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Separation of triacylglycerols containing positional isomers of hexadecenoic acids by enantiomeric liquid chromatography-mass spectrometry

Tomáš Řezanka [a,*], Jaromír Lukavský [b], Martin Rozmoš [a], Linda Nedbalová [c], Jan Jansa [a]

[a] Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Institute of Botany, Czech Academy of Sciences, Dukelská 135, 379 82 Třeboň, Czech Republic
[c] Department of Ecology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

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ABSTRACT

Triacylglycerols (TAGs) containing positional isomers of hypogeic (Hy), palmitoleic (Po), and palmitvaccenic (Pv) acids from three microorganisms (top-fermenting brewer's yeast *Saccharomyces cerevisiae*, green alga *Coccomyxa elongata*, and arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis*) were analyzed. Dozens of regioisomers and enantiomers of TAGs containing one, two or three hexadecenoic acids have been identified by means of reversed phase chromatography/mass spectrometry (RP-HPLC/MS). The regioisomers of TAGs containing two palmitic acids and any hexadecenoic acid were separated. Analysis of regioisomers of TAGs having one Pv residue showed that asymmetric molecular species such as PvPP or PPPv were dominant in *Rhizophagus*. TAGs were also analyzed on a chiral phase column and nine molecular species of TAGs containing two palmitic and any of three hexadecenoic acids were separated and identified. In the case of TAGs containing one palmitic and two hexadecenoic acids, the separation was successful only if the hexadecenoic acids were identical. Separation of TAGs containing three hexadecenoic acids was successful only if all three hexadecenoic acids were identical. Regardless of the type of TAG, it was found that TAGs in the AM fungus and containing palmitvaccenic acid bound at the *sn*-1 position of the glycerol backbone were dominant, suggesting similarity in the biosynthesis of the different TAGs. The covalent adduct chemical ionization method was used for identification of TAGs as adduct with (1-methyleneimino)-1-ethenyl ion, which reacted with double bond of the unsaturated fatty acid. Tandem MS thus makes it possible to identify TAGs containing various hexadecenoic acids.

1. Introduction

Triacylglycerols (TAGs) are the major storage lipids of all eukaryotic organisms. TAG molecules contain a glycerol backbone to which three different fatty acids (FAs) are ester-linked. The structure of TAGs affects their physicochemical properties as well as their metabolism. Various methods have been used to analyze TAGs. Currently, combination a liquid chromatography with a mass spectrometry (LC-MS) is the most commonly used approach, most frequently employing electrospray ionization (ESI) [1,2] or atmospheric pressure chemical ionization (APCI) [3,4]. Information on the total number of carbon atoms and the number of double bonds can be obtained from mass spectra, whereas the exact structure can then be determined using tandem MS. The separation of the different molecular species can be carried out in various ways, such as reversed phase chromatography, silver ion liquid chromatography, chiral chromatography [5–8], etc.

Several methods can use MS to distinguish between molecular species having different positions of double bonds in fatty acids [9]. In general, these approaches require chemical lipid derivatization (Patternò-Büchi reactions, radical-directed dissociation, reaction with ozone introduced into the source of electrospray ions, etc.). One of the possibilities is a selective reaction of ionic molecules, i.e. covalent adduct chemical ionization [10]. This analysis uses a reaction between acetonitrile-derived reaction ions and unsaturated fatty acid(s) double bonds and identification of adducts by APCI. Subsequent collision-induced dissociation of covalent adducts ions results in characteristic fragmentation and allows the assignment of the position(s) of the double bond(s). This method has previously been used to identify the position of

* Corresponding author.
E-mail address: rezanka@biomed.cas.cz (T. Řezanka).

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the double bond(s) in a number of fatty acids methyl esters (FAMEs) [11,12] as well as in TAGs [10]. An ion/molecule reaction with neutral acetonitrile produces (1-methyleneimino)-1-ethenyl ion (MIE, $CH_2 = C{=}N^+{=}CH_2$), which forms a covalent adduct with the double bond(s). Tandem mass spectra of this MIE adduct results in diagnostic fragments indicating the position of the double bond(s) in the acyls.

Separation of TAGs differing only in fatty acids having different double bond positions, such as oleic (9*Z*-18:1 (O) or 18:1n-9, respectively) and *cis*-vaccenic (11*Z*-18:1 (Va) or 18:1n-7) acids has been published only exceptionally. Lisa et al. [13] described the separation of TAGs that contained petroselinic (6*Z*-18:1 (Pe) or 18:1n-12, respectively), oleic, and *cis*-vaccenic acids but the individual TAGs only contained three identical FAs each. Further, Leskinen et al. [14] published a paper in which TAGs containing positional isomers of FAs (oleic versus vaccenic acids) were separated. The identification was performed on the basis of the similarity of retention times of prepared regioisomers and diacylglycerols (DAGs) ion intensities (the ratio $[AB]^+/([AB]^+$ and $[AA]^+$).

As can be seen from the above examples, at least several papers have been published in the field of positional isomers of octadecenoic acids. The situation is completely different for TAGs containing positional isomers of hexadecenoic acids. The analysis of fatty acids as well as triacylglycerols in yeast has previously been published [15,16]. The major FA of yeast was palmitoleic (9*Z*-16:1 (Po) or 16:1n-7) acid [17]. Several molecular species of TAGs were identified by RP-HPLC/MS and chiral HPLC/MS analysis, e.g. *sn*-PoPoP, *sn*-PPoPo, and *sn*-PoPPo (where P stands for palmitic acid, 16:0). Analysis of the arbuscular mycorrhizal (AM) fungus revealed that the majority of hexadecenoic acid was the less common palmitvaccenic acid (11*Z*-16:1 (Pv) or 16:1n-5) [18]. TAGs analysis was described only by direct nanospray infusion quadrupole time-of-flight tandem MS [19]. This method does not allow to unequivocally determine the position of double bonds in native TAGs and also does not allow to identify regioisomers and enantiomers. Nevertheless, TAGs with summary compositions corresponding to schemes 16:1/16:1/16:1, 16:1/16:1/16:0, and 16:1/16:0/16:0 with concentrations up to 3 μmol/gram fresh weight were established to be the three dominant forms of the native TAGs in the AM fungal mycelium [19].

