary material".

Results and Discussion

NARP-LC/MS-APCI of Synthetic Standards

When separating TAG using NARP-LC, the order of their elution depends on the length of the acyl chains and the number of double bonds present in the molecule. Hundreds of TAG were separated in this way. If the TAG contains in the molecule two or three different FA, then its molecule can form regioisomers or possibly enantiomers. Regioisomers, in which FA form positional isomers on glycerol backbone, can be readily separated from each other, e.g., *sn*-PPO from *sn*-POP [29]. Symmetrical TAG (e.g., *sn*-POP) are usually eluted before the asymmetric ones (*sn*-PPO). Furthermore, it is possible to identify them on the basis of the abundance of ions of the type $[M + H–R_2COOH]^+$, in which ion losing FA from the *sn*-2 is minor in the spectrum. Based on the ratio of ion abundances of $[M + H–R_{1(3)}COOH]^+$ and $[M + H–R_2COOH]^+$, this makes it possible to determine which acid is esterified to

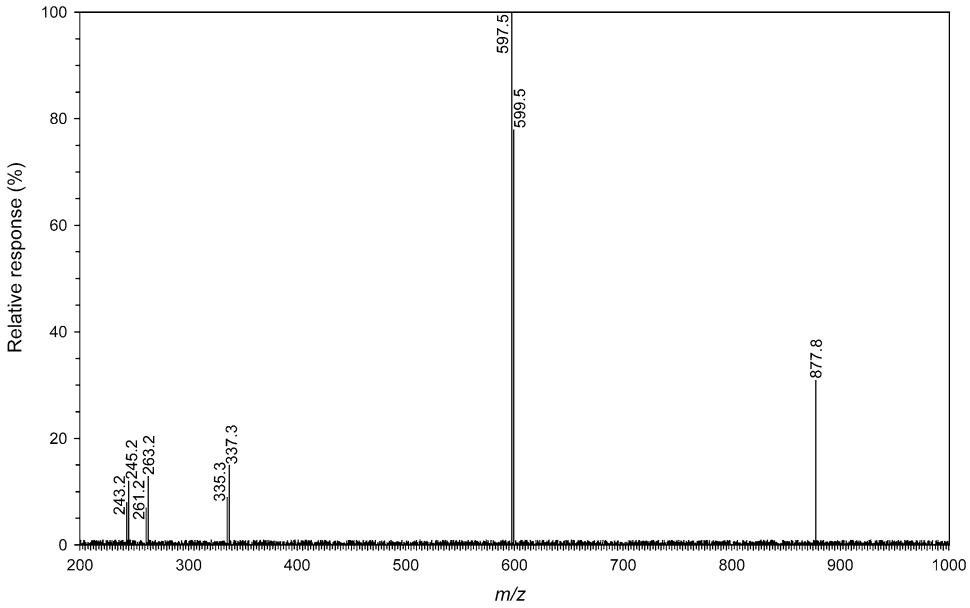

Fig. 1 Tandem mass spectrum of synthetic 1,2-dilinoleoyl-3-linolenoyl-*sn*-glycerol (*sn*-LLLn)

the primary and which to the secondary alcohol group. This fact is best illustrated by Fig. 1, which shows mass spectrum of a synthetic standard. An example is the MS^2 spectrum of the first synthesized TAG, i.e., *sn*-LLLn, in which were identified the following ions *m/z* 243.2 ($[R_3CO\text{-}18]^+$), 245.2 ($[R_1CO\text{-}18]^+$), 261.2 ($[R_3CO]^+$), 263.2 ($[R_1CO]^+$), 335.3 ($[R_3COO + 58]^+$), 337.3 ($[R_1COO + 58]^+$), 597.5 ($[M + H–R_1COOH]^+$), 599.5 ($[M + H–R_3COOH]^+$), 877.8 ($[M + H]^+$). Diagnostic ions include a pair at *m/z* 597.5 and 599.5. The less abundant ion, at 599.5 $[LL]^+$ arises by loss of linolenic acid from the molecule, whereas the more abundant ion at *m/z* 597.5, basically the sum of ions $[L_1Ln_3]^+$ and $[L_2Ln_3]^+$, is formed by loss of linoleic acid. The ratio of ions $[LL]^+$ to $[LLn]^+$ is 78:100, which clearly shows the structure of *sn*-LLLn. Other TAG, whether natural or synthetic, have been identified in a similar manner.

