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Regioisomeric Analysis of Triacylglycerols

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Animal fats and vegetable oils contain predominantly triacylglycerols (TAG), which are esters of fatty acids (FA) and glycerol. Although fatty acids in TAG usually have an even number of carbon atoms, exceptionally they could also have an odd number (e.g., TAG from certain bacteria). These bacteria biosynthesize both odd-numbered and branched FA, which can then become minor components of the milk of ruminants. The effect of consumed food can also be reflected in the contents of the FA in TAG of fish oil, since fish stands near the end of the food chain, that begins with phytoplankton.

Double bonds in the molecule of FA (and thus also in TAG) are usually in the cis (*Z*) geometrical configuration and their number in one acyl is from 0 to 6, exceptionally in certain microorganisms to 8 (e.g., the 28:8 N-3 acid from the dinoflagellate *Gymnodinium sanguineum*).

In the case of asymmetric TAG, i.e., when each of the primary hydroxyl groups is esterified with a different FA, the configuration gives rise to regioisomers (formerly called positional isomers). Warning – do not be confused with positional isomers of fatty acids, which differ in the position of double bond(s) – e.g., oleic and *cis*-vaccenic acid, α- and γ-linolenic acid, etc.). The classic case of two regioisomers of TAG is 1-palmitoyl-2,3-diolein (POO) and 1,3-dioleoyl-2-palmitin (OPO). Another possibility is the formation of enantiomers such as *sn*-POO vs. *sn*-OOP.

Instead of the usual D/L or R/S nomenclature, a special nomenclature is used for the stereochemistry of TAGs where the primary hydroxyl groups are often termed α- and α′ and the secondary β, or the "stereospecific numbering" (*sn*) system recommended by the IUPAC-IUB commission.

However, "optical activity" of these TAG is usually too low to be measurable; for example, *sn*-OPP has $[\alpha]_D^{20} = 0.00$ (*c* 7.05, $CHCl_3$) (Stamatov and Stawinski 2007). Identification of both regioisomers and enantiomers is very important because the positional distribution of FA has a strong influence on both the nutritional and the physicochemical properties of TAG. Natural TAG are biosynthesized strictly stereospecifically. Thus the proportion of POP is 100 % in sunflower oil, whereas lard has the ratio of POP to OPP 8:92 (Lisa et al. 2009b). The different physical properties include for instance different melting points of SLO and SOL, wherein S is stearic acid, O is oleic, and L is linoleic acid. The respective melting points are ~ −3 °C and ~ −18 °C. Furthermore, the structure of TAG is important for

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nutrition. In the process of digestion of fats, lipases in the intestinal tract of mammals hydrolyze TAG to form *sn*-2 monoacylglycerols and free fatty acids. The FA are released from positions 1 and 3 and monoacylglycerols are then absorbed by the cells of the small intestine. It is important to note that the content of FA alone does not provide enough information about the molecular structure of TAG.

Chemical-enzymatic and physicochemical methods have therefore been developed and will be discussed below.

Gas Chromatography and GC-MS

This method has, in our opinion, currently only a historical importance, which is confirmed by the negligible number of works using GC published in the last year when compared with the articles using HPLC. The main reason why GC ceased to be used for TAG analysis is instability (thermal decomposition) of TAG, especially unsaturated TAG (e.g., trilinolenin) at higher temperatures. Moreover, regioisomers were separated only exceptionally, see, e.g., the paper (Kemppinen and Kalo 1998) where butter oil was analyzed and BuPP from PBuP were separated (where Bu is butyric acid). This approach is not possible for TAG with longer FA.

Analysis of Regioisomers TAG by ^{13}C-NMR

^{13}C-NMR spectroscopy can be used to determine the FA at the primary and secondary hydroxyls, i.e., regioisomers but not enantiomers. FA at the *sn*-1(3) and *sn*-2 can be identified based on the chemical shifts of the ester group at ~172 ppm, where chemical shift of -COOH in FA bound to a primary hydroxyl group is about 0.4 ppm higher than the chemical shift of the same atoms in acids esterifying secondary hydroxyl.