In the third case, the fatty acid content of green alga was analyzed and hypogeic (7*Z*-16:1 (Hy) or 16:1n-9) acid was found as the minor positional isomer of hexadecenoic acid. The literature data describing the presence of this acid are relatively controversial. More than 500 occurrences of hypogeic acid in plants are recorded in a public database [20]. Unfortunately, the hypogeic acid content is usually very low except for a few plant species of the family Proteaceae [21], which occurs in the tropics and subtropics of the southern hemisphere. Besides, minor content, up to a maximum of 1 % of total FAs, was found e.g. in *Canarium ovatum, Lupinus mexicanus, Triticum aestivum*, etc. [22]. This acid has also been identified in algae, e.g. in the green algae such as *Cladophora fascicularis* (1.62 % of total FAs) [23], up to 2.2 % of total FAs in *Micractinium* sp. [24] or *Coccomyxa* sp. (the content was about 2 % of the total FAs) [25]. Interestingly, a newly isolated strain of *Coccomyxa elongata* contained 5.7 %–14.8 % of this fatty acid with a maximum recorded under the nitrogen-limiting conditions [26]. Lipidomic analysis of TAGs of the genus *Coccomyxa* has also been performed [27]. In there, 16:1/16:3/18:3 TAG was identified as a minor TAG; conversely 16:1/18:2- and 16:1/18:3-PG were major phosphatidylglycerols. No other positional isomers of hexadecenoic acid, e.g., 3(*E*)-16:1, have been identified in the TAGs. This latter acid is present in phosphatidylglycerols of photosynthetic eukaryotes [28]. In the paper by Allen et al. [29], the analysis of hydrogenated TAGs including their regioisomers was described. For C50 TAGs, the ratio of asymmetric/symmetric TAGs (e.g. PPS/PSP) decreases, while for C52 TAGs, the ratio increases.

Based on our previous experience with the analysis of TAG regioisomers and enantiomers [16,30], TAGs containing palmitoleic, *cis*-palmitvaccenic, and hypogeic acids have been identified in brewer's yeast (*Saccharomyces cerevisiae*), AM fungus (*Rhizophagus irregularis*) and green alga (*Coccomyxa elongata*) by reversed phase and/or chiral phase columns connected with an MS.

2. Material and methods

2.1. Standards

Common chemicals were purchased from Sigma-Aldrich, ltd. (Prague, Czech Republic), i.e. acetonitrile, 2-propanol, hexane, dichloromethane, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydro chloride (EDCI), *N,N*-dimethylpyridin-4-amine (DMAP), lipase from porcine pancreas (type VI-S, ≥20,000 units/mg protein), phospholipase C from *Clostridium perfringens* type XIV (150 units/mg protein), preparative TLC glass plates, silica gel 60 matrix, binder - polymeric, fluorescent indicator. The phospholipids, 1,2-dipalmitoleoyl-*sn-glycero*-3-phosphocholine (Po/Po-PC), 1,2-dipalmitoyl-*sn-glycero*-3-phospho choline (P/P-PC) were purchased from Sigma-Aldrich. 11(*Z*)-Hexadecenoic (palmitvaccenic, 16:1n-5), 9(*Z*)-hexadecenoic (palmitoleic, 16:1n-7) and 7(*Z*)-hexadecenoic (hypogeic, 16:1n-9) acids were purchased from Larodan (Solna, Sweden).

2.2. Cultivation

Top-fermenting brewer's yeast *Saccharomyces cerevisiae* was obtained from a large brewery in the Czech Republic (yearly beer output of more than 20,000,000 L) [15]. The strain *Coccomyxa elongata* (CCALA 427, GenBank: KX809908.1, isolated from a pool in the Czech Republic) was acquired from the Culture Collection of Autotrophic Organisms at the Institute of Botany, Třeboň, Czech Republic. The cultivation was described previously [31]. Briefly, the strain was cultivated in liquid circumneutral Bold's Basal Medium (BBM) [32]. The volume of algal suspension in Erlenmeyer flasks was 1000 mL. The culture was kept in a Q-Cell 200 incubator (PolLab, Poland) with continuous light of 20 μmol photons m^{-2} s^{-1} provided by a cool white 8 W fluorescent tube and the cultivation temperature was 20 °C. The strain was cultivated for 40 days and finally harvested by centrifugation at 4400 rpm for 10 min. The cultivation of *Rhizophagus irregularis* has been carried in absence of any other microorganisms in 1-liter cultivation vessels half filled with liquid MSR medium [33,34] with fourfold elevated phosphorus concentration (16.4 mg KH_2PO_4 per liter). The Ri-*T*-DNA transformed roots of *Cichorium intybus*, pre-colonized for two months by the AM fungal strain LPA9 (BEG 236, https://www.i-beg.eu/cultures/BEG236.htm), have been placed on a sterile nylon mesh (40 um) over a plastic floater. The cultivation lasted for 6 months, after which all the roots remained above the nylon mesh and the root-free AM fungal biomass freely hanging in the medium was harvested. All samples were stored frozen (-80 °C) until analysis.

2.3. Lipid extraction and isolation of TAGs

The extraction procedure was based on the method described by Bligh and Dyer [35] with modification as previously described [36]. Briefly, the frozen cells were suspended in a dichloromethane-methanol mixture (2:1) for 30 min with stirring, after which dichloromethane and water were added and the dichloromethane phase was evaporated to dryness under reduced pressure. Total lipids were fractioned by previously described methods [37]. Lipid classes were eluted from a Sep-Pak Vac Silica cartridge 35 cc (Waters; 10 g normal-phase silica) by chloroform (neutral lipids), acetone (glycolipids), and methanol (phospholipids) according to previously published procedure [38]. The solvents were removed in a stream of N_2 and TAGs were dissolved in the mobile phase (acetonitrile and 2-propanol (99:1, vol/vol)) for further analysis by LC-MS. Mixed samples of TAGs were obtained by mixing the appropriate extracts from individual microorganisms.

2.4. Preparation and analysis of methyl and 3-pyridylcarbonyl esters of fatty acids

Aliquots of the TAGs were saponified overnight in 10 % KOH in methanol at room temperature [39]. Methyl and 3-pyridylcarbonyl esters were prepared from TAGs using standard procedures. A detailed description of the preparation and the GC–MS analysis is provided in the Supplementary data. The mass spectra in the electron ionization (EI) mode were analyzed as previously described [36,40,41], see also three mass spectra of 3-pyridylcarbonyl C16 esters (Figs. S2, S3, and S4) in Supplementary data.

