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Regioisomeric and enantiomeric analysis of triacylglycerols

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ABSTRACT

A survey of useful methods for separation and identification of regioisomers and enantiomers of triacylglycerols. Gas chromatography, gas chromatography-mass spectrometry, ^{13}C NMR determination of regioisomers by enzymatic methods, and supercritical fluid chromatography are briefly surveyed, whereas a detailed description is given of the analysis of triacylglycerols by liquid chromatography, especially with silver ion (Ag^+; argentation), and nonaqueous reversed phase liquid chromatography. Special attention is paid to chiral chromatography. Details of mass spectrometry of triacylglycerols are also described, especially the identification of important triacylglycerol ions such as $[M + H\text{-}RCOOH]^+$ in atmospheric pressure chemical ionization mass spectra.

Vegetable oils and animal fats are composed mostly of triacylglycerols (TAG), which are esters of three fatty acids (FA) with glycerol. The chemical formula of TAG is RCOO-C−CH(−OOCR′)COOCR″, where R, R′, and R″ are long alkyl chains. The fatty acids RCOOH, R′COOH, and R″COOH can all be different (ABC), or two of them may be the same (AAB), or all three may be the same (AAA). The numbers of carbon atoms in the acyl chains of natural TAG are usually 16, 18, 20, or 22. This is due to their biosynthesis, where the FA in plants and animals are biosynthesized from acetyl-CoA (malonyl-CoA) and therefore contain even-numbered chains. Some bacteria and also other organisms biosynthesize FA with odd-numbered and branched chains. The milk of ruminants contains acids with an odd number of carbon atoms, for instance 15 or 17, because these acids are produced by bacteria in the rumen. Milk fat and fish oils are taken to be the most complex mixtures of TAG. The complex character of fish oil TAG is caused by two factors; one is the biosynthesis itself and the other, which is to our mind more conducive for the complexity of TAG, is the fact that the fish are almost at the end of the food chain, which begins with plankton. Fish oils therefore contain TAG obtained from food as well as biosynthesized TAG. In contrast, common vegetable oils, e.g., olive, sunflower, or canola oils, usually do not contain more than 10 fatty acids, while the fish oils have over 100 [1].

When a given TAG is asymmetrical, i.e., one of the primary hydroxyls and also the secondary hydroxyl are linked to the same FA and the next primary hydroxyl is esterified by another FA, this configuration gives rise to regioisomers (less correctly positional isomers—they should not be confused with positional isomers of FA caused by the position of double bond(s)). If the glycerol backbone is esterified by two or three different FA, then, for instance, if the two primary hydroxyls are esterified by different FA, the TAG may be optically active, i.e., may form two enantiomers. However, the "optical activity" of these TAG is usually too low to be measured. For example, *sn*-1-oleoyl-2,3-dipalmitoyl-*sn*-glycerol (*sn*-O/P/P) has $[\alpha]_D^{20} = 0.00$ (c 7.05, $CHCl_3$) [2].

Identification of regioisomers and enantiomers is important for many reasons. Positional distribution of FA on the glycerol backbone affects the technological and physical properties of fats and hence their nutritional and sensory properties. In nature, the FA is not randomly esterified to the different *sn* positions. Thus P/O/P and O/P/P in sunflower oil are present in a ratio of 100/0, whereas in lard the ratio is 8/92 [3]. Physical properties of regioisomers such as S/L/O and S/O/L (wherein S is stearic, O is oleic, and L is linoleic acid) vary substantially. The respective melting points of the individual crystalline α and β forms [4] are −2 to −4 °C and −15 to −17 °C, and −11.5 to −13 °C and −18 to −19 °C. Since they are regioisomers, both TAG have the same FA and the same molecular weight. It is quite surprising that simply changing the position of two FA on the glycerol backbone shifts the melting point by as much as 10 °C. One should note that both compounds have the same FA, and the

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List of abbreviations

^{13}C NMR	^{13}C nuclear magnetic resonance
aiMa	Anteiso-margaric acid (14-methylhexadecanoic acid)
A	Arachidonic acid
APCI-MS	Atmospheric-pressure chemical ionization–mass spectrometry
Bu	Butyric acid
C	Caproic acid
Ca	Caprylic acid
DAG	Diacylglycerol
D	Docosahexaenoic acid
Dp	Docosapentaenoic acid
E	Eicosapentaenoic acid
ESI	Electrospray ionization mass spectrometry
ECN	Equivalent carbon number
FA	Fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectrometry
Pm	Hexadecatetraenoic acid
HPLC	High-performance liquid chromatography
iMa	Iso-margaric acid (15-methylhexadecanoic acid)
L	Linoleic acid
αLn	α-Linolenic acid
γLn	γ-Linolenic acid
LC-MS	Liquid chromatography–mass spectrometry
Ma	Margaric acid (heptadecanoic acid)
MRM	Multiple reaction monitoring
M	Myristic acid
CN	Number of carbon atoms
DB	Number of double bonds
O	Oleic acid
P	Palmitic acid
Po	Palmitoleic acid
RP-HPLC/MS-APCI	Reversed phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry
Ag^+-LC	Silver ion (Ag^+; argentation) chromatography
SCF	Supercritical fluid chromatography
S	Stearic acid
St	Stearidonic acid
MS/MS	Tandem mass spectrometry
TAG	Triacylglycerol
2D-LC NARP	Two-dimensional nonaqueous reversed-phase liquid chromatography
UPC^2	Ultraperformance convergence chromatography
V	cis-Vaccenic acid

difference is caused by a mere change of the positions of the two FA, i.e., by changing acids esterified on the primary and secondary hydroxyls.

TAG molecular structure is of great importance in terms of nutritional, biochemical, and technological aspects. During the digestion of fats, lipases hydrolyze TAG mainly to free fatty acids from the *sn*-1 (3) position. The resulting 2-mono-acyl-*sn*-glycerols are then absorbed by the cells of the small intestine.

In addition, the molecular structure of the TAG has an influence on physical properties such as the crystal structure, solubility, and viscosity of the fats and oils. The FA content itself does not provide enough information about the molecular structure of TAG.

Therefore, various analytical methods are used to separate, identify, and quantify TAG on the basis of their chemical–physical properties, which is the topic of this review. We mostly discuss chromatographic and mass spectrometric methods in the analysis of triacylglycerols, as well as the techniques for stereospecific analysis, with special attention to regioisomeric and enantiomeric analysis of triacylglycerols.

Analysis of TAG by GC and GC-MS

TAG separation by GC, preferably using GC-MS, has in our opinion only historical significance. The number of publications reporting GC analysis TAG in 2015, according to the Web of Science, is less than 1% of the studies that used liquid chromatography. It should be noted that in the 1980s of the last century this was the only method that made it possible to identify the molecular species of TAG. However, even at that time, there were many issues that remain unresolved to this day. Above all is the method of injecting TAG on the column. Only two meaningful techniques were used, cold on-column injection and on-column injection at high oven temperature. Another unpleasant property of TAG is their thermal instability, which involves decomposition on the column. Thus vegetable oils such as linseed oil cannot be analyzed because the TAG containing linoleic and linolenic acids are degraded. A further problem was identification, because the commonly used electron impact ionization does not give satisfactory results. This last drawback was partially circumvented by using chemical ionization, described in some detail by Řezanka and Mareš [5]. A crucial shortcoming is that neither regioisomers nor enantiomers can be separated by GC. One of the few exceptions that have been published is the work of Kemppinen and Kalo [6], who analyzed butter oil on a polarizable phenylmethylsilicone capillary column and separated regioisomers of TAG containing, e.g., Bu/P/P and P/Bu/P or C/M/O and M/C/O (M is myristic acid). Unfortunately, as the authors themselves write, only regioisomers of TAG containing butyric (Bu) and caproic (C) acids were separated.

All these problems and drawbacks have led to the abandonment of TAG analysis using GC (GC-MS) and to the development of TAG analysis by liquid chromatography. The review by Mareš [7] describes all the problems and indicates some solutions to these problems.