Chiral-LC of Synthetic Standards

Chiral LC separations of synthetic TAG containing two linoleic acids per molecule is shown in Fig. 2. The TAG were prepared by previously described methodology, i.e., from commercially available 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine by hydrolysis with phospholipase C and subsequent esterification of the free hydroxyl in position 3 on the glycerol backbone by a corresponding PUFA. This yielded a series of homologues of 1,2-dilinoleoyl-3-acylglycerols, see Table 1 and Fig. 2. Figure 2 also shows selected-ion monitoring chromatograms (SIM) of four commercially available standards, where the LLL and LnLnLn were

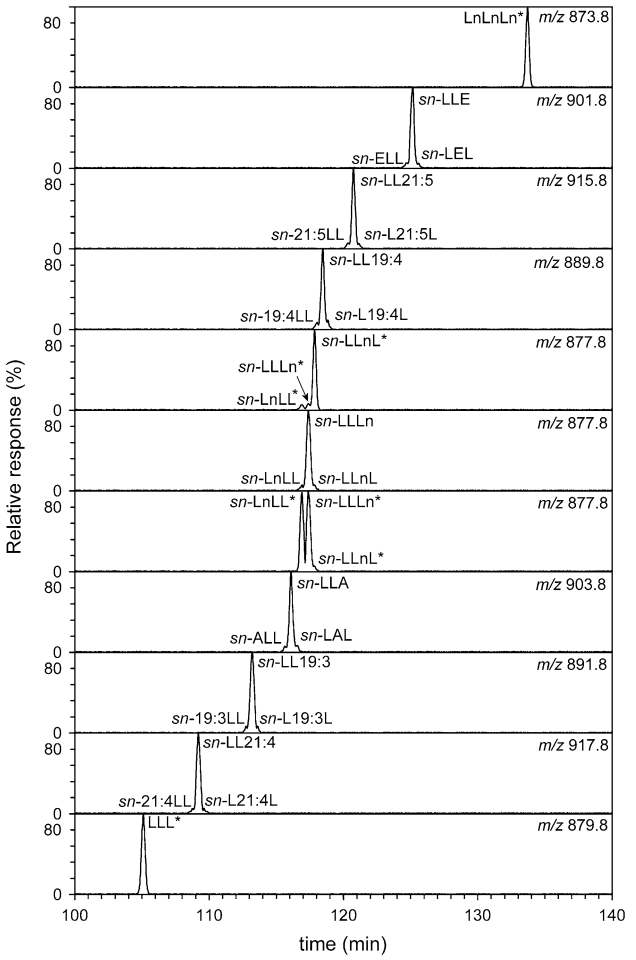

Fig. 2 Chiral LC of synthetic standards, for abbreviations see Table 1

Table 1 The ratio of enantiomers and regioisomers synthetized from *sn*-1,2- dilinoleoylglycerol and fatty acids

RT	TAG	%[a]	ACN	DB	ECN
105.1	LLL	100	54	6	42
108.9	*sn*-21:4LL	4	57	8	41
109.2	*sn*-LL21:4	91	57	8	41
109.5	*sn*-L21:4L	5	57	8	41
112.9	*sn*-19:3LL	5	55	7	41
113.1	*sn*-LL19:3	90	55	7	41
113.4	*sn*-L19:3L	5	55	7	41
115.8	*sn*-ALL	5	56	8	40
116.1	*sn*-LLA	87	56	8	40
116.4	*sn*-LAL	8	56	8	40
116.9	*sn*-LnLL	47	54	7	40
117.3	*sn*-LLLn	46	54	7	40
118.0	*sn*-LLnL	6	54	7	40
116.9	*sn*-LnLL	5	54	7	40
117.3	*sn*-LLLn	91	54	7	40
118.0	*sn*-LLnL	4	54	7	40
116.9	*sn*-LnLL	4	54	7	40
117.3	*sn*-LLLn	5	54	7	40
118.0	*sn*-LLnL	91	54	7	40
118.0	*sn*-19:4LL	7	55	8	39
118.4	*sn*-LL19:4	86	55	8	39
118.8	*sn*-L19:4L	8	55	8	39
120.4	*sn*-21:5LL	4	57	9	39
120.7	*sn*-LL21:5	91	57	9	39
121.0	*sn*-L21:5L	5	57	9	39
124.8	*sn*-ELL	5	56	9	38
125.1	*sn*-LLE	91	56	9	38
125.5	*sn*-LEL	4	56	9	38
133.8	LnLnLn	100	54	9	36