2.5. Preparation of TAGs with a mixed FA composition

1,2-Dipalmitoyl- and 1,2-dipalmitoleoyl-*sn-glycero*-3-phosphatidylcholines were treated with phospholipase C as described by Harwood [42]. A detailed description of the preparation method is provided in the Supplementary data.

The esterification of glycerol derivatives with acids has been previously described [43,44]. Briefly, diacylglycerols were reacted with free fatty acids in the presence of DMAP and EDCI to yield the corresponding triacylglycerols. The Table S1 shows the list of TAGs prepared by synthesis.

2.6. TAG analysis using reversed phase (RP)-HPLC/ESI-MS

The LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and three Luna Omega 1.6 μm, C18, 100 Å, LC Column 150 × 2.1 mm, connected in series. Although the efficiency stated by the manufacturer was not achieved, i.e. 350,000 plates/meter (see manufacturer's literature), the efficiency achieved in our case was approximately 307,000 plates/meter. Tripalmitin with t_R 92.44 min was used as an internal standard, with a flow rate of 0.90 mL/min, an injection volume of 7 μL, and column temperature of 23 °C. TAGs were separated using a solvent gradient program with acetonitrile (ACN) containing 5 mM aqueous ammonium acetate, and 2- propanol (iPrOH) containing 7 mM aqueous ammonium acetate as follows: initial ACN/iPrOH (98:2, vol/vol), a linear change to 22:78 ACN/iPrOH (vol/vol) from 4 min to 105 min, and a hold for 5 min. The composition was returned to the initial conditions over 5 min.

The high-resolution hybrid MS LTQ Orbitrap Velos (Thermo Fisher Scientific, San Jose, CA, USA) was used. APCI-MS analysis was performed in the positive FT ion mode. Mass spectra were acquired with target mass resolution of R = 90,000 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* 150–1000. Spray current was 4.0 μA, source temperature was 350 °C, and capillary temperature was 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 collision-induced dissociation (CID) experiments. The CID normalization energy of 35 % was used for the fragmentation of parental ions. The MS/MS product ions were detected in high-resolution FT mode. The calibration of the MS spectrometer was conducted using a Pierce LTQ Orbitrap negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA). The internal lock mass was used in the acquisition of mass spectra, i.e., 255.2330 Da, $[M-H]^-$ of palmitic acid in negative ESI. The mass accuracy was better than 0.9 ppm. The chemical structures of compounds were confirmed with the help of the spectral database LIPID MAPS® Lipidomics Gateway (https://www.lipidmaps.org/).

2.7. TAG analysis using chiral LC/ESI-MS

The same LC system used in the RP mode was also used for separation in chiral mode. TAGs (~1 mg/mL in mobile phase) were chromatographically separated 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) (Sigma-Aldrich, ltd.), connected in series. The mobile phase was a gradient introduced at 95 % A and 5 % B at 0 min to 45 % A and 55 % B over 105 min, and subsequently, isocratically maintained for 10 min, where A is hexane and B is a hexane, propan-2-ol (97:3% v/v) mixture [16,45]. The flow rate, column temperature, and injection volume were 0.45 mL/min, 24 °C, and 10 μL, respectively. Other conditions were the same as those used for RP-LC (see above).

2.8. Determination of geometric configuration of double bonds

A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT) infrared spectrophotometer was used for scanning infrared spectroscopy of methyl esters as neat film.

3. Results and discussion

3.1. Fatty acids

Preliminary identification, later confirmed by mass spectra, was based on elution order of FAMEs. Retention times (t_R) of three standards of methyl esters, i.e. 16:1n-5, 16:1n-7, and 16:1n-9 acids were compared with retention times of natural samples (alga, AM fungus, yeast). The mass spectra of the positional isomers of monoenoic FAMEs are almost identical [46] and therefore 3-pyridylcarbinol esters were prepared. The mass spectra of these esters are shown in Figs. S2, S3, and S4. As can be seen from these mass spectra, the low molecular weight regions, as with all 3-pyridylcarbinol esters, are prominent ions at *m/z* = 92, 108, 151 and 164. In contrast, the positional isomers of hexadecenoic acids as 3-pyridylcarbinol esters have different mass spectra. The hypogeic acid as 3-pyridylcarbinol ester has the gap of 26 Da between *m/z* 206 and 232, respectively gap of 40 Da between *m/z* 206 and 246. The 3-pyridylcarbinol ester of palmitoleic acid had a gap of 26 Da between the ions at *m/z* 234 and 260 and a gap of 40 Da between the ions at *m/z* 234 and 274, respectively. The 3-pyridylcarbinol ester of palmitvaccenic acid had a gap of 26 Da between ions at *m/z* 262 and 288 and a gap of 40 Da between ions at *m/z* 262 and 302, respectively. These mass spectra thus verified the double bond positions in the three hexadecenoic acids (Fig. S2-S4).

No easily recognizable difference between *cis* (*Z*) and *trans* (*E*) double bonds was found [47] and therefore the mass spectra of 3-pyridylcarbinyl derivatives are identical. For this reason, and to exclude the presence of the *trans* isomers, infrared spectra were used. All double bonds had a *cis* (more recently *Z*) geometric configuration, as determined using the infrared (IR) spectra [47]. The IR spectra of all FAMEs completely lacked absorption of the C—H bond in the range of 960–980 cm^{-1}, that is, absorption of the double bond with a geometric *trans* configuration (more correctly *E*).

The analysis of FAs in all three microorganisms led to essentially the same results (Table 1) as already published. Palmitoleic acid was the predominant acid of yeast, palmitvaccenic of the AM fungus, although it should be noted that trace amounts (~0.6 % of total FAs) of palmitoleic acid have also been found in the AM fungus. However, this amount is more than two orders of magnitude lower than the content of the major palmitvaccenic acid. For the green alga, all three hexadecenoic fatty acids were identified (at concentrations of 1.03 to 2.62 % of total FAs), as already reported for *Coccomyxa* sp. [25].

3.2. RP-HPLC/MS of TAGs

A method based on the elution order of TAGs has been used several times for preliminary identification [30,48]. However, this method based on ECN (ECN = ACN-2 × DB (ECN = effective carbon number; ACN = acyl carbon number; DB = number of double bonds)) was not

Table 1
Fatty acid content from three microorganisms (top-fermenting brewer's yeast *Saccharomyces cerevisiae*, green alga *Coccomyxa elongata*, and arbuscular mycorrhizal fungus *Rhizophagus irregularis*).