Analysis of TAG regioisomers by ^{13}C NMR

^{13}C NMR spectroscopy is another possible way to identify the regioisomers, i.e., acids esterified in the α (*sn*-1/3) and the β (*sn*-2) positions [8]. Based on the chemical shifts of the ester group, i.e., about δ 172 ppm, it is possible to distinguish bonds on the primary hydroxyl (δC_1 value of an acid esterified at the primary hydroxyl group is about 0.4 ppm higher; e.g., the values for triolein are δ 173.215 and δ 172.807 ppm). The method has a big disadvantage in that, unless the regioisomers have been separated beforehand, it always measures the sum of FA bound to the hydroxyl(s). Enantiomers of TAG cannot be determined by ^{13}C NMR.

Determination of regioisomers by enzymatic methods

Determination of the FA at the *sn*-2 and thus identification of regioisomers can be also done using lipases, e.g., pancreatic lipase or the mold *Rhizopus arrhizus* lipase. The fatty acids are hydrolyzed

from primary hydroxyls of TAG to give 2-mono-acyl-*sn*-glycerols. These can be further chemically hydrolyzed and FA at the *sn*-2 can be determined. The method has two main drawbacks—without separation of the molecular species of TAG it determines all the FA on the primary and all FA on secondary hydroxyls, and it does not allow the determination of enantiomers. The method of using a sequence of reactions, including enzymatic, is shown in Fig. 1. Although the preparation of TAG involves racemization (inter- or intramolecular esterification), by selecting appropriate reaction conditions [9], the TAG can be obtained with enantiomeric purity of over 90%, which is sufficient for the identification of individual enantiomers.

Supercritical fluid chromatography

Supercritical fluid chromatography (SCF) is a method that uses a pressurized gas, usually CO_2, above its critical temperature and critical pressure as a mobile phase. Both packed and capillary columns were used in the analysis of TAG by SFC. TAG were usually eluted from the capillary column by supercritical carbon dioxide and detected by mass spectrometry (MS). A better separation is ensured by programming the temperature and pressure, and also by the use of modifiers, i.e., different mixtures of organic solvents (methanol, acetonitrile, etc.), which increase the mobile phase polarity or density or deactivate and/or solvate or induce swelling of the stationary phase. Triacylglycerols were separated based on FA chain length using a reverse phase column. However, the resolution obtained by SFC was not as good as that using high-performance liquid chromatography (HPLC). A completely new approach to the analysis of TAG has therefore been described in the last few years, which represents a “rediscovered” supercritical fluid chromatography, now called Ultraperformance Convergence Chromatography (UPC^2) [10].

The great advantage of SFC is substantial shortening of the time of analysis. Thus Lee [11] analyzed TAG soybean oil in 8 min, even though regioisomers were not separated. A packed column, i.e., a sulfonic silica-based strong cation exchanger (Nucleosil 100-5) loaded with silver ions, was used to separate sunflower oil TAG, and again regioisomers were not separated but were identified by APCI-MS [12]. Only recently were regioisomers not only identified but also chromatographically separated [13]. The authors managed to separate, for instance, S/P/Ln (tR 23.33 min) and S/Ln/P (tR 17.96 min) and also other (P/P/Ln-P/Ln/P, P/P/L-P/L/P, P/P/O-P/O/P, S/P/Ln-S/Ln/P, S/P/O-S/O/P, S/S/O-S/O/S) regioisomers from palm and canola oils. The proof of structure was performed using multiple reaction monitoring (MRM-ESI) based on the abundances of ions of the type $[DAG]^+$.

In conclusion, we believe that the potential of SFC or the newly developed UPC^2 has not yet been exhausted. To our current knowledge, enantiomers of TAG have not yet been separated.

Silver ion (Ag^+; argentation) chromatography

Separation of substances having multiple bonds (double bond(s) of acyl chains in the case of TAG) is based primarily on the interaction of Π electrons of double bond(s) with silver ions, better with electrons in *d* orbitals. Though these interactions are relatively weak and reversible, they nevertheless allow the separation of TAG on a chromatographic column packed with an Ag^+-based stationary phase. Separation depends primarily on the number of double bond(s), on their distribution among the acyl groups of the same molecule, on the configuration and position of double bond(s), and on whether the FA is bonded to a primary or a secondary hydroxyl. The last of these tenets enables the separation of regioisomers. The stationary phase used exclusively nowadays is one in which phenylsulfonic acid groups (strong cation exchangers) are chemically bonded to silica. Though such a stationary phase can be homemade [14], these columns are commercially available and can be used immediately after purchase. As mentioned above, one of the main mechanisms of retention is based on the interaction of the Π electrons of the double bond(s). The separation process often involves other interactions, such as the formation of chelate complexes. This is manifested, for instance, by TAG containing one linoleic acyl being retained more strongly than TAG with two oleic acyls, although the number of double bonds is the same. Other cases of complex formation have been described by Nikolova-

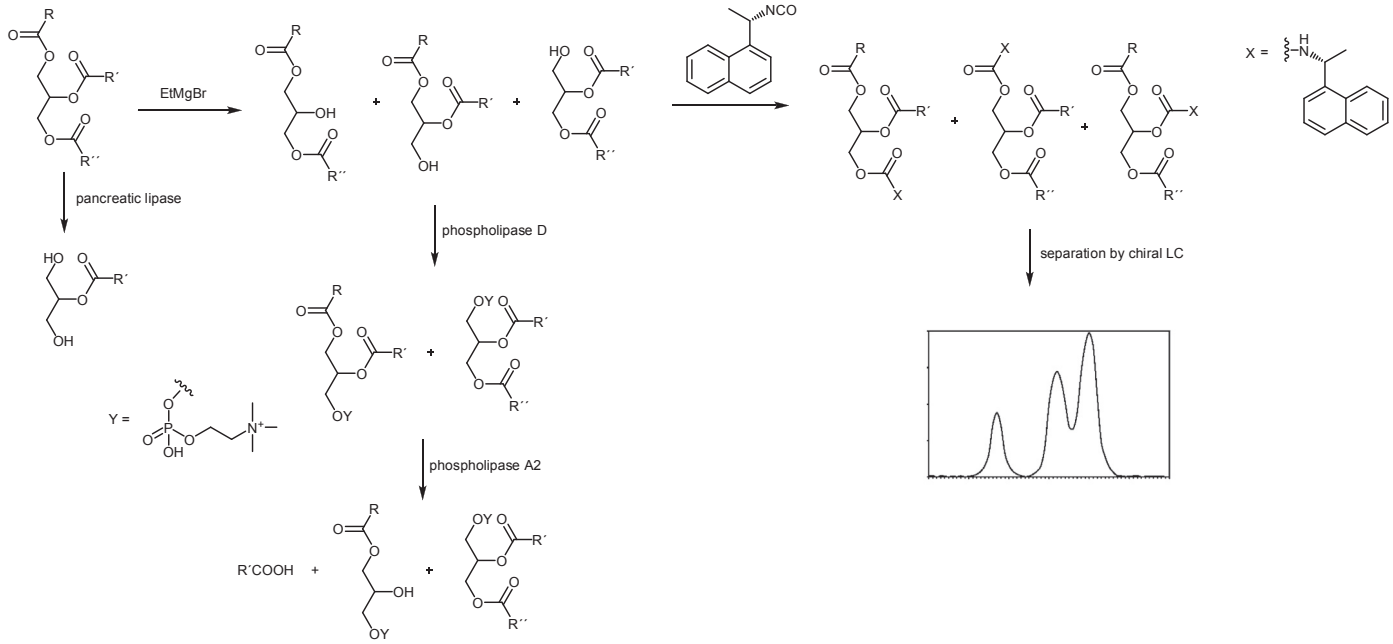

Fig.1. Determination of TAG structure by specific enzymes and by combination with chemical reactions.

Damyanova [15]. The chromatography of regioisomeric TAG, which is our case here, involves predominantly steric effects, where the double bonds of acyl groups bonded to the primary hydroxyls of the glycerol backbone (*sn*-1 and/or *sn*-3) are sterically less hindered and therefore form stable complexes with Ag^+.

With few exceptions, Ag^+-LC has been used to analyze only vegetable oils [16] or animal fats [17] with a maximum of 11 double bonds [3]. While the separation of two regioisomers, i.e., O/Ln/Ln and Ln/O/Ln, presented no problems, regioisomers with the same number of carbon atoms and with one more double bond, i.e., L/Ln/Ln and Ln/L/Ln, were separated with considerable difficulty [3]. One of the most complex natural oils—fish oil—was analyzed only exceptionally by silver ion LC, and these studies are relatively old. One-dimensional LC has not yet been used to identify single molecular species in TAGs with more than 10 double bonds [18,19]. Only Beccaria [20] identified molecular species of D/D/D, D/D/E, D/D/St, and D/E/E from menhaden oil using two-dimensional LC, but not their regioisomers and enantiomers. Therefore, all currently known studies describe the separation and identification of regioisomeric TAG only up to 8 double bonds.