RT retention time (min), *ACN* acyl carbon number, *DB* double bonds, *ECN* equivalent carbon number

[a] Percentages are calculated from the MS chromatogram

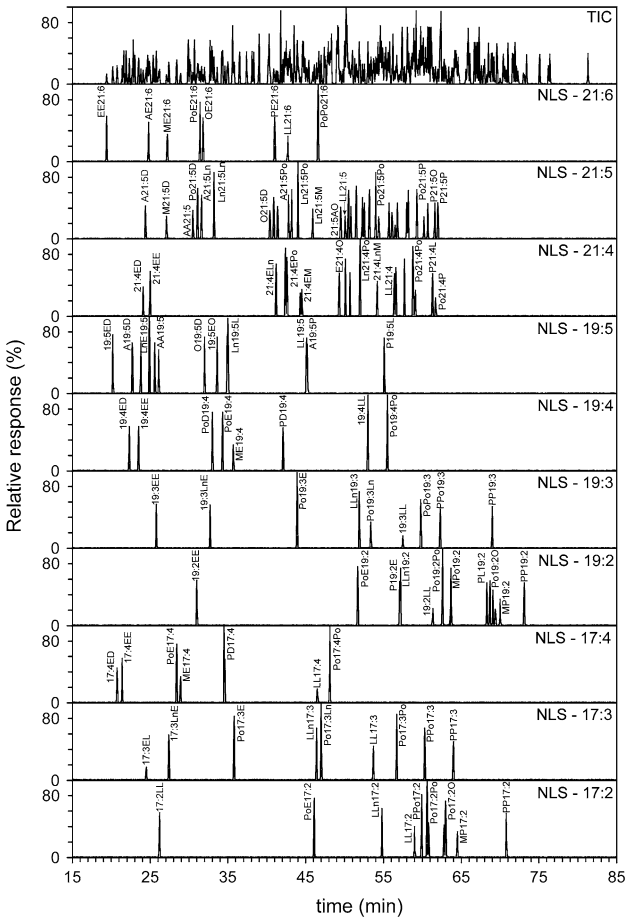

Fig. 3 RP-LC/APCI-MS chromatogram total ion current (TIC) of the TAG from the protozoan *K. quartana.* TAG containing odd-chain fatty acids as revealed by the neutral loss scan for $[M + H]^+$—the appropriate odd FA. In the abbreviations, e.g., EE, AE, or PoE, E stands for eicosapentaenoic, A arachidonic and Po palmitoleic acids, which implies that, for example. EE21:6 is a triacylglycerol containing two eicosapentaenoic acids and one heneicosahexaenoic acid

measured only as reference samples. Two molecular species, i.e., dilinoleoyl-linolenoylglycerol (LLLn and/or LnLL) show a clear and predictable behavior. Commercially available LLLn is a mixture of two enantiomers, i.e., *sn*-LLLn and *sn*-LnLL in the 1:1 ratio corresponding to given data. Interesting is the minor proportion of the symmetric regioisomer (LLnL) generated probably during hydrolysis by phospholipase C wherein the small of amount of 1,3-linoleyl-*sn*-glycerol is formed. The same holds for the symmetric regioisomer (LLnL) which is composed of both enantiomers in an amount of about 5 %. All *sn*-3-acyl-1,2-dilinoleines that we prepared exhibit high stereospecific purity. The content of the desired enantiomer is around 90 % of the total sum of TAG, see Table 1 and Fig. 2. Based on previously published work [28, 30], we again used the column packed with chiral oligosaccharide, i.e., β-cyclodextrin modified by tris-3,5-dimethylphenylcarbamate. TAG retention time then depends on the number of double bonds in fatty acids bound to the glycerol backbone. It increases with an increasing number of double bonds in the acyl chains and, conversely, decreases with increasing length of the acyl chains. If the TAG is composed of three different fatty acids, then it may be composed of up to six molecular species. Their identification essentially depends on the availability of standards (individual enantiomers), which cannot always be separated from each other [18, 28], so we did not deal with this issue here.