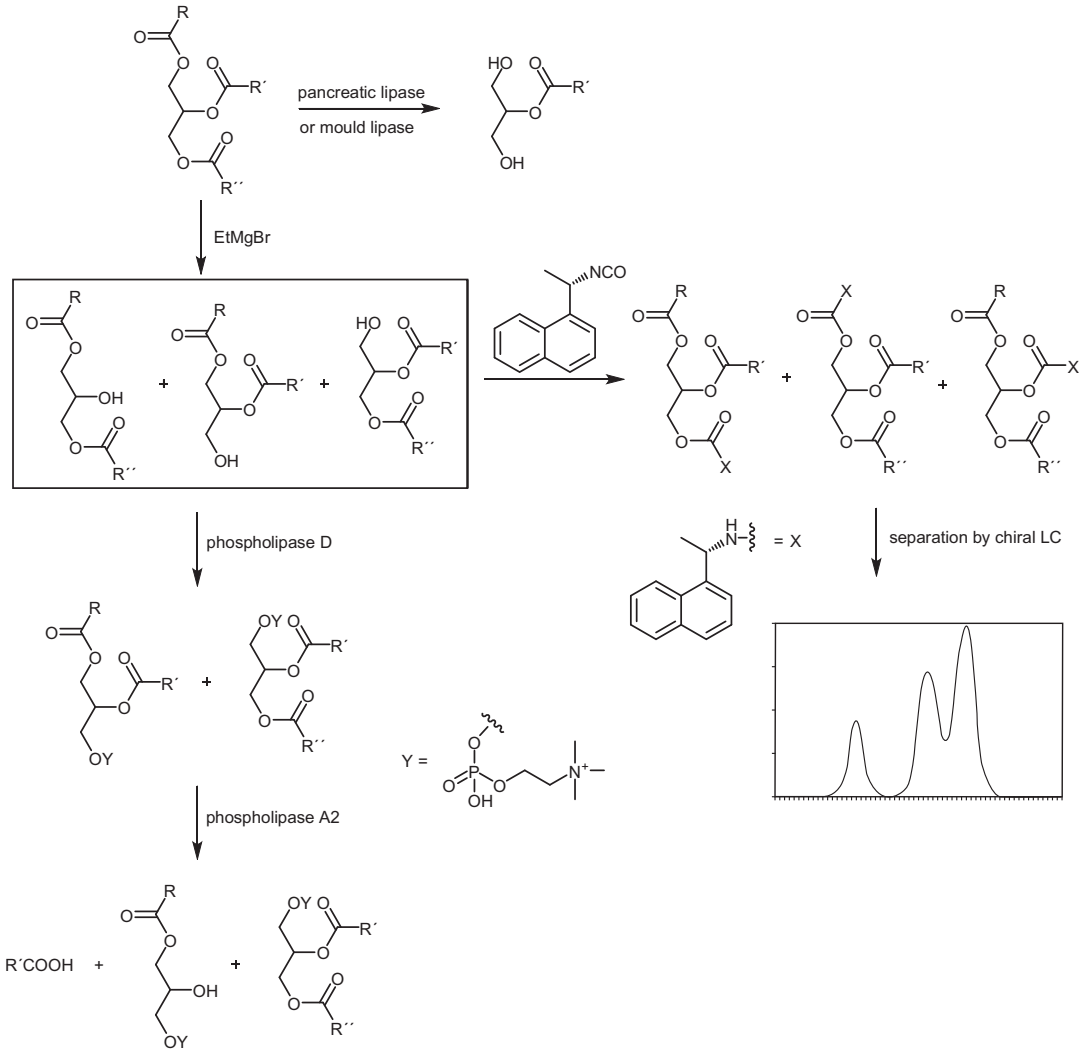
Determination by Enzymatic Methods

Analysis of TAG can be performed by specific enzymes or by a combination with chemical reactions, which is described in Fig. 1. It should be noted that neither ^{13}C-NMR nor enzymatic methods allow the determination of individual molecular species without their prior separation. Only the sum of the acids on the glycerol backbone is determined.