One of the first papers dealing with silver ion LC of both synthetic TAG and vegetable oils described the analysis of oil from the seeds of cloudberry, evening primrose, borage, alpine currant, and black currant. Synthetic standards were separated, e.g., αLn/αLn/αLn from γLn/γLn/γLn and O/γLn/O from O/O/γLn. Unfortunately, the authors did not observe the separation of regioisomers of seed oil TAG. Identification of regioisomers by atmospheric-pressure chemical ionization MS (APCI-MS) was based on the ratio of $[M\text{-}R_iCOO]^+$ to $[M\text{-}R_2COO]^+$ ions [21].

Lísa [3] analyzed TAG containing four acids—P, O, L, and Ln—i.e. TAG with up to 8 double bonds; individual regioisomers were separated almost without problems. Their study shows how necessary it is to use standards and also MS for identifying individual molecular species of TAG. Randomization of PPP and LnLnLn yields six molecular species. While a symmetrical TAG with three double bonds, i.e., PLnP, has a lower retention time than asymmetric (LnPP), an opposite retention order is found with TAG with six double bonds, LnLnP (asymmetric TAG) being eluted before the symmetric counterpart (LnPLn). The same work describes the determination of the ratio of regioisomers in two natural samples, sunflower oil and lard, and, as expected, the vegetable oil and animal fat differed dramatically in the proportion of individual regioisomers—the L/L/P—L/P/L ratio was 97:3 in the oil and 9:91 in the lard.

Ready separation and identification of regioisomers of TAG (S/S/O-S/O/S, S/L/S-S/S/L, S/O/O-O/S/O, etc.) from interesterified palm oil by Ag^+-LC-electrospray mass spectrometry (ESI-MS) has been described [22].

Off-line two-dimensional chromatography (2D) consisting of nonaqueous reversed-phase LC (NARP-LC) and Ag^+-LC was used to analyze TAG of rice oil. APCI-MS was employed to detect the ratio of regioisomers of some TAG; for instance, the L/L/O—L/O/L ratio was 86:14 [23].

The regioisomeric structures of TAG αLn/L/L and γLn/L/L in black currant seed oil were determined by Ag^+-LC/APCI-MS and Ag^+-LC/ESI-MS/MS techniques (MS/MS is tandem mass spectrometry). It was found that with both acids (αLn and γLn), the abundance of the asymmetric TAG (γLn/L/L and L/L/γLn) was always higher than that of the symmetrical (L/γLn/L). The same holds true even more for the TAG with αLn (αLn/L/L and L/L/αLn versus L/αLn/L). Ions of the type $[M + H\text{-acid}]^+$ (APCI) and ions $[M+^{109}Ag\text{-acid}]^+$ (ESI-MS/MS) were used to determine the ratio of regioisomers [24]. An off-line 2D chromatographic method—NARP-LC in the first dimension and Ag^+-LC in the second dimension—enabled the separation of TAG from black currant oil. The TAG with up to eight double bonds, e.g., Ln/Ln/L and Ln/L/Ln, were again separated by Ag^+-LC and identified by positive-ion APCI [25].

The separation of symmetrical disaturated triacylglycerols, predominantly nonsymmetrical triacylglycerol isomers S/S/O, S/S/L, and S/S/Ln and symmetrical regioisomers, by Ag^+-LC on a semipreparative scale was concerned [26].

Ag^+-LC/APCI-MS was used for the separation of regioisomeric TAG acquired by randomization of P/P/P, S/S/S, A/A/A, O/O/O, L/L/L, Ln/Ln/Ln, and γLn/γLn/γLn. The resulting TAG were separated on three columns connected in series, and APCI spectra were measured in five different mass spectrometers. On the basis of the abundance of $[M + H\text{-}R_iCOOH]^+$ ions, individual separated regioisomers of TAG were identified. However, some TAG, such as O/Ln/Ln and O/γLn/Ln or O/Ln/γLn and O/γLn/γLn, defied clear separation. The retention time was increasing in regioisomers having more unsaturated fatty acyls in the *sn*-1/3 positions, which means that O/O/Ln has a longer retention time than O/Ln/O [17].

Off-line 2D-LC NARP and Ag^+-LC with APCI-MS were used to analyze peanut oil, which is characterized by a small amount of Ln. In the second dimension (after separation of the mixture by NARP-LC), individual regioisomers of the TAG were identified by mass spectra using $[M + H\text{-RCOOH}]^+$ ions [1]. 2D chromatography, both on-line and off-line, in this case Ag^+-SFC and NARP-LC in conjunction with a mass spectrometer (APCI), was used to analyze the molecular species of TAG from fish oil (menhaden oil) [27]. One of the few drawbacks was the long time needed for separation. Ag^+-LC was employed to separate TAG into 18 fractions (up to 18 double bonds) taking nearly 3 h. The individual fractions were further separated and identified by LC-NARP and identified by APCI-MS. For instance, three molecular species, D/E/D, D/D/E, and D/Dp/D, were identified in fraction 17. A total of 324 TAG including regioisomers were identified. This work is one of the most complex studies identifying regioisomers of TAG in fish oil. As the authors point out, the number of actually present TAG is much higher, as more than 100 FA were found in menhaden oil by GC-MS [28], which would, in accordance with Table 1, point to the presence of some half a million TAG excluding enantiomers [27].

Natural TAG having up to 12 double bonds were separated from the snow alga *Chloromonas* by Ag^+-LC/APCI-MS (Pm/Pm/Pm, Pm/Pm/St, Pm/St/St, and St/St/St, where Pm is 4,7,10,13—16:4 and St is 6,9,12,15—18:4). Two TAG, Pm/Pm/St and Pm/St/St, were partially separated into individual regioisomers (Pm/Pm/St and Pm/St/Pm, and Pm/St/St and St/Pm/St) that were identified by APCI-MS [29].

The use of silver ion LC allows the separation of TAG by the number of double bonds. The separation has been routinely carried out only with common vegetable oils, but it should be noted that oils from hemp seed, borage, various currants, etc., present problems. This is reflected in the fact that the separation of, e.g., St/Ln/Ln from Ln/St/Ln has not been performed. As an example of successful separations may serve the separation of regioisomers of Ln/Ln/Ln and/or γLn/γLn/γLn in vegetable oils in which the number of double bonds reaches a maximum of 9. Thus the regioisomers may have a maximum of 8 double bonds, e.g., L/Ln/Ln versus Ln/L/Ln, possibly in combination with γLn. For people working in the field, these oils can be separated and identified with almost no problem. Dozens of vegetable oils are commonly used both for nutrition and for industrial purposes, e.g., production of biodiesel. Oils in which TAG with more than 10 double bonds predominate have analyzed only rarely and with difficulty (oils containing stearidonic acid). The situation is even worse with oils such as fish oils. 2D chromatography currently appears to be the method of choice, preferably in an off-line arrangement.

Table 1
The number of possible TAGs.

Description	Number of possible TAGs	Number of possible TAGs for $y = 10$
Without isomers	$x = (y^3 + 3y^2 + 2y)/6$	220
Without enantiomers	$x = (y^2 + y^3)/2$	550
All isomers	$x = y^3$	1000

Note: x is the number of TAGs; y is the number of FAs in TAGs.

Nonaqueous reversed-phase liquid chromatography

The resolution of molecular species of TAG by a NARP-LC in connection with a mass spectrometer and usually positive or negative APCI or ESI has been described many times. Separation of TAG on a reversed phase is one of the common and very frequent methods of TAG analysis. The efficiency of separation of TAG on a reversed phase depends mainly on the length of the chains of the three fatty acyl moieties, where each double bond reduces the effective chain length by ~2 carbon atoms, thereby separating the so-called critical pairs.