We observed that the order of elution of either odd or even TAG depends on the esterification of respective FA on the glycerol backbone. If we have two regioisomers, i.e., two TAG of the type *sn*-AAB (*sn*-BAA), wherein A and B are FA, then always the first eluted from the column is

asymmetric TAG (*sn*-AAB or *sn*-BAA) followed by the symmetric TAG (*sn*-ABA). If an enantiomer is esterified by more unsaturated PUFA in position *sn*-1, then the first eluted is *sn*-LnLL followed by *sn*-LLLn. However, this order cannot be generalized since a minor modification of the stationary phase, i.e., substitution of methyl groups for chlorine in the 3-position (i.e., 3,5-dimethyl-phenyl-carbamate *versus* 3-chloro-4-methyl-phenyl-carbamate) reverses the order of elution. This is explained by the fact that chlorine is electron-withdrawing [31].

The above rule holds only for a given combination of the stationary and mobile phase and cannot be generalized. However, what remains is a very difficult and challenging task, i.e., to synthesize at least one enantiomer for each pair of enantiomers. As already described, the mass spectra of both enantiomers are almost identical and it is not possible to decide on their basis which is the enantiomer in question. The same applies to the optical properties such as optical rotation or ORD (optical rotatory dispersion), as their values approach zero. For instance, for 1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol the $[a]_D^{20} = 0.00$ (c 7.05, $CHCl_3$) [32].

NARP-LC/MS-APCI of Natural Samples

From *K. quartana* and *M. alpina* (cultivated on pentadecane and heptadecane) were isolated total lipids by standard methods, see "Materials and Methods". The lipids from the protozoan were then used to separate TAG, which were further chromatographed by NARP-LC (Fig. 3). We identified all TAG having an abundance higher than 0.05 % of total TAG.

The methyl esters of fatty acids (FAME) of TAG from all cultivations were analyzed by GC–MS. The acids were identified and the positions of double bonds in PUFA were determined in their picolinyl esters. Mass spectra of two odd picolinyl esters of PUFA are shown in (Fig. 5S and 6S). The spectra clearly show several gaps which unambiguously confirm the structure of the 5,8,11,14,17-nonadecapentaenoic acid (19:5ω2). The picolinyl ester exhibits a series of five gaps of 26 Da between *m/z* 178–204, 218–244, 258–284, 298–324, and 338–364, which fully confirms the above structure, see also the structural formula given in (Fig. 5S). The latter acid, i.e., an all-*Z*-4,7,10,13,16-heneicosapentaenoic acid (21:5ω5), see (Fig. 6S), was identified in a similar manner.

MS^2 then identified a total of 266 molecular species of TAG. Although this number seems high in comparison with published data [33], which usually report on fewer than a hundred TAG, it is negligible when compared with the theoretical number of possible TAG. Table 1S (Supplements) shows that the number of FAME identified and quantified in *K. quartana* is 34, which means 34^3 possible TAG and more than 39,000 possible molecular species.

The NLS (neutral-loss scan) method used for easier identification odd TAG greatly simplified further identification, see also Fig. 3. This figure shows the proportion of individual TAG in *K. quartana*, i.e., a microorganism producing odd FA, based on total ion current (TIC) and NLS of odd TAG.

Tandem MS and neutral loss fragments were employed to identify corresponding odd TAG and determine their structure, including the number of carbon atoms and number of double bonds. The NLS revealed that some peaks were inseparable by LC, although the two columns in series had a total of about 26,000 plates/250 mm theoretical plates. The peaks contained up to three isobaric molecular species that were further separated as regioisomers or enantiomers.