FAME	fungus	±SD[a]	yeast	±SD	alga	±SD
12:0	0.0	–	4.0	0.7	0.0	–
14:0	0.1	0.1	1.7	0.6	0.0	–
16:0	12.6	1.4	17.5	1.3	12.1	1.2
16:1n5	76.8	1.9	0.0	–	5.8	1.1
16:1n7	0.6	0.2	41.7	1.8	4.9	0.7
16:1n9	0.0	–	0.0	–	2.7	0.4
16:2n6	0.0	–	0.0	–	1.1	0.3
16:3n4	0.0	–	0.0	–	2.4	0.5
16:3n3	0.0	–	0.0	–	6.2	0.4
18:0	0.3	0.1	17.8	1.3	0.4	0.2
18:1n7	1.9	0.4	0.0	–	3.8	1.0
18:1n9	1.3	0.3	15.2	0.9	17.2	1.6
18:2n6	0.5	0.2	0.0	–	20.5	2.1
18:3n6	0.0	–	0.0	–	0.1	0.1
18:3n3	3.3	0.5	0.0	–	21.8	0.9
18:4n3	0.0	–	0.0	–	0.8	0.2
20:0	0.0	–	1.1	0.3	0.0	–
20:1n9	0.1	0.1	0.0	–	0.0	–
20:3	0.6	0.2	0.0	–	0.0	–
20:4	0.9	0.2	0.0	–	0.2	–
20:5	1.0		0.0	–	0.0	–

[a] Mean ± S.D. from three measurements, i.e. relative %.

successful, probably because the individual TAGs were all structurally very similar, i.e. they differed only in the position of the double bond in the individual acyls. Nevertheless, dozens of molecular species were separated, mainly due to the use of connection of three reversed phase columns in series with the efficiency of ~ 307,000 plates/meter, using tripalmitin as an internal standard.

The separation of total TAGs from the three natural sources (top-fermenting brewer's yeast *S. cerevisiae*, green alga *C. elongata*, and mycorrhizal fungus *R. irregularis*) is shown in Fig. 1. $[DAG]^+$ ions resulting from loss of neutral RCOOH + NH_3 from $[M + NH_4]^+$ in the interval 500–600 Da were used for identification.

If the ECN of TAGs differed by two, the separation was easy. If the ECNs were equal for the two molecular species, then it depended on the identity of acyls bound to the glycerol backbone. Separation of triunsaturated TAGs, e.g., HyHyHy, PoPoPo, and PvPvPv, where all three acyls are identical, was performed on a base line. In contrast, the separation of PvPvPo, PoPvPv, PvPoPv, PvPoPo, and PoPvPo completely failed (Fig. S1), mainly due to the lack of available synthetic standards. In contrast, the separation of regioisomers of TAGs containing two palmitic acids and one of any hexadecenoic acids was successful (Fig. 2) although it was not on the base line. As was described several times [30,48], the tandem of MS enantiomers are identical. On the contrary, the tandem MS of the regioisomers are different [49]. It is clear (Table S2) that in the case of a symmetric TAG (16:0/16:1/16:0) the ratio of $[DAG]^+$ ions $[16:0/16:1]^+$ (549.4879 Da) and $[16:0/16:0]^+$ (551.5036 Da) was about 100:40, while for asymmetric TAG (16:0/16:0/16:1), the ratio of the respective ions was 100:75. Again, a similar ratio was found for the two regioisomers 16:0/16:1/16:1 (asymmetric, $[DAG]^+$ ion ratio 77:100) and 16:1/16:0/16:1 (symmetric) ion ratio 37:100, respectively). The ion abundance ratios $[DAGs]^+$ were measured for the respective regioisomers (five analyzes). The average abundance values and ± SD of the individual regioisomers are given in Table S2. By comparing both the measured values from five regioisomer analyzes containing one or two hexadecenoic acids, the realistic abundance of symmetric versus asymmetric TAGs (Table S2) is quite obvious. It is also clear from this table that in the case of positional isomers of hexadecenoic acids (Po versus Pv), i.e. those that differ in the position of the double bond on the carbon chain, the ratio of ions $[DAGs]^+$ is not affected by the position of the double bond(s).

The Fig. 1 included in an earlier study [15] are showing the mass of PoPPo and PoPoP regioisomers, which differ only in the abundance of ions $[PoP]^+$ vs $[PoPo]^+$. In the case of symmetric TAG (PoPPo), the ion abundance ratio of $[PoPo]^+$ vs $[PPo]^+$ is about 100:32, in the case of asymmetric TAG (PoPoP), the abundance ratio of the respective ions is about 100:75. Again, different abundances of $[PP]^+$ and $[PPo]^+$ ions were found in the current study, with their ratio being 35: 100 for PPoP and 78: 100 for PPPo. From both of these examples, it is clear that the rule formulated by Mottram et al. [49] more than 20 years ago still applies.

As expected from our results with yeast TAG analysis [15,16], the separation of TAGs containing palmitic and palmitoleic acid was