Critical pairs are those TAG that have the same equivalent carbon number (ECN), which is defined as ECN = CN − (2 × DB), where CN is the number of carbon atoms and DB is the number of double bond(s). As an example, we can mention two TAG, O/O/O 48 = 54 − (2 × 3) and P/P/P 48 = 48 − (2 × 0). The position of the double bond(s) is also important, as seen in studies concerned with the separation of TAG with oleic and cis-vaccenic acids or α- and γ-linolenic acids.

Many different types of reverse phases with different chain lengths have been tested, but the C18 phase is used almost exclusively in practice. Phases C8 and C30 did not bring any significant improvements in separation. This is understandable, since most natural TAG comprise even-numbered FA in the range C16–C22, and a stationary phase that is similar to the chain length of the acyl chains of the separated TAG maximizes interaction and has the greatest efficiency. Column length or its effectiveness (number of theoretical plates) plays an essential role in the separation. However, increasing the length of the column, especially when combining several columns in series, also increases the retention time, so it is advisable to compromise between efficiency and retention time. For example, in separating branched chain TAG (containing iso- and/or anteiso-heptadecanoic acids), it took hours to elute them from the column [30].

Today's modern mass spectrometers are capable of analyzing small quantities of substances, so it is preferable to use columns 2.1 mm or even less in diameter because of lower solvent consumption. Solvent mixtures with acetonitrile and different modifiers, e.g., isopropanol or acetone, are most commonly used as a mobile phase, predominantly for gradient elution. A number of studies deal with optimizing the separation, e.g., by selecting a suitable stationary phase [31], mobile phase [32], length of the column [31], or column temperature [31]. Currently the only suitable detector is MS, which is indispensable for identifying regioisomers.

One of the first papers dealing with separation and identification of regioisomers, published 20 years ago by Kalo [33], describes separation of TAG generated by randomization of three TAG, tributyrin, trilaurin, tristearin, using gas chromatography and also RP-HPLC on a C18 column. Individual regioisomers, for instance S/Bu/S and S/S/Bu, were identified after separation by liquid chromatography using ^{1}H NMR.

One of the most complex animal fats in terms of the number of fatty acids and thus TAG is butterfat. Common butter contains FA with a wide range of chain lengths (most of all natural fats) including odd-chain and unsaturated FA. Kalo [34,35] attempted separation and identification of regioisomers, which was successful with TAG having short- and long-chain FA (S/Bu/S versus S/S/Bu or O/Bu/Bu versus Bu/O/Bu).

A study describing the analysis of butterfat using normal-phase LC/ESI-MS was published by Kalo [36]. It describes the analysis of hundreds of TAG including the separation and identification of regioisomers (P/Bu/Bu versus Bu/P/Bu or 19:0/O/S versus O/19:0/S). TAG containing odd chains (O/P/23:1) were identified but TAG containing branched chain fatty acid(s) were not.

The most frequently analyzed were vegetable oils and TAG in them, mainly for reasons of easy accessibility of these oils and also because of their importance for the food industry and technological uses.

Momchilova [32,37] described separation of regioisomers of P/O/P-P/P/O, P/L/P-P/P/L, P/E/P-P/P/E, and P/D/P-P/P/D with acetonitrile as the mobile phase modified by methanol, ethanol, 2-propanol, 1-propanol, acetone, or dichloromethane. The retention times were very long, often hundreds of minutes. The latter publication reported the separation of additional regioisomers, for instance P/Ln/P-P/P/Ln and P/A/P-P/P/A. The retention times again reached hundreds of minutes. According to the authors, retention times could be significantly shortened by using a gradient; this was fully confirmed in later studies.

Kuroda [38] separated two regioisomeric TAG, O/P/O and O/O/P, at different temperatures with a recycled HPLC system. The study brought no breakthrough; the analysis again took hundreds of minutes and the number of cycles was at least 5.

One theoretical and two practical uses of two LC-MS for separation of regioisomers of TAG were published by Mottram and Evershed [39] and Mottram [40,41]. The first publication of Mottram is considered pioneering in this field and first defined the rule "The neutral loss of FA from the *sn*-2 position provides the fragment ion, i.e., $[M + H\text{-}RCOOH]^+$, with a lower relative abundance in comparison to *sn*-1 and *sn*-3 positions". This rule applies regardless of the type of instrument and also regardless of the type of the analyzed TAG; see, e.g., Holčapek [15]. While in the first two studies regioisomers were not separated, the third, published three years later, could make use of a highly efficient column that made it possible to separate the regioisomeric O/P/O-O/O/P, S/O/S-S/S/O, etc., found in animal (beef, chicken, lamb, and pork) fats. The rarely used propionitrile was employed for elution. All regioisomers were identified by the abundance of $[M + H\text{-}RCOOH]^+$ ions, $[AA]^+$ and $[AB]^+$.

Two regioisomers, L/L/O and L/O/L, were precisely determined in vegetable oils (grape seed, olive, pumpkin seed, soybean, sunflower, and wheat germ oils) using a calibration curve made of pure TAG using LC/APCI-MS in selected ion monitoring (SIM) mode based on the relative abundances of the $[LL]^+$ and $[LO]^+$ diacylglycerol fragment ions $[M + H\text{-}RCOOH]^+$ [42]. It was found that all oils except olive oil always contain L/L/O-L/O/L in a certain proportion (grape seed, sunflower, pumpkin seed, soybean, and wheat germ oils accounted for the sum of the total molecular species equal to 44.2, 26.8, 16.7, 15.9, and 13.9%, respectively) [42], which confirms the hypothesis that the different TAG in vegetable oils are synthesized stereospecifically.

The representation of the molecular species of TAG was

Table 2
Selected examples of TAG analyzed by Ag-LC and NARP-LC.

Reference	Source	Type of analysis	Types of TAG
[27]	Snow alga *Chloromonas*	Ag^+-LC/APCI-MS	Pm/Pm/St – Pm/St/Pm, Pm/St/St – St/Pm/St
[25]	Menhaden oil	Ag^+-SFC/APCI	18 fractions (up to 18 double bonds)
[1]	Peanut oil	Ag^+-LC/APCI-MS	P/O/O-O/P/O
[15]	Synthetic TAG	Ag^+-LC/APCI-MS	O/Ln/Ln – O/γLn/Ln, O/Ln/γLn – O/γLn/γLn
[24]	Synthetic TAG	Ag^+-LC	S/S/O-S/O/S, S/S/L-S/L/S
[23]	Black currant seed oil	Ag^+-LC/APCI-MS and Ag^+-LC/ESI-MS/MS	αLn/L/L – γLn/L/L
[21]	Rice oil	Ag^+-LC/APCI-MS	L/L/O-L/O/L
[3]	Sunflower oil and lard	Ag^+-LC/APCI-MS	L/L/P-L/P/L
[19]	Synthetic TAG	APCI-MS	O/γLn/O – O/O/γLn
[52]	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
[51]	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
[28,50]	Synthetic and natural TAG	NARP-LC/APCI-MS	P/Ma/Ma – P/aiMa/aiMa, P/Ma/Ma – P/iMa/iMa
[49]	Bacterium *R. Erythropolis*	NARP-LC/APCI-MS	Po/15:0/Po – 15:0/Po/Po, O/Ma/O – Ma/O/O
[48]	Bacterium *R. Erythropolis* and plant *D. vulgaris*	RP-HPLC/APCI-MS	P/P/P13 – P/P13/P, P/P/P14 – P/P14/P
[44]	Green algal TAG after hydrogenation	RP-LC/ESI-MS/MS	P/P/S-P/S/P, S/S/P-S/P/S
[42,43]	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,
[41]	Beef, pork, and chicken fats, palm oil, and 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
[40]	Grape seed, sunflower, pumpkin seed, soybean, and wheat germ oils	LC/APCI-MS	L/L/O-L/O/L
[37–39]	Beef, chicken, lamb, and pork fats	RP-HPLC/APCI-MS	O/P/O-O/O/P, S/O/S-S/S/O
[36]	Synthetic TAG	recycle HPLC system	O/P/O – O/O/P
[30]	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
[35]	Synthetic TAG	NARP-LC	P/Ln/P-P/P/Ln, P/A/P-P/P/A
[34]	Butterfat	normal-phase LC/ESI-MS	P/Bu/Bu – Bu/P/Bu, 19:0/O/S – O/19:0/S
[32,33]	Butterfat	normal-phase LC/ESI-MS	S/Bu/S – S/S/Bu, O/Bu/Bu – Bu/O/Bu

