Journal of Chromatography A 1623 (2020) 461161

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of triacylglycerols containing allenic and acetylenic fatty acids by enantiomeric liquid chromatography-mass spectrometry

Andrea Palyzová, Tomáš Řezanka*

Institute of Microbiology, the Czech Academy of Sciences, 142 20 Prague 4, Vídeňská 1083, Czech Republic

ARTICLE INFO

Article history:
Received 28 February 2020
Revised 20 April 2020
Accepted 22 April 2020
Available online 28 April 2020

Keywords:
Triacylglycerols
Acetylenic fatty acids
Allenic fatty acids
Ximenynic acid
Crepenynic acid
Enantiomeric separation

ABSTRACT

Triacylglycerols (TAGs) containing less common fatty acids (FAs) were isolated from the seeds of three plants (*Santalum album*, *Crepis foetida*, and *Leucas aspera*). These FAs had allenic (laballenic acid, Lb) and acetylenic (crepenynic, C; ximenynic acids, Xi) bonds. TAGs were analyzed on reversed-phase and chiral columns. High-resolution tandem mass spectrometry identified TAGs by positive electrospray ionization (ESI^+). Twenty-two molecular species of TAGs isolated from the seed oil of *Santalum album* were separated by RP-HPLC and chiral HPLC methods and identified by positive electrospray ionization tandem MS detection (ESI^+-MS). Two major enantiomers, i.e., *sn*-OOLb and *sn*-LLLb (O represents oleic acid; and L represents linoleic acid), were synthesized from the appropriate phosphatidylcholines. This allowed the identification of enantiomers after separation by chiral chromatography by tandem mass spectrometry. Similarly, TAGs from the seeds of *Crepis foetida*, and *Leucas aspera* were analyzed by reversed-phase chromatography and identified by mass spectrometry. Four enantiomers (*sn*-OOC, *sn*-LLC, *sn*-OOXi, and *sn*-LLXi) were synthesized. A total of six and three enantiomers of TAGs containing crepenynic and ximenynic acids, respectively, were identified by chiral column analysis. The retention times of TAGs containing allenic and acetylenic bonds were always greater on the reversed-phase column than TAGs with the same number of carbon atoms and the same unsaturation (e.g., LLL versus LLLb). From the chiral column, the regioisomers and enantiomers were eluted in the order of symmetric-asymmetric-asymmetric (i.e., *sn*-OCO, *sn*-COO, and *sn*-OOC). Through tandem mass spectrometry, we were able to identify and distinguish regioisomer $[DAG]^+$-type ions, i.e., $[M–NH_4–NH_3–RCOOH]^+$, that can be considered diagnostic. Unfortunately, enantiomers and TAGs with the same numbers of carbon atoms and the same unsaturation levels have identical mass spectra, such as LLL and LLLb.

1. Introduction

During ontogenesis, higher plants accumulate storage substances, such as lipids (plant oils). These storage substances have widespread applications; plant storage organs have been used as food or animal feed and as raw materials for various industrial products. Vegetable oils are the most important part of the human diet, providing a significant amount of energy and serving as sources of essential fatty acids (FAs). All vegetable oils are triacylglycerols (TAGs), which are esters of glycerol, wherein all hydroxyl groups are esterified by FAs. TAGs often include palmitic (16:0, P), stearic (18:0, S), oleic (9–18:1, O), linoleic (9,12–18:2, L) and α-linolenic (9,12,15–18:3, Ln) acids. These five FAs have even numbers of carbon atoms and are the most widespread in nature. Minor and unusual FAs differ in at least one double bond position or configuration or include cumulated double (allenic) or triple bonds or other functional groups.