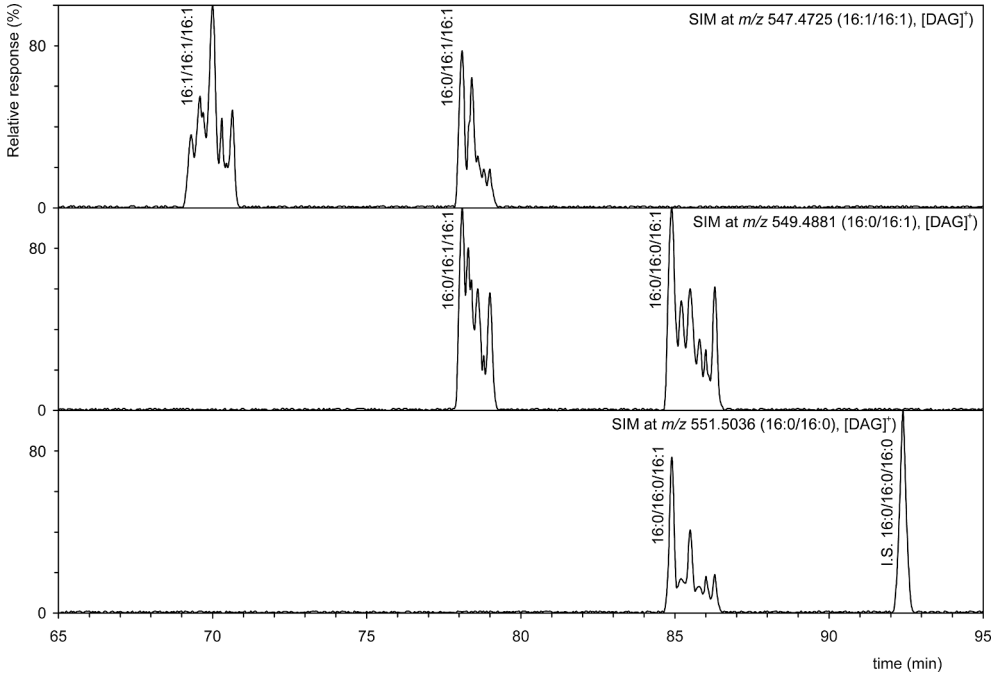

Fig. 1. The reversed phase separation of mixture of TAGs on three reversed phase columns connected in series from top-fermenting brewer's yeast *Saccharomyces cerevisiae*, green alga *Coccomyxa elongata*, and mycorrhizal fungus *Rhizophagus irregularis*.

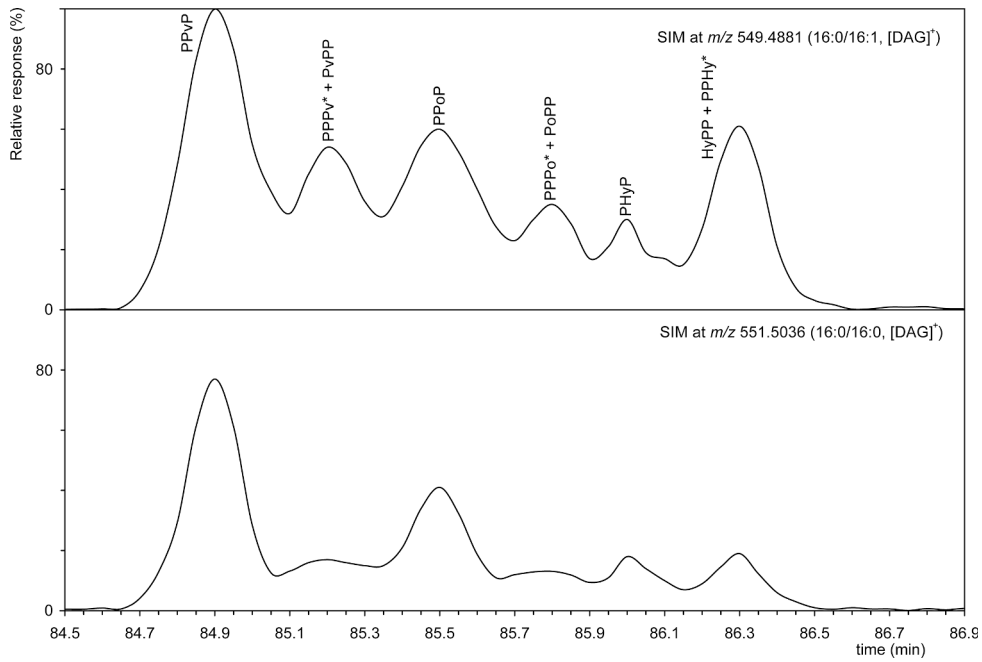

Fig. 2. The reversed phase separation of TAGs mixture containing two palmitic and one hexadecenoic acid (hypogeic, palmitoleic, and palmitvaccenic acids). $[DAG]^+$ ions resulting from loss of neutral RCOOH + NH_3 from $[M + NH_4]^+$ in the interval 500–600 Da were used for identification. Peaks marked with an asterisk were synthesized.

successful, see Table 2. It was similar to the AM fungus where the only major hexadecenoic acid was the palmitvaccenic acid. Because palmitoleic acid concentrations were two orders of magnitude lower than palmitvaccenic acid, TAGs containing both hexadecenoic acids in the same molecule were not identified in the AM fungus, unlike in the algae, where the content of all three fatty acids was comparable.

Our results are in accordance with previously published papers [15,16], but thanks to analysis by reversed phase, it was possible to determine that the majority diunsaturated regioisomers of the AM fungus were asymmetric TAGs (i.e. PvPvP + PPvPv versus PvPPv, and they were in ratio 77:39) (Table 2). In contrast, monounsaturated TAGs (PPPv + PvPP vs PPvP) were present in a ratio 53:10. These findings suggest that asymmetric TAGs are preferentially biosynthesized, see also below in the subchapter on chiral analysis. Very similar results were actually obtained by the previously published analysis of TAGs from *S. cerevisiae* [15,16], where the ratio of asymmetric (PPPo or PoPP) versus symmetric (PPoP) TAGs in monounsaturated TAGs was around 15–17 to 1, in diunsaturated TAGs (PoPoP or PPoPo) versus (PoPPo), it was 65–94 to 10. It is clear from the results that despite taxonomically distant relationship between yeast and the AM fungus, biosynthesis of asymmetric regioisomers is preferred in both of these organisms. Unfortunately, in the previously published papers [19,50], neither reversed phase analysis nor chiral analysis were carried out, thus determination of the ratio of regioisomers and enantiomers was not at all possible. In those previous studies, direct infusion nanospray tandem MS confirmed the presence of three TAGs, i.e. 16:1/16:1/16:1, 16:0/16:1/16:1, and 16:0/16:0/16:1 in wild type of *R. irregularis* [50]. Also in the paper of Wewer et al. [19] the same TAGs were identified in the extraradical mycelium of *R. irregularis* using a quadrupole time-of-flight tandem MS.