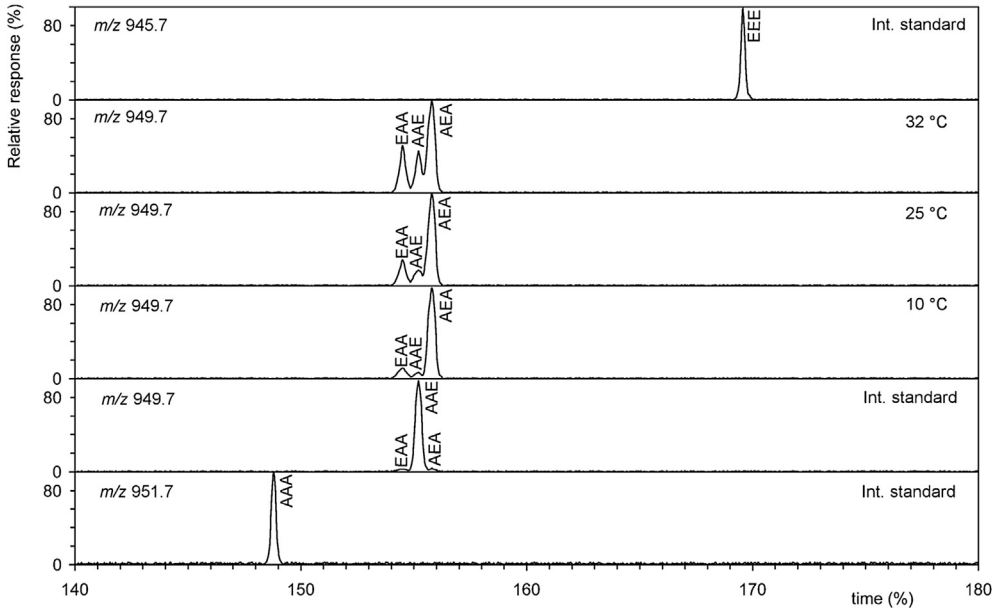

Fig.2. Chiral-LC of TAGs (SIM—selected ion monitoring) from alga *Trachydiscus minutus*, cultivated at various temperatures (10, 25, and 32 °C).

determined in vegetable oils (palm oil, cocoa butter) and animal fats (beef, pork, and chicken fats) on the basis of calibration curves prepared from seven pairs of regioisomers [43]. The difference in relative abundances of fragment ions of the type $[AA]^+$ versus $[AB]^+$ was used to determine each regioisomer in an AAB/ABA pair, again without HPLC separation.

Leskinen [44,45] identified regioisomers (e.g., L/O/L-L/L/O, O/L/O-L/O/O, O/P/O-P/O/O, and P/O/P-P/P/O) in lard, rapeseed, sunflower seed, palm, black currant seed, and sea buckthorn pulp oils using both APCI and ESI, based on the abundance of the ion $[M\text{-}H\text{-}RCOOH\text{-}100]^-$. The various regioisomers were merely determined by mass spectrometry, not chromatographically separated. The authors, however, managed to separate TAG containing positional isomers of FA, i.e., oleic and cis-vaccenic acid (Po/V/Po and Po/O/Po), from sea buckthorn pulp oil, or αLn/L/L and γLn/L/L from black currant seed oil.

Determination of regioisomers using RP-LC/ESI-MS/MS with multiple reaction monitoring (MRM) was employed, e.g., in specific quantification of TAG in lipid extracts of algae of two genera, *Chlamydomonas reinhardtii* and *Coccomyxa subellipsoidea*, for the

Table 3
Survey of literature data on the separation of TAGs on chiral columns.

Reference	Column type	Mobile phase; temperature/infusion solvent/ matrix	Ion source analyzer	Mass range (*m*/*z*)	Samples matrix
[55]	Chiracel OF cellulose-tris-(4-chlorphenylcarbamate)	Hexane/iproh (200/1 or 400/1)	UV, 210 nm		
	Chiracel OD cellulose-tris-(3,5-dimethylphenylcarbamate)	Hexane/iproh (200/1 or 400/1)	UV, 210 nm		
[56]	Two chiral Lux Cellulose-1	Gradient 90% hexane and 10% hexane/iproh (99/1, *v*/*v*)	Positive-ion APCI mode	50 −1200	TAG-regioisomers + enantiomers of hazelnut oil + human plasma
[57]	Chiralpak IA amylose tris-(3,5-dimethylphenyl carbamate)	Hexane/ethanol (100/0.5, *v*/*v*); or hexane/iproh (100/0.5, *v*/*v*) or methanol or acetonitrile	Positive-ion APCI mode	500 −1000	TAG-enantiomers
[58]	Chiracel OD-RH (or OD-H) cellulose tris-(3,5-dimethylphenylcarbamate)	Methanol	Positive-ion APCI mode	500 −1000	TAG-enantiomers from marine fish and mammals
[63]	Astec cyclobond I 2000 DMP 3,5-dimethylphenyl carbamate modified β-cyclodextrin	99:1 methanol:10 mm ammonium acetate	Positive-ion APCI mode	200 −1000	Diatom *Phaeodactylum tricornutum* + synthetic standards
[65]	Two Astec cyclobond I 2000 DMP 3,5-dimethylphenyl carbamate modified β-cyclodextrin	Gradient 90% hexane and 10% hexane/iproh (99:2, *v*/*v*) to 50% hexane and 50% hexane/iproh 120 min	Positive-ion APCI mode	200 −1100	11 yeast strains
[64]	Two Astec cyclobond I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin)	Gradient 90% hexane and 10% hexane/iproh (99:2, *v*/*v*) to 50% hexane and 50% hexane/iproh 180 min	Positive-ion APCI mode	200 −1100	Microalgae
[67]	Astec cyclobond I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin)	Gradient 90% hexane and 10% hexane/iproh (99:2, *v*/*v*) to 50% hexane and 50% hexane/iproh 120 min	Positive-ion APCI mode	200 −1100	TAGs in different strains of the green alga *Stichococcus bacillaris*
[68]	two Astec cyclobond I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin)	Gradient 90% hexane and 10% hexane/iproh (99:2, *v*/*v*) to 50% hexane and 50% hexane/iproh 180 min	Positive-ion APCI mode	200 −1100	TAG-regioisomers + enantiomers of C16, C18 and C20 PUFAs
[69]	Two Astec cyclobond I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin)	Gradient 90% hexane and 10% hexane/iPrOH(99:2, *v*/*v*) to 50% hexane and 50% hexane/iPrOH 180 min	Positive-ion APCI mode	200 −1000	TAG-regioisomers + enantiomers of *Trachydiscus minutus* (PUFAs)
[70]	Astec cyclobond I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin)	Gradient 90% hexane and 10% hexane/iPrOH(99:2, *v*/*v*) to 50% hexane and 50% hexane/iPrOH 180 min	Positive-ion APCI mode	200 −1100	Odd-TAG regioisomers + enantiomers of *Khawkinea quartana* + *Mortierella alpina*

purpose of determining the evolutionary divergence in regioisometry among green microalgae [46].

Separation of TAG containing PUFAs from tuna and algae was performed on two C18 columns in series. Individual regioisomers were determined by high-resolution tandem MS, but were not separated using liquid chromatography [47]. Some studies performed the analysis of regioisomeric TAG using MS alone, including high-resolution tandem MS [48]. Byrdwell [49] published a study on the theory of abundance of TAG-derived ions.

TAG having odd-numbered omega-phenyl alkanoic acids from seeds of the flower plant *Dracunculus vulgaris* and TAGs from the bacterium *Rhodococcus erythropolis* prepared by precursor-directed biosynthesis from phenylalanine and having the corresponding even-numbered omega-phenyl alkanoic acids were separated by RP-HPLC/MS-APCI [50]. Regioisomers P/P/P13–P/P13/P and P/P/P14–P/P14/P were determined (but not separated) based on abundance ratios of ions of the type $[DAG]^+$ at 551 and 585 Da, and at 551 and 599 Da, respectively.