Liquid Chromatography-Mass Spectrometry

The following liquid chromatography-mass spectrometry (LC-MS) methods allow direct separation and identification of individual molecular species of TAG. Regioisomers can be separated either by argentation liquid chromatography (Ag^+-LC) or nonaqueous reversed-phase chromatography (NARP) (Kalo and Kemppinen 2012) and determined preferably by mass spectrometry (Murphy and Axelsen 2011) with atmospheric pressure chemical ionization (APCI) (Byrdwell 2001, 2005; Mottram 2005; Rezanka and Sigler 2007) or electrospray ionization (ESI) (Cozzolino and De Giulio 2011). Enantiomers are usually separated on a column with a chiral stationary phase. It is necessary to use at least one of the enantiomers as an internal standard (the standards are usually not commercially available and have to be synthesized, which is not a trivial matter), because the mass spectra of both enantiomers are identical.

Besides Ag^+-LC and NARP-LC, supercritical fluid chromatography (SFC) is also used for the separation of regioisomers. SFC is based on the principle of behavior of supercritical carbon dioxide, which is often used together with the admixture of modifiers (mixture of organic solvents). Of all the above mentioned methods, SFC is the least frequently used. However, it has been employed to separate TAG regioisomers (Lee et al. 2014), for instance SPLn and SLnP. Their structure was then determined by MS.



Regioisomeric Analysis of Triacylglycerols, Fig. 1 Determination of TAG structure by specific enzymes and by a combination with chemical reactions

Argentation (Silver Ion) High Performance Liquid Chromatography (Ag^+-LC)

Ag^+-LC is based on the interaction of π-electrons of the double bonds in FA molecules with *d*-electrons of silver bonded to the carrier (ion exchanger). TAG are eluted from the column primarily according to the number of double bonds; the greater the number of double bond(s), the longer the retention time. This method can separate regioisomers, since the double bond(s) in the acyl bound to the primary hydroxyl groups are less sterically hindered and therefore have greater affinity for silver ions. Usually, only vegetable oils and animal fats are analyzed, because the method is usable for a maximum of ~10 double bonds in the TAG. Fish oils cannot be analyzed by this method, because they contain TAG with up to 18 double bonds (3x docosahexaenoic acid).

Fish oil or TAG of a similar composition from algae (phytoplankton) have not yet been separated, only the ratios of regioisomers were determined by mass spectrometry or the regioisomers were determined after hydrogenation of TAG. To our current knowledge no separation has as yet been performed of, e.g., StLnLn and LnStLn, i.e., two regioisomers of TAG found as a minor component of certain vegetable oils (borage, hemp, different currant oils, etc.), while regioisomers of alpha and gamma-linolenic acids were successfully separated. In conclusion, the analysis of TAG isolated from most vegetable oils and animal fats presents no problem, if it is performed by an experienced researcher. Examples of the analyzed oils are given in Table 1.

Nonaqueous Reversed Phase Liquid Chromatography (LC-NARP)

LC-NARP in connection with mass spectrometry has been used in innumerable studies. Separation process comprises interaction of the stationary phase, mostly RP-18, with TAG molecules. Stationary phases with a different chain length (C8, C28, etc.) have been tested, but these phases did not bring any significant improvement in the separation. This is explained by the fact that the conventional TAG contain even-numbered FA with chain lengths C16-C22, i.e., approximately equal to those of the stationary phase. Column efficiency is a very important factor for the separation of regioisomers. Better column efficiency can be achieved by combining several columns in series. However, this arrangement extends the retention time of the eluted TAG up to several hours. TAG are primarily separated according to the equivalent carbon number (ECN), which is defined as $ECN = CN - (2 \times DB)$, where CN is the number of carbon atoms and DB is the number of double bond(s). TAG having the same ECN, for instance the above regioisomers, may be separated on an efficient column since the separation depends also on other contributory effects such as the position of double bonds, as seen, e.g., in the separation of α- and γ-linolenic acids, see Table 1. A series of studies have dealt with the optimization of separation by selecting a suitable stationary phase (Lisa et al. 2009a), mobile phase (Momchilova et al. 2004), length of the column (Lisa et al. 2009a), column temperature (Lisa et al. 2009a), etc.