TAGs number in the several tens and include hundreds of molecular species depending on the number of fatty acids. A factor that increases the number of molecular species of TAGs is the presence of regioisomers and enantiomers. For example, triacylglycerols in which only two FAs are sterically bound, e.g., oleic and linoleic acids, exist in eight isomers (OOO, LLL, LLO, OLL, LOL, OOL, LOO, OLO). Regioisomers may be separated on a reversed-phase column or by Ag^+-HPLC. Their identification is possible using tandem mass spectrometry [1,2]. In contrast, enantiomers exhibit the same tandem mass spectra but can be separated on a chiral phase column [3]. The separation of TAGs that occur in commercially available plant seeds has already been described [4]. However, the analyses of TAGs containing less common or unusual FAs have rarely been reported. Examples include odd [5], long [6], cyclo[7], C16 [8] or C18 [9] PUFAs, edible fats and oils, [10], and enantiomeric separations of asymmetric TAGs by recycle HPLC [11].

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

https://doi.org/10.1016/j.chroma.2020.461161
0021-9673/

Based on our previous experience with analyses of regioisomers and enantiomers of TAGs, two reversed-phase (RP18) columns coupled in series and a chiral phase column based on tris-3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin were used. TAGs have been identified in three vegetable oils that contained significant amounts of laballenic (*Leucas aspera*), stearolic, ximenynic (also called santalbic acid) (*Santalum album*), and crepenynic acids (*Crepis foetida*) by LC-MS in positive ESI mode using high-resolution tandem MS.

2. Experimental methods

2.1. Standards

Common chemicals were purchased from Sigma-Aldrich, Ltd. (Prague, Czech Republic). The phospholipids, 1,2-dilinoleyl-*sn*–glycero–3-phosphatidylcholine (18:2/18:2-PC) and 1,2-dioleoyl-*sn*–glycero–3-phosphatidylcholine (18:1/18:1-PC), were purchased from Sigma–Aldrich, Ltd. Stearolic (S8268) and ximenynic (CDS018698) acids (Sigma–Aldrich, Ltd.) were purchased previously. Seeds of three plants, *Santalum album, Crepis foetida* subsp. *foetida*, and *Leucas aspera* Bronze Flamingo, were purchased from a local garden center. The seeds were not sourced from natural collections.

2.2. Lipid extraction and isolation of TAGs

Seeds were powdered and extracted thoroughly with light petroleum ether to yield oils. Briefly, the plant oils were applied to Sep-Pak Cartridges Vac 35 cc (Waters, Prague, Czech Republic) with 10 g of an aminopropylsilica-based polar bonded phase, and neutral lipids were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (70:30% v/v). Solvents were evaporated, and the oil samples were dissolved in an acetonitrile, propan-2-ol, hexane mixture (33:33:33% v/v/v), which was injected into an HPLC column [12]. Oil from the seeds of *Santalum album* was used for laballenic acid preparation.

2.3. Preparation of laballenic acid

TAGs were saponified with aqueous ethanolic KOH solution (1 M) for 24 h at room temperature. After acidification, the mixture was extracted three times with hexane, washed with water and, after drying over anhydrous sodium sulfate, evaporated to dryness. FAMEs were prepared by refluxing free fatty acids for 1 h in excess methanol under catalytic treatment with sulfuric acid. Total FAMEs were separated on argentation TLC plates, 20 × 20 cm, 250 µm layer, 15% $AgNO_3$/silica gel plate (Miles Scientific (previously Analtech), Newark, DE, USA). Hexane–diethyl ether 80:20% v/v was used as the elution phase. Methylated laballenate was scraped and extracted with diethyl ether, saponified again with ethanolic KOH solution, and free fatty acid was used to synthesize the appropriate TAGs.

2.4. Preparation of TAGs with a mixed FA composition

1,2-Dioleoyl- and 1,2-dilinoleoyl-*sn*–glycero–3-phosphatidylcholines were treated with phospholipase C (Type I from *C. perfringens* (*C. welchii*)) as described by Christie [13]. A detailed description of the preparation method is provided in the Supplementary Information.

The esterification of glycerol derivatives with acids has been previously described [14,15]. Briefly, diacylglycerols were reacted with free fatty acids in the presence of 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) to yield the corresponding triacylglycerols (see the Supplementary Information).