Odd- and branched-chain fatty acids of the iso and anteiso series occur in many bacteria as the major FA. They are also present in milk fat, which is largely derived from rumen bacteria. We have focused on the analysis of TAG with odd FA and with branched-chain FA. *R. erythropolis* was chosen as a model organism that produces both odd and also iso- and anteiso-FA. Both separation by NARP-LC and identification of regioisomeric TAG with odd FA, i.e., Po/15:0/Po and 15:0/Po/Po, O/Ma/O and Ma/O/O, etc., by positive APCI has been published [51].

NARP-LC was used in another study to separate three synthetic TAG (Ma/Ma/Ma, iMa/iMa/iMa, and aiMa/aiMa/aiMa) and other TAG such as P/Ma/Ma and P/aiMa/aiMa or P/Ma/Ma and P/iMa/iMa [30]. In the case of a natural mixture the separation was much worse, and only TAG with linear and branched FA could be separated. Moreover, APCI mass spectra of TAG with linear and branched FA are not identical, as was later confirmed [52].

Algae and cyanobacteria as representatives of photosynthetic lower microorganisms have significantly different metabolisms, which is reflected in the biosynthesis of fatty acids. Both groups are biosynthesizing primarily PUFAs whose biosynthesis does not occur in higher, mainly flowering plants. An example is the entire group of mainly C16 and C18 PUFAs, i.e., FA with two to four double bonds (16:3n-3 and 16:3n-4 or 18:4 and 18:5 acids), and of course all C20 and C22 PUFA such as arachidonic, eicosapentaenoic, and docosahexaenoic acids. These acids are absent in higher plants without genetic manipulation.

We have therefore focused our work on these PUFAs. TAG having these acids were separated by NARP-LC and identified by MS; see Table 2.

Separation of regioisomers (P/P/C-P/C/P, P/P/O-P/O/P, P/O/O-O/P/O, and P/P/D-P/D/P) was performed by LC/APCI-MS/MS for both standards and for TAG of milk from various mammals and also for cheeses [53].

Separation of regioisomers depending on column temperature, e.g., 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, and P/E/E-E/P/E on a C28 column has been described [54].

Enantiomers

Analysis of enantiomers of TAG is usually carried out by classical methods combining several reaction steps; see Fig. 1. Although the sequence of reactions and the separation of the individual derivatives were successful, an unsolved problem still remained: individual FA were determined in positions sn-1, sn-2, and sn-3 but if the TAG had not been previously separated, the FA were determined depending only on the complexity of the starting mixture. In other words, if a mixture of TAG was taken into the reaction, total FA were determined in each sn position, not in each TAG. Only the use of chiral LC-TAG (Fig. 2) made it possible to separate and identify individual molecular species—enantiomers. The first studies pointing to the possibility of separation of enantiomers of TAG were published over 10 years ago, but they were performed only with semisynthetic derivatives (i.e., TAG not normally found in nature), such as TAG having two caprylic acids and eicosapentaenoic acid (Ca/Ca/E versus E/Ca/Ca) [55]; see also Table 3. It was necessary to wait over 10 years until the advent of chiral LC and its application to a natural mixture—hazelnut oil and TAG from human plasma [56].

The separation is most commonly performed on a stationary phase based on cellulose-tris-(3,5-dimethylphenylcarbamate) or cellulose-tris-(3-chloro-4-methylphenyl carbamate). Hexane-2-propanol or methanol can be used as a mobile phase [57]. In the case of the enantiomers it is always necessary to obtain a standard of at least one of the two possible enantiomers, because the order of elution from the column is practically impossible to predict. Just a minor modification of the stationary phase, e.g., Cl replacement for methyl (cellulose-tris-(3,5-dimethylphenylcarbamate) versus cellulose-tris-(3-chloro-4-methylphenyl carbamate)) suffices to reverse the order of elution [58].

In other publications, the authors focused on microbial oils from bacteria [59] and eukaryotic microorganisms producing TAG, which are widely used in the food industry and with potential use as third-generation biodiesel [60]. Thousands of articles have been devoted to the overproduction of TAG in microorganisms, mainly using genetic manipulation and optimization of culture conditions; however, only dozens deal with the analysis of microbial oils, i.e., TAG identification. A brief overview of some interesting sources containing unusual TAG is shown below.

Many of our studies [29,61–70]—see Table 3—have described the separation of regioisomers and enantiomers from algae, cyanobacteria, and fungi, in other words from lower organisms. These organisms are characterized by much richer and more branched fatty acid biosynthesis than higher plants. Especially algae produce both even- and odd-numbered PUFAs, which, save for three PUFA (α-18:3 and γ-18:3 and −18:4), are not found in higher plants. These are mainly 16:3, 16:4, 18:5, 20:4, 20:5, and 22:6 acids (e.g., Figs. 3S–7S), to name just the major ones. Table 3 gives the conditions under which the separation and identification of regioisomers and enantiomers was carried out, as well as their sources. We would like to mention, e.g., the separation of odd-numbered PUFA from fungi and protozoa, which involved the isolation and identification of both regioisomers and enantiomers of odd-numbered C19 and C21 PUFA that occur as minor FA in nature [71,72].

Our results may serve to defining the rule that the isomer with the unsaturated acyl residue in either the 1- or 3-position is retained more strongly on the column than the respective 2-isomer. This phenomenon is assumed to be due to the fact that TAGs with two neighboring saturated acyl residues (i.e., 1,2 or 2,3) are retained more strongly than the respective 1,3-isomers due to the interaction of the stationary phase with double bonds.

Conclusions

Finally, we would like to highlight a few basic ideas that should be taken into account in the separation and identification of regioisomers and enantiomers of TAG. Regioisomers can be separated readily on a sufficiently effective column. Efficiency is achieved primarily by combining several columns in series; this, however, increases the retention time. It is therefore appropriate to optimize both the resolution and the duration of analysis. Separation of regioisomers before the assay appears to be much more suitable than their determination based on the ion abundances in MS. If both regioisomers are available, then one can very accurately determine the representation of individual regioisomers from the calibration curve (TAG mixtures in several ratios). If the calibration curve is missing, then one can only roughly estimate the ratio of regioisomers. In the case of the enantiomers, it is necessary to have at least one of them. We can thus state, may be somewhat surprisingly, that the limiting factor is not an effective column or a low-power mass spectrometer, but the lack of standards. This implies that regioisomers can be determined by means of mass spectra, since they have different intensities of $[RCOO\text{-}M]^+$ ions, while enantiomers have exactly the same MS spectra, which is not surprising.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2016.05.028.

References

[1] J. Hu, F. Wei, X.Y. Dong, X. Lv, M.L. Jiang, G.M. Li, H. Chen, Characterization and quantification of triacylglycerols in peanut oil by off-line comprehensive two-dimensional liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry, J. Sep. Sci. 36 (2013) 288–300.
[2] S.D. Stamatov, J. Stawinski, Regioselective and stereospecific acylation across oxirane- and silyloxy systems as a novel strategy to the synthesis of enantiomerically pure mono-, di- and triglycerides, Org. Biomol. Chem. 5 (2007) 3787–3800.
[3] M. Lísa, H. Velínská, M. Holčapek, Regioisomeric characterization of triacylglycerols using silver-ion HPLC/MS and randomization synthesis of standards, Anal. Chem. 81 (2009) 3903–3910.
[4] A.G. Marangoni, Fat Crystal Networks, CRC Press, Boca Raton, FL, USA, 2004. ISBN 9781420030549.
[5] T. Řezanka, P. Mareš, Determination of plant triacylglycerols using capillary gas-chromatography, high-performance liquid-chromatography and mass-spectrometry, J. Chromatogr. 542 (1991) 145–159.
[6] A. Kemppinen, P. Kalo, Analysis of sn-1(3)- and sn-2-short-chain acyl isomers of triacylglycerols in butteroil by gas-liquid chromatography, J. Am. Oil Chem. Soc. 75 (1998) 91–100.
[7] P. Mareš, High-temperature capillary gas-liquid chromatography of triacylglycerols and other intact lipids, Progr. Lip. Res. 27 (1988) 107–133.
[8] P. Blaise, R. Wolff, M. Farines, Regiospecific study of vegetable oil triacylglycerols by chemical cleavage and high-resolution NMR C-13, OCL-Ol, Corps Gras Li 4 (1997) 135–141.
[9] K. Mori, Pheromone synthesis. Part 253: Synthesis of the racemates and enantiomers of triglycerides of male *Drosophila* fruit flies with special emphasis on the preparation of enantiomerically pure 1-monoglycerides, Tetrahedron 68 (2012) 8441–8449.
[10] Q. Zhou, B. Gao, X. Zhang, Y. Xu, H. Shi, L.L. Yu, Chemical profiling of triacylglycerols and diacylglycerols in cow milk fat by ultra-performance convergence chromatography combined with a quadrupole time-of-flight mass spectrometry, Food Chem. 143 (2014) 199–204.
[11] J.W. Lee, T. Uchikata, A. Matsubara, T. Nakamura, E. Fukusaki, T. Bamba, Application of supercritical fluid chromatography/mass spectrometry to lipid profiling of soybean, J. Biosci. Bioeng. 113 (2012) 262–268.
[12] P. Sandra, A. Medvedovici, Y. Zhao, F. David, Characterization of triglycerides in vegetable oils by silver-ion packed-column supercritical fluid chromatography coupled to mass spectroscopy with atmospheric pressure chemical