Enantiomers

As mentioned above, TAG having various FA bound to primary hydroxyls of glycerol backbone form enantiomers. Until recently, their determination was very time- and material-consuming (see Fig. 1), because a combination of chemical and enzymatic methods was used. However, a serious problem still remains. If the TAG are not preseparated to single isomers, only total FA bound at positions *sn*-1, *sn*-2, and *sn*-3 will be determined. Identification of individual enantiomers is possible only when chiral stationary phases are used. The most commonly used stationary phases are cellulose-tris-(3,5-dimethylphenylcarbamate) or cellulose-tris-(3-chloro-4-methylphenyl carbamate) and mixtures of hexane/*i*-PrOH or methanol as mobile phases. It is necessary to use standards to verify the structure of the enantiomers, because even a little change in the structure of the stationary phase is known to cause a change in elution (order) of TAG (Nagai et al. 2013). Examples of some enantiomer analyzes are shown in Fig. 2 and Table 2, which also presents our study on the enantiomers, mostly from algae. In contrast to the usual vegetable oils, algae produce a much broader range of polyunsaturated FA (PUFA), especially 16:2–16:4, 18:5, 20:4, 20:5, 22:6, etc. Based on our experience with the analysis of enantiomers we attempted to define the following rule: an enantiomer of TAG having on primary carbon more unsaturated FA has a longer retention time than the *sn*-2 isomer (see Figs. 3, 4, and 5, for regioisomers and enantiomers of TAG containing arachidonic and eicosapentaenoic acids).

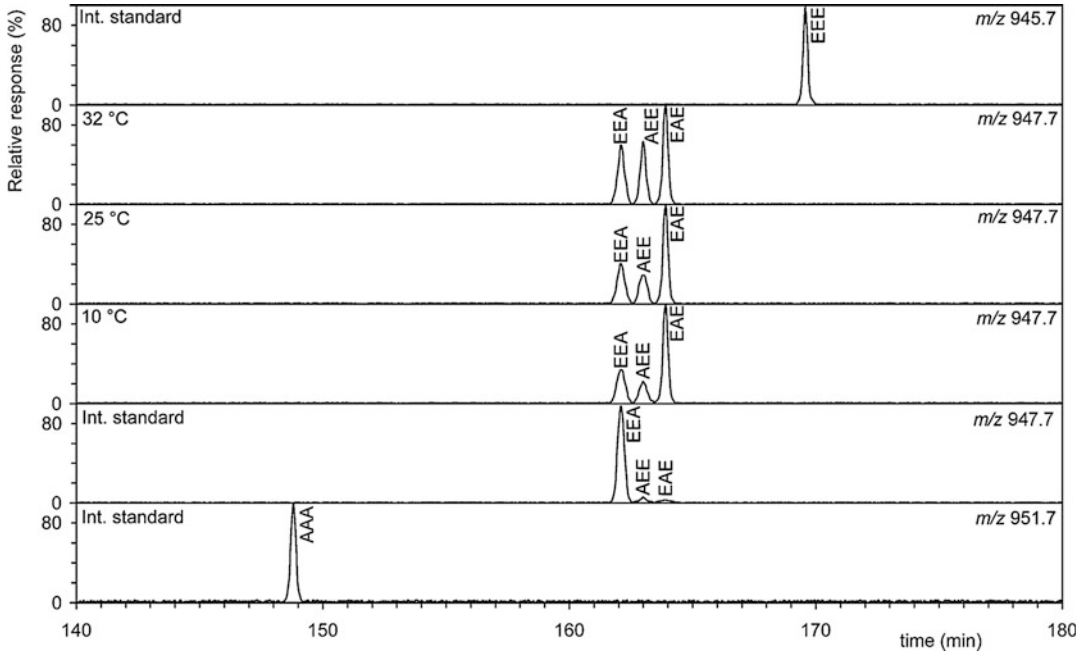
Regioisomeric Analysis of Triacylglycerols, Table 1 Selected examples of TAG analyses by Ag-LC and NARP-LC