Compared with published syntheses requiring five reaction steps [16,17,3], the above-described method advantageously requires only two reaction steps. Additionally, one of the two steps is enzymatic hydrolysis, which guarantees mild conditions and can therefore be used for FA-labile TAGs.

2.5. Analysis of fatty acids

Fatty acid methyl esters (FAMEs) were prepared from neutral lipids using a standard procedure with sodium methoxide [18]. A detailed description of the preparation of 3-pyridylcarbonyl esters is provided in the Supplementary Information.

The GC–MS analysis of FAME and 3-pyridylcarbonyl ester mixtures was performed using a Finnigan/MAT 1020 B instrument (Bremen, Germany) in electron ionization (EI) mode, and the esters were identified as previously described [19,20,21] (see Supplementary Information).

2.6. TAG analysis using RP-HPLC/ESI–MS

TAGs were analyzed using an LC–MS system with two RP-HPLC columns connected in series (see Supplementary Information). LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high-resolution hybrid mass spectrometer, was combined with a liquid chromatograph (see Supplementary Information). This instrument was used for both tandem mass spectrometry and selected ion monitoring (SIM).

2.7. TAG analysis using chiral LC/ESI–MS

The same LC system used in the reversed-phase 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 [22,23]. 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.

3. Results and discussion

3.1. Allenic fatty acids

One of the most widespread C18 allene FAs, i.e., laballenic acid (5,6-octadecadienoic acid, 5,6–18:2, Lb) has been isolated and identified primarily in seeds of plants of the Labiateae family [24,25,26]. Although the allenic group is responsible for considerable optical activity, no effects have been reported on the separation of TAGs by chiral chromatography (see below). Additionally, a higher homolog, i.e., phlomic acid (7,8-eicosadienoic acid, 7,8–20:2, Ph) was isolated from seeds of the same family. In the seeds of the genus *Leucas* (formerly *Phlomis*), up to 10 FAs were identified, the majority of which were palmitic (16:0, P), oleic (9–18:1, O), linoleic (9,12–18:2, L) and laballenic acids. The contents of all FAs, including minorities, are listed in Table 1S (Supplementary Information). Except in the Labiateae family, allenic acids occur rarely [24,27].

Although allenic FAs are less widespread in nature, several papers have described the identification of fatty acid methyl esters (FAMEs) and 3-pyridyl carbonyl (formerly called picolinyl) esters (Christie et al. [28] published the key article). Allenic FAMEs are