ionization and coordination ion spray, J. Chromatogr. A 974 (2002) 231–241.
[13] J.W. Lee, T. Nagai, N. Gotoh, E. Fukusaki, T. Bamba, Profiling of regioisomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 966 (2014) 193–199.
[14] W.W. Christie, A stable silver-loaded column for the separation of lipids by high-performance liquid-chromatography, J. High. Resolut. Chrom. Chrom. Comm. 10 (1987) 148–150.
[15] B. Nikolova-Damyanova, Retention of lipids in silver ion high-performance liquid chromatography: facts and assumptions, J. Chromatogr. A 1216 (2009) 1815–1824.
[16] M. Lísa, M. Holčapek, M. Boháč, Statistical evaluation of triacylglycerol composition in plant oils based on high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry data, J. Agric. Food Chem. 57 (2009) 6888–6898.
[17] M. Holčapek, H. Dvořáková, M. Lísa, A.J. Girón, P. Sandra, J. Cvačka, Regioisomeric analysis of triacylglycerols using silver-ion liquid chromatography-atmospheric pressure chemical ionization mass spectrometry: comparison of five different mass analyzers, J. Chromatogr. A 1217 (2010) 8186–8194.
[18] P. Laakso, W.W. Christie, J. Pettersen, Analysis of North Atlantic and Baltic fish oil triacylglycerols by high-performance liquid chromatography with a silver ion column, Lipids 25 (1990) 284–291.
[19] W. Christie, Separation of molecular species of triacylglycerols by high-performance liquid chromatography with a silver ion column, J. Chromatogr. A 454 (1988) 273–284.
[20] M. Beccaria, R. Costa, G. Sullini, E. Grasso, F. Cacciola, P. Dugo, L. Mondello, Determination of the triacylglycerol fraction in fish oil by comprehensive liquid chromatography techniques with the support of gas chromatography and mass spectrometry data, Anal. Bioanal. Chem. 407 (2015) 5211–5225.
[21] P. Laakso, P. Voutilainen, Analysis of triacylglycerols by silver-ion high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry, Lipids 31 (1996) 1311–1322.
[22] P.J.W. Schuyl, T. de Joode, M.A. Vasconcellos, G.S.M.J.E. Duchateau, Silver-phase high-performance liquid chromatography electrospray mass spectrometry of triacylglycerols, J. Chromatogr. A 810 (1998) 53–61.
[23] P. Dugo, O. Favoino, P.Q. Tranchida, G. Dugo, L. Mondello, Off-line coupling of non-aqueous reversed-phase and silver ion high-performance liquid chromatography–mass spectrometry for the characterization of rice oil triacylglycerol positional isomers, J. Chromatogr. A 1041 (2004) 135–142.
[24] H. Leskinen, J.-P. Suomela, J. Pinta, H. Kallio, Regioisomeric structure determination of α-and γ-linolenoyldilinoleoylglycerol in blackcurrant seed oil by silver ion high-performance liquid chromatography and mass spectrometry, Anal. Chem. 80 (2008) 5788–5793.
[25] M. Holčapek, H. Velínská, M. Lísa, P. Česla, Orthogonality of silver-ion and non-aqueous reversed-phase HPLC/MS in the analysis of complex natural mixtures of triacylglycerols, J. Sep. Sci. 32 (2009) 3672–3680.
[26] R.O. Adlof, G.R. List, Synthesis and analysis of symmetrical and nonsymmetrical disaturated/monounsaturated triacylglycerols, J. Agric. Food Chem. 51 (2003) 2096–2099.
[27] I. Francois, S. Pereira Ados, P. Sandra, Considerations on comprehensive and off-line supercritical fluid chromatography x reversed-phase liquid chromatography for the analysis of triacylglycerols in fish oil, J. Sep. Sci. 33 (2010) 1504–1512.
[28] A.R. Fardin-Kia, P. Delmonte, J.K.G. Kramer, G. Jahreis, K. Kuhnt, V. Santercole, J.I. Rader, Separation of the fatty acids in menhaden oil as methyl esters with a highly polar ionic liquid gas chromatographic column and identification by time of flight mass spectrometry, Lipids 48 (2013) 1279–1295.
[29] T. Řezanka, L. Nedbalová, L. Procházková, K. Sigler, Lipidomic profiling of snow algae by ESI-MS and silver-LC/APCI-MS, Phytochemistry 100 (2014) 34–42.
[30] O. Schreiberová, T. Krulikovská, K. Sigler, A. Čejková, T. Řezanka, RP-HPLC/MS-APCI analysis of branched chain TAG prepared by precursor-directed biosynthesis with *Rhodococcus erythropolis*, Lipids 45 (2010) 743–756.
[31] M. Lísa, M. Holčapek, H. Sovová, Comparison of various types of stationary phases in non-aqueous reversed-phase high-performance liquid chromatography-mass spectrometry of glycerolipids in blackcurrant oil and its enzymatic hydrolysis mixture, J. Chromatogr. A 1216 (2009) 8371–8378.
[32] S. Momchilova, K. Tsuji, Y. Itabashi, B. Nikolova-Damyanova, A. Kuksis, Resolution of triacylglycerol positional isomers by reversed-phase high-performance liquid chromatography, J. Sep. Sci. 27 (2004) 1033–1036.
[33] P. Kalo, A. Kemppinen, I. Kilpelainen, Determination of positional distribution of butyryl groups in milkfat triacylglycerols, triacylglycerol mixtures, and isolated positional isomers of triacylglycerols by gas chromatography and H-1 nuclear magnetic resonance spectroscopy, Lipids 31 (1996) 331–336.
[34] P. Kalo, A. Kemppinen, V. Ollilainen, A. Kuksis, Analysis of regioisomers of short-chain triacylglycerols by normal phase liquid chromatography-electrospray tandem mass spectrometry, Int. J. Mass Spectrom. 229 (2003) 167–180.
[35] P. Kalo, A. Kemppinen, V. Ollilainen, A. Kuksis, Regiospecific determination of short-chain triacylglycerols in butterfat by normal-phase HPLC with on-line electrospray-tandem mass spectrometry, Lipids 39 (2004) 915–928.
[36] P. Kalo, A. Kemppinen, V. Ollilainen, Determination of triacylglycerols in butterfat by normal-phase HPLC and electrospray-tandem mass spectrometry, Lipids 44 (2009) 169–195.
[37] S. Momchilova, Y. Itabashi, B. Nikolova-Damyanova, A. Kuksis, Regioselective separation of isomeric triacylglycerols by reversed-phase high-performance liquid chromatography: Stationary phase and mobile phase effects, J. Sep. Sci. 29 (2006) 2578–2583.
[38] I. Kuroda, T. Nagai, H. Mizobe, N. Yoshimura, N. Gotoh, S. Wada, HPLC separation of triacylglycerol positional isomers on a polymeric ODS column, Anal. Sci. 24 (2008) 865–869.
[39] H.R. Mottram, R.P. Evershed, Structure analysis of triacylglycerol positional isomers using atmospheric pressure chemical ionisation mass spectrometry, Tetrahedron Lett. 37 (1996) 8593–8596.
[40] H.R. Mottram, S.E. Woodbury, R.P. Evershed, Identification of triacylglycerol positional isomers present in vegetable oils by high performance liquid chromatography atmospheric pressure chemical ionization mass spectrometry, Rapid Commun. Mass Spectrom. 11 (1997) 1240–1252.
[41] H.R. Mottram, Z.M. Crossman, R.P. Evershed, Regiospecific characterisation of the triacylglycerols in animal fats using high performance liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry, Analyst 126 (2001) 1018–1024.
[42] A. Jakab, I. Jablonkai, E. Forgacs, Quantification of the ratio of positional isomer dilinoleoyl-oleoyl glycerols in vegetable oils, Rapid Commun. Mass Spectrom. 17 (2003) 2295–2302.
[43] L. Fauconnot, J. Hau, J.M. Aeschlimann, L.B. Fay, F. Dionisi, Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry, Rapid Commun. Mass Spectrom. 18 (2004) 218–224.
[44] H. Leskinen, J.P. Suomela, H. Kallio, Quantification of triacylglycerol regioisomers in oils and fat using different mass spectrometric and liquid chromatographic methods, Rapid Commun. Mass Spectrom. 21 (2007) 2361–2373.
[45] H.M. Leskinen, J.P. Suomela, H.P. Kallio, Quantification of triacylglycerol regioisomers by ultra-high-performance liquid chromatography and ammonia negative ion atmospheric pressure chemical ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 1–5.
[46] J.W. Allen, C.C. DiRusso, P.N. Black, Triglyceride quantification by catalytic saturation and LC-MS/MS reveals an evolutionary divergence in regioisometry among green microalgae, Algal Res. 5 (2014) 23–31.
[47] C. Baiocchi, C. Medana, F. Dal Bello, V. Giancotti, R. Aigotti, D. Gastaldi, Analysis of regioisomers of polyunsaturated triacylglycerols in marine matrices by HPLC/HRMS, Food Chem. 166 (2015) 551–560.
[48] K. Nagy, L. Sandoz, F. Destaillats, O. Schafer, Mapping the regioisomeric distribution of fatty acids in triacylglycerols by hybrid mass spectrometry, J. Lipid Res. 54 (2013) 290–305.
[49] W.C. Byrdwell, The simulacrum system as a construct for mass spectrometry of triacylglycerols and others, Lipids 51 (2016) 211–227.
[50] T. Řezanka, O. Schreiberová, A. Čejková, K. Sigler, The genus *Dracunculus* – A source of triacylglycerols containing odd-numbered ω-phenyl fatty acids, Phytochemistry 72 (2011) 1914–1926.
[51] T. Řezanka, O. Schreiberová, T. Krulikovská, J. Masák, K. Sigler, RP-HPLC/MS-APCI analysis of odd-chain TAGs from *Rhodococcus erythropolis* including some regioisomers, Chem. Phys. Lipids 163 (2010) 373–380.
[52] M. Lísa, K. Netušilová, L. Franěk, H. Dvořáková, V. Vrkoslav, M. Holčapek, Characterization of fatty acid and triacylglycerol composition in animal fats using silver-ion and non-aqueous reversed-phase high-performance liquid chromatography/mass spectrometry and gas chromatography/flame ionization detection, J. Chromatogr. A 1218 (2011) 7499–7510.
[53] N. Gotoh, Y. Matsumoto, T. Nagai, H. Mizobe, K. Yoshinaga, K. Kojima, I. Kuroda, Y. Kitamura, T. Shimizu, H. Ishida, S. Wada, Actual ratio of triacylglycerol positional isomers in milk and cheese, J. Oleo Sci. 61 (2012) 173–180.
[54] T. Nagai, N. Gotoh, H. Mizobe, K. Yoshinaga, K. Kojima, Y. Matsumoto, S. Wada, Rapid separation of triacylglycerol positional isomers binding two saturated fatty acids using octacocyl silylation column, J. Oleo Sci. 60 (2011) 345–350.
[55] Y. Iwasaki, M. Yasui, T. Ishikawa, R. Irimescu, K. Hata, T. Yamane, Optical resolution of asymmetric triacylglycerols by chiral-phase high-performance liquid chromatography, J. Chromatogr. A 905 (2001) 111–118.
[56] M. Lísa, M. Holčapek, Characterization of triacylglycerol enantiomers using chiral HPLC/APCI-MS and synthesis of enantiomeric triacylglycerols, Anal. Chem. 85 (2013) 1852–1859.
[57] Y. Itabashi, Chiral separation of glycerolipids by high-performance liquid chromatography, J. Lipid Nutr. 21 (2012) 27–34.
[58] T. Nagai, Y. Matsumoto, Y. Jiang, K. Ishikawa, T. Wakatabe, H. Mizobe, K. Yoshinaga, K. Kojima, I. Kuroda, T. Saito, Actual ratios of triacylglycerol positional isomers and enantiomers comprising saturated fatty acids and highly unsaturated fatty acids in fishes and marine mammals, J. Oleo Sci. 62 (2013) 1009–1015.
[59] H.M. Alvarez, A. Steinbuchel, Triacylglycerols in prokaryotic microorganisms, Appl. Microbiol. Biotechnol. 60 (2002) 367–376.
[60] G. Dragone, B. Fernandes, A. Vicente, J.A. Teixeira, Current research, technology and education topics in applied microbiology and microbial biotechnology, in: A.M. Vilas (Ed.), Third Generation Biofuels from Microalgae, Formatex Research Center, Badajoz, 2010, pp. 1355–1366.
[61] T. Řezanka, J. Lukavský, L. Nedbalová, K. Sigler, Effect of nitrogen and phosphorus starvation on the polyunsaturated triacylglycerol composition, including positional isomer distribution, in the alga *Trachydiscus minutus*, Phytochemistry 72 (2011) 2342–2351.
[62] T. Řezanka, J. Lukavský, L. Siřišťová, K. Sigler, Regioisomer separation and