Reference	Source	Type of analysis	Types of TAG
(Rezanka et al. 2014b)	Snow algae *Chloromonas*	Ag^+-LC/APCI-MS	Pm/Pm/St – Pm/St/Pm, Pm/St/St – St/Pm/St
(Francois et al. 2010)	Menhaden oil	Ag^+-SFC/APCI	18 fractions (up to 18 double bonds)
(Hu et al. 2013)	Peanut oil	Ag^+-LC/APCI-MS	P/O/O-O/P/O
(Holcapek et al. 2010)	Synthetic TAG	Ag^+-LC/APCI-MS	O/Ln/Ln – O/γLn/Ln, O/Ln/γLn – O/γLn/γLn
(Adlof and List 2003)	Synthetic TAG	Ag^+-LC	S/S/O-S/O/S, S/S/L-S/L/S
(Holcapek et al. 2009)	Blackcurrant seed oil	Ag^+-LC/APCI-MS and Ag^+-LC/ESI-MS/MS	αLn/L/L – γLn/L/L
(Dugo et al. 2004)	Rice oil	Ag^+-LC/APCI-MS	L/L/O-L/O/L
(Lisa et al. 2009b)	Sunflower oil and lard	Ag^+-LC/APCI-MS	L/L/P-L/P/L
(Laakso and Voutilainen 1996)	Synthetic TAG	APCI-MS	O/γLn/O – O/O/γLn
(Nagai et al. 2011)	Synthetic TAG	C28 reverse-phase NARP-LC/APCI-MS	P/P/C-P/C/P, P/P/O-P/O/P, O/P/O-P/O/O, P/P/D-P/D/P, P/D/D-D/P/D, P/P/E-P/E/P, P/E/E-E/P/E
(Gotoh et al. 2012)	Milk from various mammals	LC/APCI-MS/MS	P/P/C-P/C/P, P/P/O-P/O/P, P/O/O-O/P/O, P/P/D-P/D/P
(Schreiberova et al. 2010; Lisa et al. 2011)	Synthetic and natural TAG	NARP-LC/APCI-MS	P/Ma/Ma – P/aiMa/aiMa, P/Ma/Ma – P/iMa/iMa
(Rezanka et al. 2010)	Bacterium *Rhodococcus erythropolis*	NARP-LC/APCI-MS	Po/15:0/Po – 15:0/Po/Po, O/Ma/O – Ma/O/O
(Rezanka et al. 2011)	Bacterium *R. erythropolis* and plant *Dracunculus vulgaris*	RP-HPLC/APCI-MS	P/P/P13 – P/P13/P, P/P/P14 – P/P14/P
(Allen et al. 2014)	Green algal TAG after hydrogenation	RP-LC/ESI-MS/MS	P/P/S-P/S/P, S/S/P-S/P/S
(Leskinen et al. 2007, 2010)	Lard, rapeseed, sunflower seed, palm, black currant seed, and sea buckthorn pulp oils	APCI and ESI	L/O/L-L/L/O, O/L/O-L/O/O, O/P/O-P/O/O, P/O/P-P/P/O,
(Fauconnot et al. 2004)	Beef, pork, and chicken fats and palm oil, cocoa butter	RP-HPLC/APCI-MS	P/P/S-P/S/P, P/P/O-P/O/P, S/S/O-S/O/S, P/O/O-O/P/O, S/O/O-O/S/O, P/P/L-P/L/P, L/L/S-L/S/L
(Jakab et al. 2003)	Grape seed, sunflower, pumpkin seed, soybean, and wheat germ oils	LC/APCI-MS	L/L/O-L/O/L
(Mottram and Evershed 1996; Mottram et al. 1997; Mottram et al. 2001)	Beef, chicken, lamb, and pork fats	RP-HPLC/APCI-MS	O/P/O-O/O/P, S/O/S-S/S/O

(*continued*)

Regioisomeric Analysis of Triacylglycerols, Table 1 (continued)