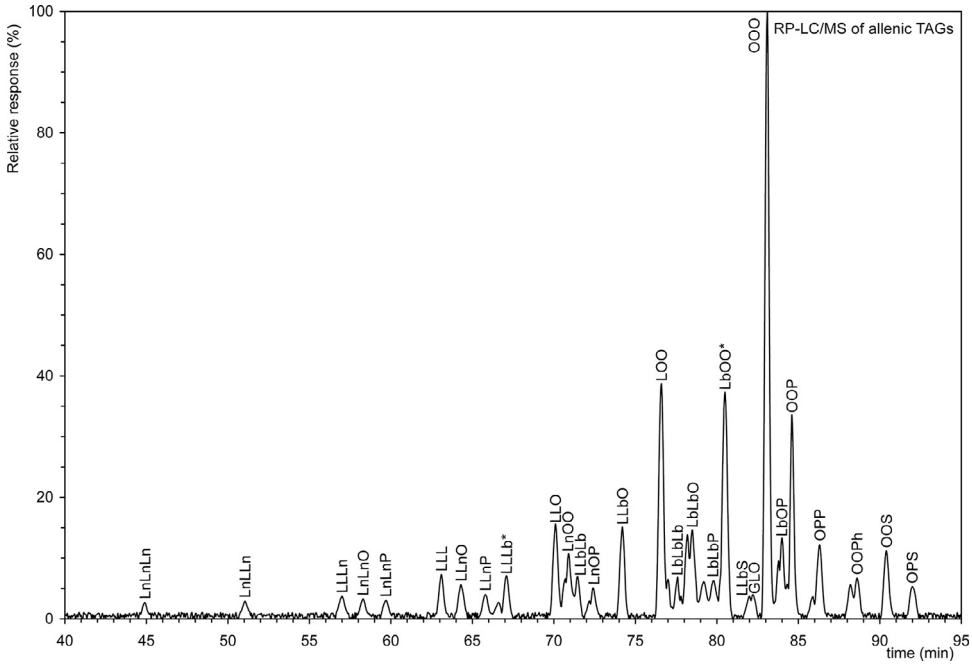

Fig. 1. Reverse phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP-LC/ESI–MS) chromatogram from 40 to 95 min of total ion current (TIC) of the TAGs from the *Leucas aspera* seeds. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e., ions resulting loss of neutral $RCOOH+NH_3$ from $[M+NH_4]^+$ in the interval from 480 to 680 Da. The $[DAG]^+$ type ion was always the base peak.

unique and are similar to acetylenic FAs (see below). The cumulated double bond system in allenic FAMEs migrates less under electron impact conditions and therefore, the mass spectra are characterized by $[M]^+$ and $[M-31]^+$ ions and three ions resulting from β bond cleavage to the allenic bond. For complete identification, 3-pyridyl carbonyl esters were prepared and analyzed. Figs. 1S and 2S show two mass spectra of the derivatives of laballenic and phlomic acids. Diagnostic ions, as in the case of FAMEs, are the ions formed by the β cleavage of a single bond to the allenic group.

3.2. Preparation of laballenic acid

The preparation of laballenic acid has been described in the literature [29]. Briefly, oil was saponified, and FAMEs were prepared in a large excess of methanol with H_2SO_4. The mixture of FAMEs was separated by argentation TLC chromatography. The allenic ester has an Rf higher than methyl oleate [30], meaning that it forms a band between methyl stearate and methyl oleate. The appropriate band was extracted from the TLC plate, and the corresponding methyl ester was saponified with aqueous ethanolic KOH. The free acid was used to synthesize two stereospecific TAGs (see Experimental methods).

3.3. Reversed-phase TAGs containing allenic fatty acids

TAG molecular species analysis was performed on two C18 reversed-phase columns coupled in series. Table 2S lists the tR and abundances of individual TAG molecular species. Although laballenic acid has two double bonds (like linoleic acid), the retention times of the TAGs were different (e.g., tR TAGs in Table 2S). Replacement of linoleyl acyl with laballenyl acyl resulted in increased tR in our samples and in published retention times [31,32]. For example, the differences among LLO, LLbO, and LbLbO were 3.8 and 4.1 min. Similarly, in the LLL, LLLb, LLbLb, and LbLbLb series, the values were 3.7, 4.1, and 5.7 min. TAGs containing allenic acids always had higher tR values than the corresponding isomeric TAGs containing linoleic acid (Fig. 1).

The contributions of two cumulative double bonds in laballenic acid at specific retention times were smaller than those of two methylene interrupted double bonds. In other words, the allenic double bond is less polar than methylene interrupted double bonds. The elution sequence from the RP-18 chromatography column was nearly identical to published data. The order of elution of the TAGs in Seher and Fiebig [31] and the tR of our sample showed a 100% match. Minor differences were observed for the TAGs from *Phlomis tuberosum* [32]; however, the authors used a relatively unusual mobile phase, i.e., butanol:acetonitrile:hexane. It should be noted that the differences were at most hundredths of a minute, e.g., 16.03 versus 16.04 min for LOP-LbLbO and 17.51 versus 17.50 for LPP-LbLbP. Because we prepared two TAGs, i.e., *sn*-LLLb and *sn*-OOLb, we were able to compare natural TAGs with synthetic TAGs by RP-HPLC and tandem MS.