Table 2
Separation and identification of TAGs from *Coccomyxa elongata, Rhizophagus irregularis,* and *Saccharomyces cerevisiae*) by RP-LC/MS-ESI.

t_R (min)	$[DAG]^{+,a}$ 551.5036	$[DAG]^{+,a}$ 549.4881	$[DAG]^{+,a}$ 547.4725	Molecular species
69.32	0	0	36	PvPvPv*[b]
69.64	0	0	55	PvPvPo or PoPvPv + PvPoPv
69.73	0	0	47	PoPoPv* or PvPoPo + PoPvPo
70.05	0	0	100	PoPoPo*
70.32	0	0	44	PoPoHy*+HyPoPo
70.49	0	0	22	PoHyPo
70.68	0	0	48	HyHyHy*
78.13	0	100	77	PvPvP + PPvPv*
78.29	0	80	39	PvPPv*
78.45	0	64	64	PoPoP + PPoPo
78.58	0	60	25	PoPPo
78.84	0	27	19	PHyHy + HyHyP
79.01	0	58	19	HyPHy
84.88	77	100	0	PPPv*+PvPP
85.23	17	54	0	PPvP
85.54	41	60	0	PPPo*+PoPP
85.77	13	35	0	PPoP
86.03	18	30	0	HyPP + PPHy
86.28	19	61	0	PHyP
92.44	100	0	0	PPP*

[a] relative abundance of appropriate ion.
[b] peaks marked with an asterisk were synthesized.

Analysis of algal TAGs after their hydrogenation showed that two pairs of regioisomers were present (PPS vs PSP and SSP vs SPS, where P is palmitic acid and S is stearic acid). The structure of regioisomers before hydrogenation cannot be determined from the previous data [29]. Another paper [27] analyzed TAGs and polar lipids in the alga *C. melkonianii*. Thirty TAGs have been identified, including 16:1/16:3/18:3. Unfortunately, in this case, TAGs were not properly separated and only TAGs differing in ECN were identified, i.e. TAGs of type 50:7, 52:9, 54:6, etc. The analysis on both reversed phase and chiral columns presented in this current study provided an almost complete view of the structure of both regioisomers and enantiomers of TAGs in the alga *C. elongata*.

3.3. Chiral analysis

Fig. 3 shows four chromatograms of TAGs containing palmitic and hexadecenoic acids. Table 3 describes the results of the analysis of TAGs from three natural sources (alga, AM fungus, yeast) obtained by analysis on two chiral columns connected in series; besides, peak resolution was

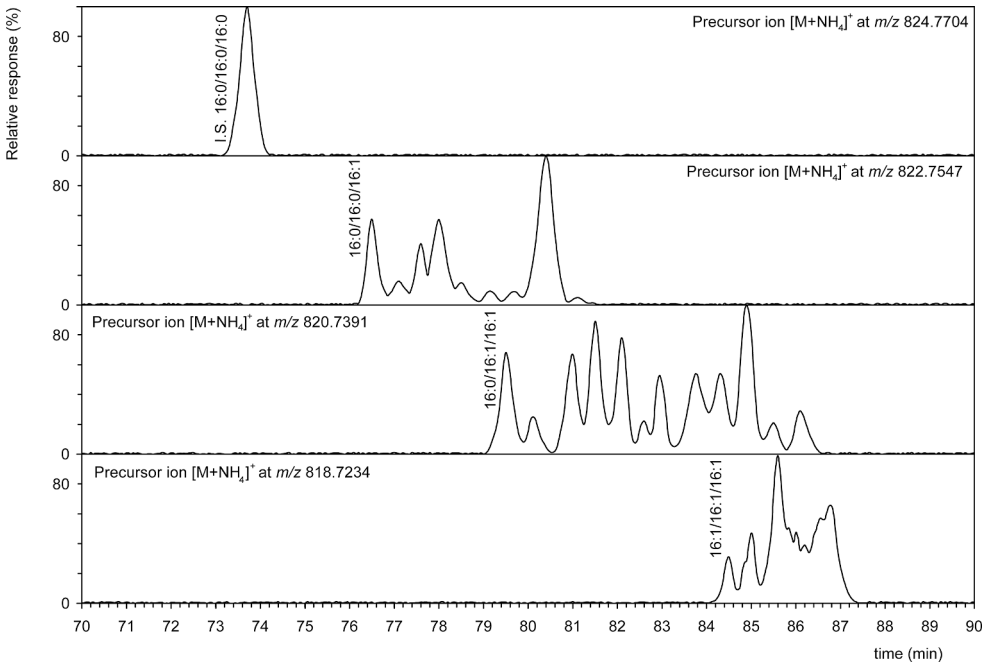

Fig. 3. Separation of TAGs from three natural sources (algae, fungus, yeast) obtained by analysis on two chiral columns connected in series.

Table 3
Separation and identification of TAGs from *Coccomyxa elongata*, *Rhizophagus irregularis,* and *Saccharomyces cerevisiae*) by chiral-LC/MS-ESI.

t_R (min)	Abundance	Molecular species	R_s[c]
73.72	100[a]	PPP*[b]	
76.54	58	PHyP	
77.11	16	**HyPP**	**0.8**
77.67	41	**PPHy***	
78.05	57	PPoP	
78.56	15	**PoPP**	**0.8**
79.03	4	**PPPo***	
79.69	9	PPvP	
80.45	100	**PvPP**	**1.0**
81.14	5	**PPPv***	
79.48	68	**HyPHy**	**1.4**
80.16	25	**PHyHy**	
81.02	67	HyHyP	
81.53	89	PoHyP + PHyPo + HyPoP + PoPHy + HyPPo + PPoHy	
82.11	78	PoPPo	
82.58	22	**PPoPo**	**0.6**
82.94	49	**PoPoP***	
83.83	53	PoPPv + PoPvP	
84.38	54	PvPPo + PPoPv + PPvPo + PvPoP	
84.96	21	PvPPv	
85.53	100	**PPvPv**	**0.9**
86.11	29	**PvPvP**	
84.58	31	HyHyHy*	
84.85	27	HyPoPo + PoHyPo	
85.04	47	PoPoHy*	
85.63	99	PoPoPo*	
85.85	50	PoPoPv*	
86.05	44	U1	
86.20	39	U2	
86.45	49	U3	
86.55	57	U4	
86.82	65	PvPvPv*	

[a] relative abundance of appropriate ion.
[b] peaks marked with an asterisk were synthesized.
[c] $R_s = (t_{R2}\text{-}t_{R1})/((w_{b1} + w_{b2})/2)$ (resolution).

added to Table 3.

Four precursor ions of the type $[M + NH_4]^+$ at different values were used for identification. Synthetically prepared tripalmitin at *m/z* 824.7704 was used as a standard. The other three chromatograms show the separation of TAGs containing one, two and three hexadecenoic acids. Mixtures were prepared by mixing extracted lipids from the three organisms (yeast, AM fungus and alga), see Materials and Methods.