Reference	Source	Type of analysis	Types of TAG
(Kuroda et al. 2008)	Synthetic TAG	Recycle HPLC system	O/P/O – O/O/P
(Momchilova et al. 2004)	Synthetic TAG	NARP-LC	P/O/P-P/P/O, P/L/P-P/P/L, P/E/P-P/P/E, P/D/P-P/P/D
(Momchilova et al. 2006)	Synthetic TAG	NARP-LC	P/Ln/P-P/P/Ln, P/A/P-P/P/A
(Kalo et al. 2009)	Butterfat	Normal-phase LC/ESI-MS	P/Bu/Bu – Bu/P/Bu, 19:0/O/S – O/19:0/S
(Kalo et al. 2003, 2004)	Butterfat	Normal-phase LC/ESI-MS	S/Bu/S – S/S/Bu, O/Bu/Bu – Bu/O/Bu



Regioisomeric Analysis of Triacylglycerols, Fig. 2 Chiral-LC of TAGs (SIM – selected ion monitoring) from algae *Trachydiscus minutus*, cultivated at various temperatures (10 °C, 25 °C, and 32 °C)

Conclusion

In conclusion, LC-MS represents the most frequently used method for separation and analysis of regioisomers and enantiomers of TAG. However, there are several circumstances that should be taken into account. Although regioisomers of TAG could be separated by combining several columns in series, this setup increases retention time. It is therefore necessary to optimize both resolution and retention time. The exact ratio of individual regioisomers could be determined very precisely from the calibration curve, if both regioisomers are available. When separating enantiomers it is necessary to have at least one of them. It can be thus stated that limiting factor of TAG analyses is not ineffective column or low-end mass spectrometer but lack of standards.