3.4. Chiral LC and tandem mass spectra of TAGs containing allenic fatty acids

An article describing the chiral chromatography of TAGs containing at least one FA with allene bonds has not yet been published. A shortened tR would be possible by increasing the efficiency of the column, e.g., by using smaller particles. Fig. 2 shows the chromatogram of two synthetic TAGs (*sn*-OOLb and *sn*-LLLb) and a real *Leucas tuberosa* mixture. From published data [33] obtained by pancreatic lipase, saturated FAs (P and S) almost exclusively represented the *sn*-1 position and linolenic acid at the *sn*-3 position. Furthermore, the authors reported that 12% of Lb was esterified at the *sn*-2 position and the remaining 88% at the *sn*-1(3) positions. Because we followed chiral chromatography with analyses by negative ESI tandem MS, our results were more detailed.

Allenic bonds can interact with π electrons in the stationary phase; CH_2 groups of acyls can likely utilize Van der Waals forces between two methyl groups (stationary phase). For example, Ianni et al. [34] reported π-π interactions in C=C bonds; thus, the tR prediction for individual enantiomers is almost impossible. Therefore, two synthetic TAGs were prepared, i.e., *sn*-OOLb and *sn*-LLLb.

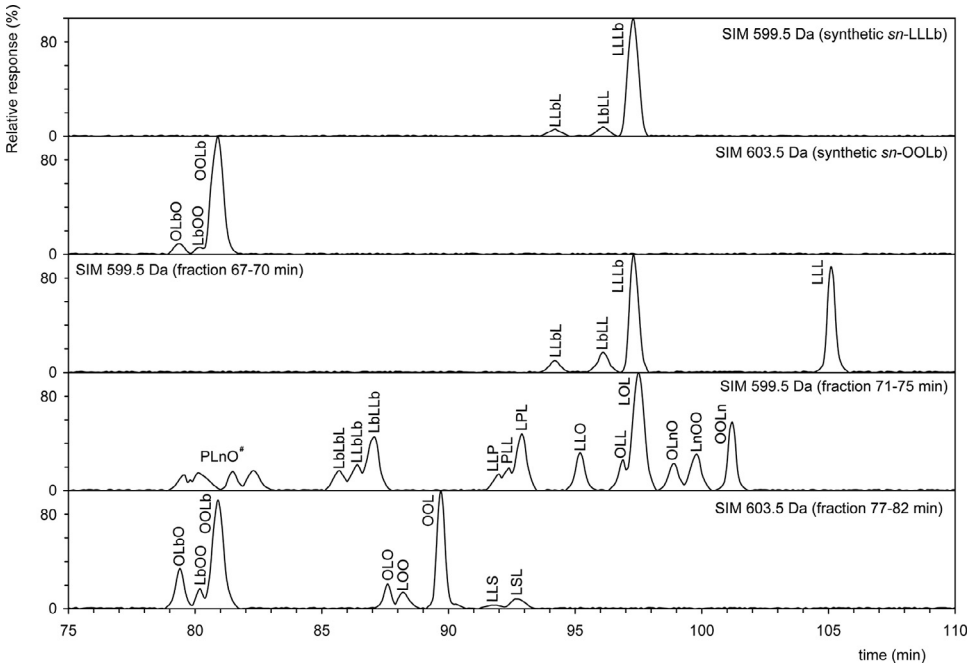

Fig. 2. Chiral-LC/MS selected-ion monitoring (SIM) chromatograms of the two ions (599.5 and 603.5 Da). PLnO# is unidentifiable mixture of six isomers.

This allowed for the prediction of the elution order of the enantiomers from the chiral column, which were further identified by tandem MS (see below).

For TAGs having four degrees of unsaturation (two double and one allenic bonds), the order of elution of the enantiomers was as follows. Symmetric TAG (*sn*-OLbO) was eluted first, followed by two asymmetric TAGs (*sn*-LbOO and *sn*-OOLb), in agreement with published data. Lisa and Holcapek [3] described the elution profile of TAGs with four degrees of unsaturation in order of symmetric-asymmetric-asymmetric TAGs, i.e., OLO-LOO-OOL.