Further, Fig. 4 shows the separation of all nine possible molecular species of TAGs containing two palmitic and one of the three hexadecenoic acids (Hy, Po, Pv). Using two columns with a chiral phase, connected in series, it was possible to separate all nine molecular species, although not on the base line. However, it was possible to determine the structure of each TAGs by synthetically prepared standards (marked with an asterisk in Fig. 4) and by the adducts with MIE. In the case of TAGs with one palmitic acid and two hexadecenoic acids, the situation was much more complicated. TAGs of yeast and the AM fungus, i.e. for molecular species containing one palmitic acid and two of any but the same hexadecenoic acids, it was not a problem to separate all nine TAGs again. Unfortunately, the combination of palmitic acid and two different hexadecenoic acid residues led to complete loss of separation (Fig. 5). For example, a peak having t_R at 81.52 min contained six molecular species of three FAs (P, Po, Hy), peaks with t_R at 83.78 and 84.27 min contained two and four molecular species, respectively. Proof of the structure of these mixtures was always performed after collection of peaks, transesterification to FAMEs and their analysis by GC–MS. Regioisomers were also analyzed by tandem MS.

In the case of TAGs with three hexadecenoic acids, where the number of theoretically possible molecular species is twenty-seven, the separation of mixed TAGs, i.e. those containing three different hexadecenoic acids, was unsuccessful (Fig. S1). It was only possible to separate three synthetic standards that contained all three residues of the same acids in the molecule (i.e., HyHyHy, PoPoPo, and PvPvPv). One synthetic standard (PoPoHy) was obtained with two different hexadecenoic acids, for which a mathematical simulation of the two peaks separation at t_R 84.87 min (HyPoPo and PoHyPo) and t_R 85.02 min (PoPoHy) was performed. Based on the calculation, it was found that the two columns connected in series had 55,600 theoretical plates. Approximately 667,000 theoretical plates would be needed to separate the above two peaks on the base line, which is twelve times more. To achieve such separation power is currently unrealistic.

Chiral analysis of TAGs was performed on yeast only in previously published research [15,16]. For *Rhizophagus* and *Coccomyxa*, at least to

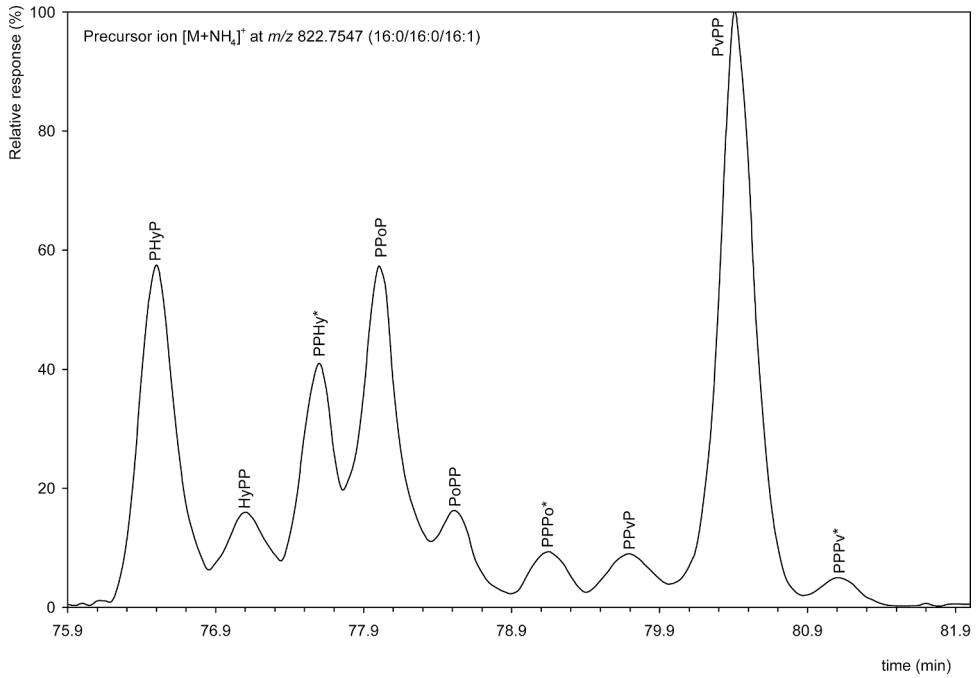

Fig. 4. Chiral separation of regioisomers and enantiomers of TAGs containing two palmitic and one hexadecenoic acids. Peaks marked with an asterisk were synthesized.