The major odd TAG identified in the protozoan *K. quartana* were primarily those that contained 21:5 acid, i.e., a total of 35 molecular species, see Table 1S. Minor TAG were those containing 19:4 and 21:6 acids (8 molecular species each) and 17:4 acid (7 molecular species). Altogether almost half (126 of 265) of all TAG were minor odd TAG. None of the natural TAG contained two odd FA and were therefore not even-chain ones. None of them also contained three PUFA.

Similarly, the extraction of *M. alpina* cultured on both hydrocarbons (pentadecane and heptadecane) yielded a mixture of TAG containing odd FAME. Figure 4 shows a mixture of TAG separated after cultivation on pentadecane. The difference between the cultivation of *M. alpina* on pentadecane and heptadecane is not striking, though the cells cultivated on heptadecane do not practically contain 15:0 and 15:1 acids. The other two tested strains, i.e., *M. elongata* and *M. humilis* essentially lack biotransformation ability (data not shown), which is in line with published data [15], wherein also the species *M. alpina* appeared to be the best biotransformer.

In essence, the cultivation of *M. alpina* on hydrocarbons yielded a mixture of odd and even TAG enriched with C17 and slightly less with C19 PUFA. Pentadecenoic acid arising from pentadecane was not further desaturated to PUFA, so that only 15:0 and 15:1 acids were identified. Analysis of these TAG containing pentadecanoic and pentadecenoic acids has been previously described [8, 9] in detail and we therefore have not dealt with it in this article. We were unable to isolate C21 or longer odd PUFA, which is consistent with previously published data [15]. The mold elongase probably cannot extend the C19 chain to C21 acid chain. For this reason, even C22 PUFA commonly occurring in nature have not been described in the *M. alpina*.

The rich variety of chain lengths and unsaturation of TAG in the *K. quartana* is caused mainly by the fact that this genus, in accordance with the published data [34],

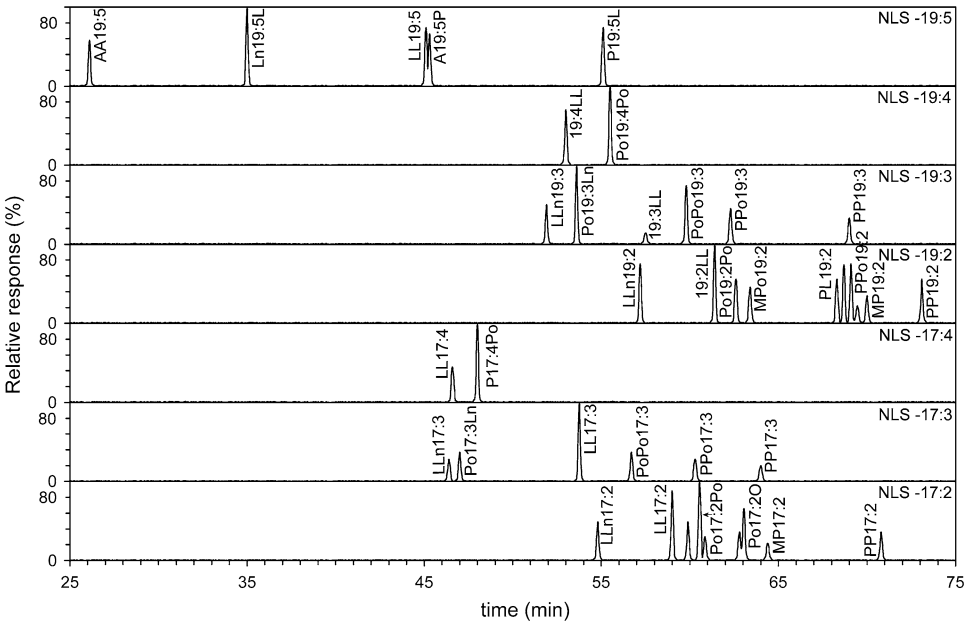

Fig. 4 RP-LC/APCI-MS chromatogram neutral loss scans of the TAG from the mold *M. alpina*