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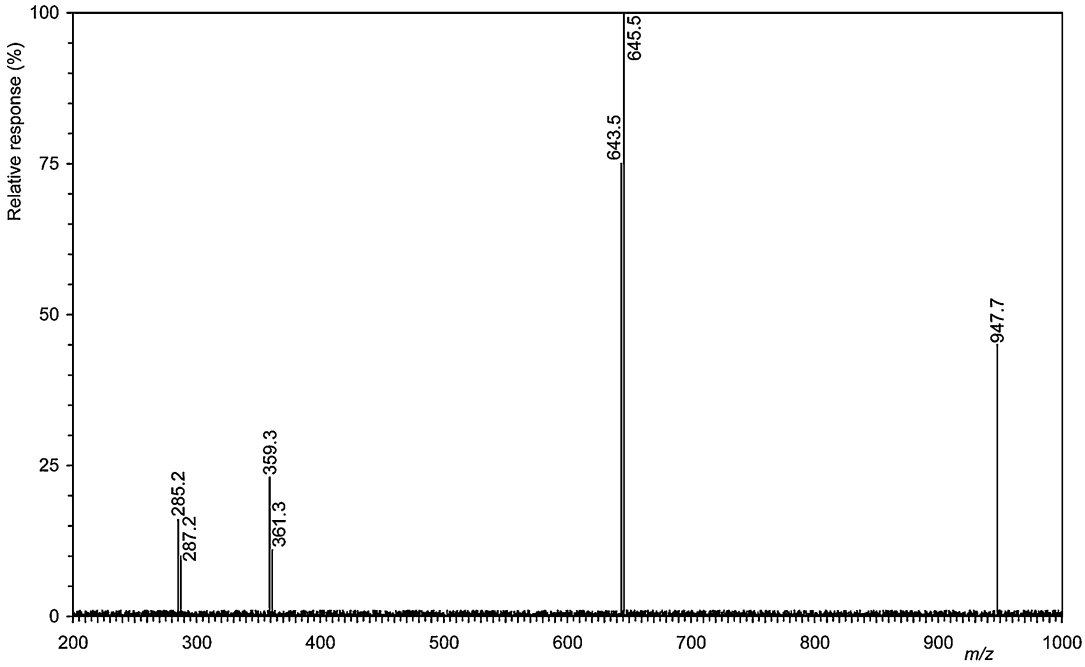
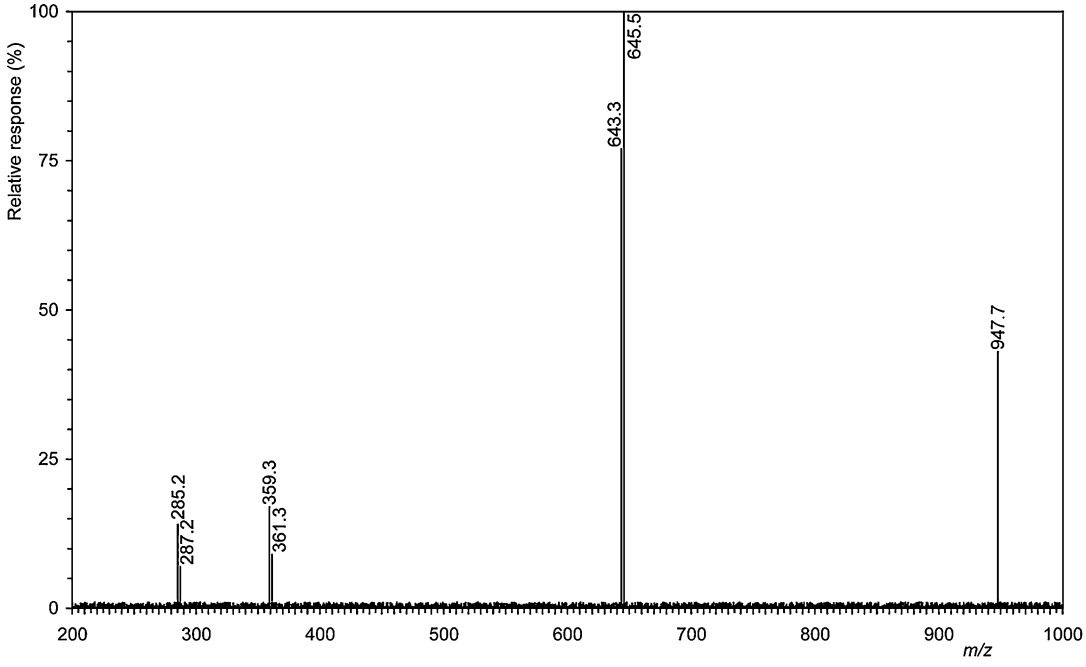
Regioisomeric Analysis of Triacylglycerols, Table 2 Survey of literature data on the separation of TAGs on chiral columns (flow 0.5 – 1.0 ml; ion source APCI or UV 210 nm*)