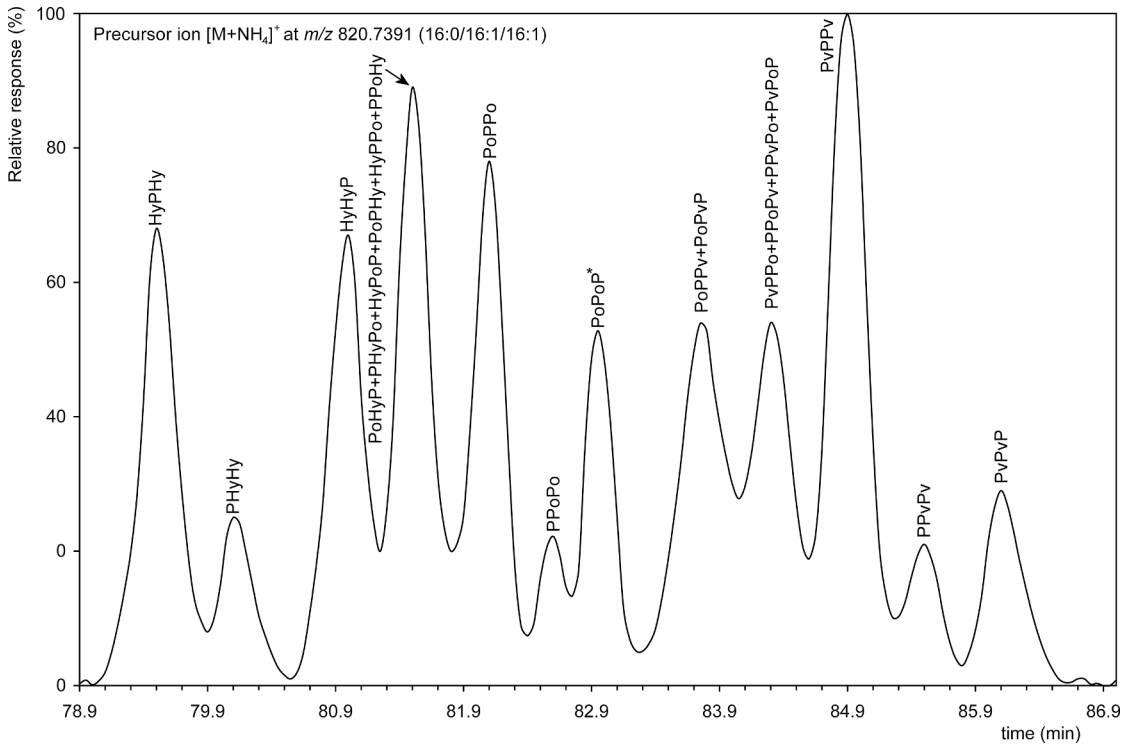

Fig. 5. Chiral separation of one palmitic acid and any two hexadecenoic acids. Peak marked with an asterisk was synthesized.

the best of our knowledge, the separation of TAGs by chiral chromatography has not yet been published. When separating the natural TAGs mixture from the *Rhizophagus*, TAGs containing one or two Pv were separated and identified based on tandem MS and with agreement of t_R of synthetically prepared standards.

Asymmetric TAG (PvPP) is the majority in TAGs with one palmitvaccenic acid in *Rhizophagus* and the same is true for TAGs with two palmitvaccenic acids (i.e. asymmetric PvPvP). It should be noted that in both cases, palmitvaccenic acid is always bound at the *sn*-1 position, suggesting that some biosynthetic steps of these different molecular species are shared. Unfortunately, biomass of other species of AM fungi is currently not available in large enough amounts and purity to further elucidate the biosynthesis of both regioisomers and enantiomers of TAGs in this specific group of soil organisms belonging to a basal fungal lineage, similarly to Mucoromycota and Mortierellomycota [51]. With respect to the latter, palmitoleic acid has previously been identified in

three strains of *Mortierella alpina* only as a minor FA, making up to 0.21 % of total FAs [52]. Nevertheless, stereospecific analysis of TAGs showed that depending on the strain, the *sn*-2 position of the glycerol backbone was preferentially esterified by Po in two strains (called E and F), but in contrast, in the strain called A, Po was predominantly present in the *sn*-1 position. Interestingly, Po was not found in position *sn*-3. *Mortierella alpina* was cultured on medium that contained only odd-numbered hydrocarbons (pentadecane or heptadecane) and two molecular species of the TAGs (PPPo and PPoPo) were identified in the mycelium [53]. Also in another paper [54], palmitoleic acid was found as a minor component of TAGs, e.g. 16:1/20:4/20:4, but exact determination of regioisomers or enantiomers was not performed.

3.4. Covalent adduct chemical ionization tandem MS

The tandem MS of six TAGs, both prepared by organic synthesis (*sn*-PPPo, *sn*-PPPv, *sn*-PPHy, *sn*-PoPoP) and those isolated from natural material (HyHyP, PvPvP), is shown in Fig. S5. The hash-marked ions are diagnostic ions (Fig. S6) that could be used to determine the position of the acyl double bond(s) in the TAG molecule. It is clear from their structures that, for example, in the case of TAG with two palmitic acids and one hexadecenoic acid, the values of the respective triplets always differ by ~ 28 Da (2 × CH_2 group). Similar results were found for TAGs containing one palmitic and two hexadecenoic acids.

An example is the tandem MS of two synthetically prepared TAGs (PPPo and PoPoP). In both cases, the two triplets of diagnostic ions are identical. In tandem MS, there are only differences in $[M + 54]^+$ ions, which is understandable because PoPoP contains two double bonds in the molecule. The effect of the second double bond in PoPoP versus PPPo is also in the presence of the ion at *m/z* 600.4986. Unfortunately, the resolution of the two regioisomers (PPoP and PPPo) failed in tandem MS and no different abundances of ions were found to distinguish the regioisomers. This is in contrast to the tandem MS of ions $[M + NH_4]^+$. These results are fully in line with the previously published observations [10].

4. Conclusion

From the above examples, it is clear that the rule formulated by Mottram et al. [49] more than 20 years ago still applies. This rule describes the tandem mass spectra of regioisomers, respectively describes the abundance of ions of the type [DAG] + caused by the loss of RCOOH + NH_3 from $[M + NH_3]^+$. Based on this abundance (e.g., Figs. 3 and 4), it is possible to distinguish between regioisomers and i.e. type of acyls are bound to the primary and secondary hydroxyl of the glycerol backbone. This means that it does not depend on the position of the double bond in the hexadecenoic acid(s), but only on the position of the acyl bound to the glycerol backbone. It should also be noted that the abundance ratio of $[DAG]^+$ ions does not correlate with the position of the double bond in hexadecenoic acid(s).

Our detailed analyses of TAG isomers in the AM fungus is particularly novel and relevant to the biology of this obligate biotrophic microorganism. The AM fungi are oleaginous microorganisms, living in close association with most terrestrial plants [55], and storing large amounts of their energy reserves in neutral lipids [19,56,57]. Unusually high abundance of palmitvaccenic acid in their biomass, particularly in the neutral lipids, resulted in using this particular compound as a proxy to estimate their biomass in soil and roots since decades [58]. Interestingly, the AM fungi seem to lack *de novo* synthesis of fatty acids, but contain fatty acid desaturase forming palmitvaccenic acid from palmitic acid [18,19,56], of which the latter is supplied by the host plant [50]. This specific desaturation pathway seem only be present in the Glomeromycota and absent in Mortierellomycota and Mucoromycota [18]. Our analyses revealed that palmitvaccenic acid is nearly always bound to *sn*-1 position of the glycerol backbone in the different TAGs, which indicates a common but as yet unknown biosynthetic mechanism involved in formation of the different TAGs in the AM fungi. Whether this has any biological (e.g., fitness) meaning or simply is a consequence of evolution of the specific energetic metabolism is the AM fungi, remains still to be elucidated – in spite of at least two decades efforts to uncover details of lipid metabolism in AM fungi [59–61].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123401.

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