identification of triacylglycerols containing vaccenic and oleic acids, and α- and γ-linolenic acids, in thermophilic cyanobacteria *Mastigocladus laminosus* and *Tolypothrix* sp. Phytochemistry 78 (2012) 147–155.
[63] T. Řezanka, J. Lukavský, L. Nedbalová, I. Kolouchová, K. Sigler, Effect of starvation on the distribution of positional isomers and enantiomers of triacylglycerol in the diatom *Phaeodactylum tricornutum*, Phytochemistry 80 (2012) 17–27.
[64] T. Řezanka, D. Matoulková, I. Kolouchová, J. Masák, K. Sigler, Brewer's yeast as a new source of palmitoleic acid—analysis of triacylglycerols by LC–MS, J. Am. Oil Chem. Soc. 90 (2013) 1327–1342.
[65] T. Řezanka, I. Kolouchová, A. Čejková, T. Cajthaml, K. Sigler, Identification of regioisomers and enantiomers of triacylglycerols in different yeasts using reversed- and chiral-phase LC-MS, J. Sep. Sci. 36 (2013) 3310–3320.
[66] T. Řezanka, J. Lukavský, L. Nedbalová, K. Sigler, Production of structured triacylglycerols from microalgae, Phytochemistry 104 (2014) 95–104.
[67] T. Řezanka, L. Nedbalová, K. Sigler, Comparative analysis of triacylglycerols from different *Stichococcus* strains by RP-HPLC/APCI-MS and chiral HPLC, J. Appl. Phycol. 27 (2015) 685–696.
[68] T. Řezanka, K. Sigler, Separation of enantiomeric triacylglycerols by chiral-phase HPLC, Lipids 49 (2014) 1251–1260.
[69] T. Řezanka, J. Lukavský, K. Sigler, L. Nedbalová, M. Vítova, Temperature dependence of production of structured triacylglycerols in the alga *Trachydiscus minutus*, Phytochemistry 110 (2015) 37–45.
[70] T. Řezanka, M. Vítova, A. Nováková, K. Sigler, Separation and identification of odd chain triacylglycerols of the protozoan *Khawkinea quartana* and the mold *Mortierella alpina* using LC-MS, Lipids 50 (2015) 811–820.
[71] T. Řezanka, K. Sigler, Odd-numbered very-long-chain fatty acids from the microbial, animal and plant kingdoms, Prog. Lipid Res. 48 (2009) 206–238.
[72] K.J.L. Chang, M.P. Mansour, G.A. Dunstan, S.I. Blackburn, A. Koutoulis, P.D. Nichols, Odd-chain polyunsaturated fatty acids in thraustochytrids, Phytochemistry 72 (2011) 1460–1465.