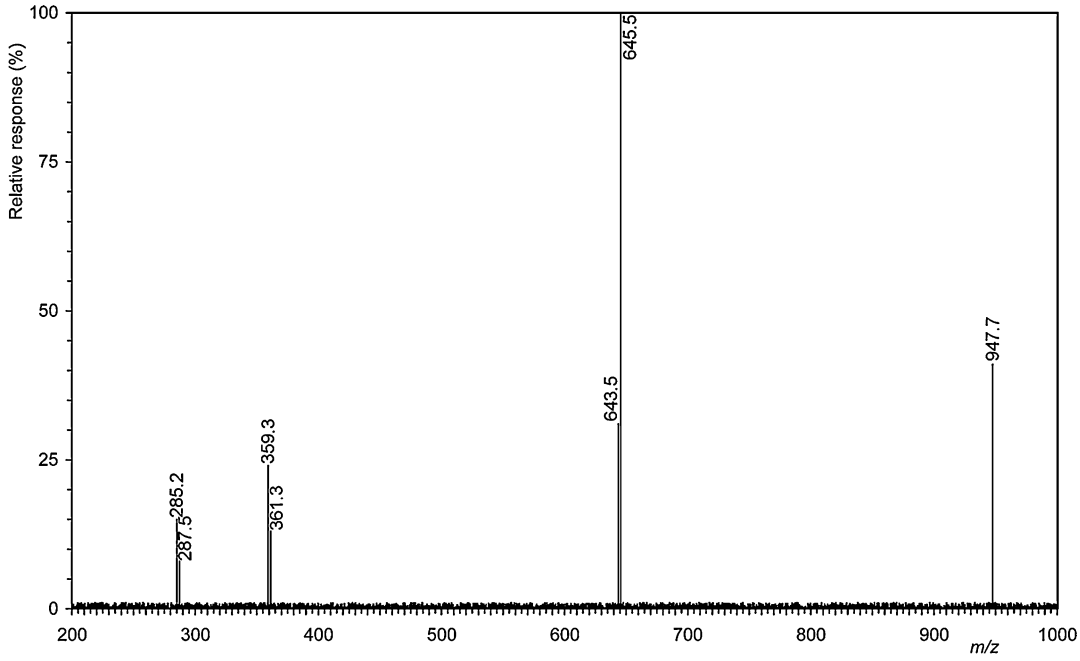
Literature	Column type	Mobile phase; temperature/ infusion solvent/matrix	Samples matrix
(Iwasaki et al. 2001)	Chiracel OF cellulose-tris-(4-chlorphenylcarbamate)	Hexane/*i*-PrOH(200/1 or 400/1)	*
(Iwasaki et al. 2001)	Chiracel OD cellulose-tris-(3,5-dimethylphenylcarbamate)	Hexane/*i*-PrOH(200/1 or 400/1)	*
(Lisa and Holcapek 2013)	Two chiral Lux Cellulose-1	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99/1, *v*/*v*)	TAG-regioisomers + enantiomers of hazelnut oil + human plasma
(Itabashi 2012)	Chiralpak IA amylose tris-(3,5-dimethylphenyl carbamate)	Hexane/ethanol (100/0.5, *v*/*v*); or hexane/*i*-PrOH (100/0.5, *v*/*v*) or methanol or acetonitrile	TAG-enantiomers
(Nagai et al. 2013)	Chiracel OD-RH (or OD-H) cellulose-tris-(3,5-dimethylphenylcarbamate)	Methanol	TAG-enantiomers from marine fish and mammals
(Rezanka et al. 2012)	CYCLOBOND™ I 2000 DMP 3,5-dimethylphenyl carbamate modified β-cyclodextrin	99:1 methanol:10 mM ammonium acetate	Diatom *Phaeodactylum tricornutum* + synthetic standards
(Rezanka et al. 2013)	Two CYCLOBOND™ I 2000 DMP 3,5-dimethylphenyl carbamate modified β-cyclodextrin	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 120 min	11 yeast strains
(Rezanka et al. 2014a)	Two Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin)	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 180 min	Microalgae
(Rezanka et al. 2015b)	Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin)	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 120 min	TAGs in different strains of the green algae *Stichococcus bacillaris*
(Rezanka and Sigler 2014)	Two Astec cyclobondTM I 2000 DMP (3,5-dimethylphenyl-carbamate modified β-cyclodextrin)	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 180 min	TAG-regioisomers + enantiomers of C16, C18 and C20 PUFAs
(Rezanka et al. 2015a)	Two Astec cyclobond™ I 2000 DMP (3,5-dimethylphenyl-carbamate modified β-cyclodextrin)	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 180 min	TAG-regioisomers + enantiomers of *Trachydiscus minutus* (PUFAs)
(Rezanka et al. 2015c)	Astec cyclobond™ I 2000 DMP (3,5-dimethylphenyl-carbamate modified β-cyclodextrin)	Gradient 90 % hexane and 10 % hexane/*i*-PrOH (99:2, *v*/*v*) to 50 % hexane and 50 % hexane/*i*-PrOH, 180 min	Odd-TAG regioisomers + enantiomers of *Khawkinea quartana* + *Mortierella alpina*



Regioisomeric Analysis of Triacylglycerols, Fig. 3 Tandem mass spectrum of synthetic EEA TAG



Regioisomeric Analysis of Triacylglycerols, Fig. 4 Tandem mass spectrum of natural AEE TAG



Regioisomeric Analysis of Triacylglycerols, Fig. 5 Tandem mass spectrum of natural EAE TAG

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