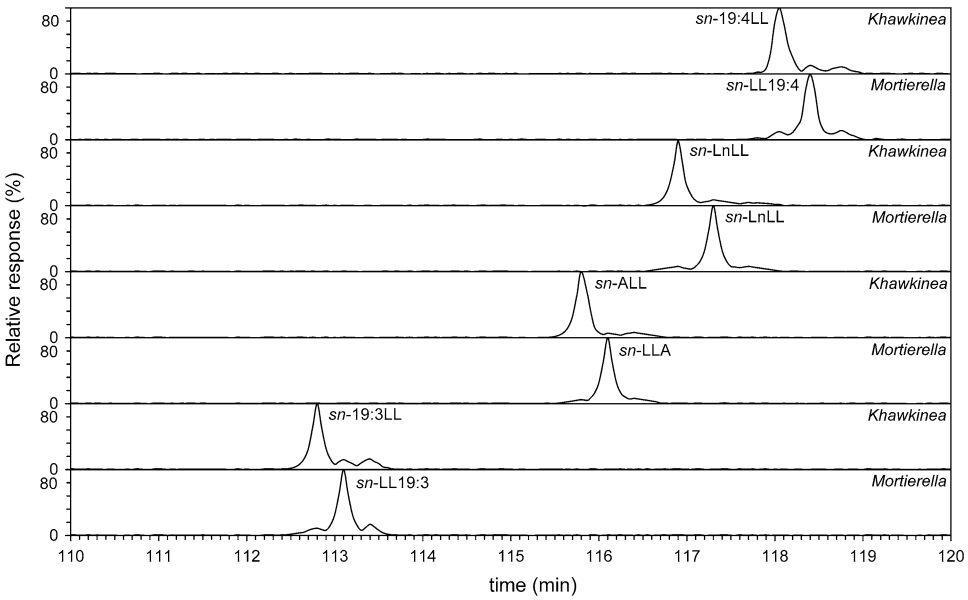

Fig. 5 The enantiomeric purity of biosynthesized TAG from protozoan *K. Quartana* and the mold *M. alpina*. The analysis was performed based on a selected-ion monitoring (SIM) chromatogram, see Fig. 2 for the respective PUFA

contains odd PUFA. At present we can only speculate on their biosynthesis based on published data.

Comparison of both the production and the proportion of molecular species of odd TAG cannot be done, because (see "Introduction") the publications describing the presence of odd TAG in microorganisms are missing.

Chiral LC of Natural Samples

As described above, the separation of enantiomers of TAG is not yet completely common. Based on the knowledge of the retention times of synthesized standards and mass spectra we have identified a homologous series of 3-acyl-1,2-dilinoleines. Real samples also contained these molecular species, see Fig. 5. Analysis of 3-acyl-1,2-dilinoleates in both the protozoan *K. quartana* and the mold *M. alpina* showed that while 3-acyl-1,2-dilinoleines predominate in the protozoan, 1-acyl-2,3-dilinoleines are major in the mold. This is best shown in Fig. 5. The regioisomers and enantiomers were identified based on the mass spectrum and the identity of retention times with synthetically prepared standards.

Figure 5 amply illustrates that in both cases the enantiomeric purity of biosynthesized TAG is higher in

even-numbered TAG regardless of whether the TAG is synthesized by the protozoan or by the mold. This can be explained by the fact that even-chain PUFA are more common substrates of acyltransferases responsible for the biosynthesis of TAG than the odd-chain PUFA and thus odd TAG are less physiological, or at least less common, i.e., less frequent, than even TAG.

Conclusion

We used for the separation of TAG two methods (NARP-LC and chiral-LC) and the synthesis of appropriate standards allowed us to identify regioisomers and enantiomers of both even and odd TAG. We were able to show that the biosynthesis of odd TAG is strictly stereospecific and depends on the production microorganism (mold *versus* protozoan), one enantiomer predominating in the protozoan and the other in the mold. Second, it was proved that even TAG are synthesized in a higher optical purity, which can be explained by a higher affinity of acyltransferases to the respective substrate, i.e., to even PUFA.

Acknowledgments The research was supported by GACR projects P503/11/0215 and 14-00227S, and by Institutional Research Concept RVO61388971.

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