The same order of elution, i.e., symmetric-asymmetric-asymmetric, was observed in TAGs with six degrees of unsaturation, i.e., *sn*-LLbL, *sn*-LbLL, *sn*-LLLb. In this case, the order of elution was different from that of Lisa and Holcapek [3] for six degrees of unsaturation for dilinolenoylstearin, i.e., LnLnS-SLnLn-LnSLn (asymmetric-asymmetric-symmetric). These different elution times could be explained by the presence of allenic bonds. The preference of the FAs at each position is not fully understood, e.g., Lisa and Holcapek [3] reported the proportions of OLO, LOO, and OOL in hazelnut oil at a ratio of 46:39:15, i.e., without significant preference for any of the three-glycerol backbone positions. Similarly, as reported in our previously published articles, there were no significant preferences for any of the three positions. For example, in the case of odd FAs, the majority of FAs was *sn*-LL19:4 in mold *Mortierella*, whereas in the protozoan *Khawkinea,* the majority of FAs was another isomer, *sn*-19:4LL [5]. Very long chain FAs were the main FAs in vegetable oil (*Ximenia americana*), green alga (*Botryococcus braunii*) and brewer yeast (*Saccharomyces pastorianus*), e.g., nervonic acid prefers the *sn*-1 position [6]. In the case of unusual FAs, such as cycloFAs, symmetrical TAG (OStO) was preferred for TAGs with sterculic acid (St), whereas for TAG containing chaulmoogric acid (Cg), the enantiomer of OCgCg was dominant [7].

TIC was filtered, yielding $[DAG]^+$ ions, i.e., ions resulting from the loss of neutral RCOOH + NH_3 from $[M+NH_4]^+$ in the interval from 596 to 604 Da. The $[DAG]^+$ ions represent the base peak. The mass spectra of TAGs containing laballenic acids were not distinguishable from the mass spectra of TAGs containing linoleic acids (Fig. 3).

The mass spectra of TAGs containing oleic and laballenic acids are presented in Fig. 4. The tandem mass spectra of *sn*-OOL and *sn*-OOLb (synthetic standard) were identical. Thus, it is only possible to determine the type of regioisomers of symmetric versus asymmetric TAGs (*sn*-OLbO, *sn*-LbOO/*sn*-OOLb), i.e., TAGs in pairs with the same molecular mass, e.g., OLO and LLO/OLL, respectively. The enantiomers of *sn*-OOLb and *sn*-LbOO showed identical tandem mass spectra (Fig. 4).

3.5. Acetylenic fatty acids

Nonconjugated acetylenic FAs, such as stearolic or crepenynic acids, have been identified in various genera, e.g., in the Santalaceae (*Santalum*) and Compositae (genus *Crepis*) families [27,35]. Ximenynic (santalbic) has been identified in the seed fat of *Santalum album* [27,35].

The seed oils of a number of Santalaceae species are rich in acetylenic fatty acids [36]. An article on genetically engineered *Arabidopsis thaliana* utilized genes from *Crepis alpin*a; unfortunately, the percentage of acetylenic FAs accumulated in neutral lipids in the transgenic plant was 5–35 times less than that of wild-type accumulating plants [37].

The mass spectrum of stearolate (ethyl octadec-9-ynoate) differs from the mass spectrum of methyl ester of linoleic acid (methyl 9,12-octadecadieonate) having the same molecular mass. The molecular ion at *m/z* 294 was detected, and two key ions at *m/z* 152 and at *m/z* 196 were observed. The ions at *m/z* 166 and 210 were stabilized by the rearrangement of the conjugated dienoic system. Similarly, other methylene-interrupted ene-ynoic FAs, such as crepenynic, are the major components of seed oils, such as those of *Crepis* [38]. The mass spectrum of methyl ester includes diagnostic ion at *m/z* 236, resulting from triple bond cleavage. Conjugated ene-ynoic FAs include ximenynic, which is a common component of some oils, e.g. ximenia oil [39] or oil from *Santalum album* [40]. In the mass spectrum of methyl ester of ximenynic acid, a char-

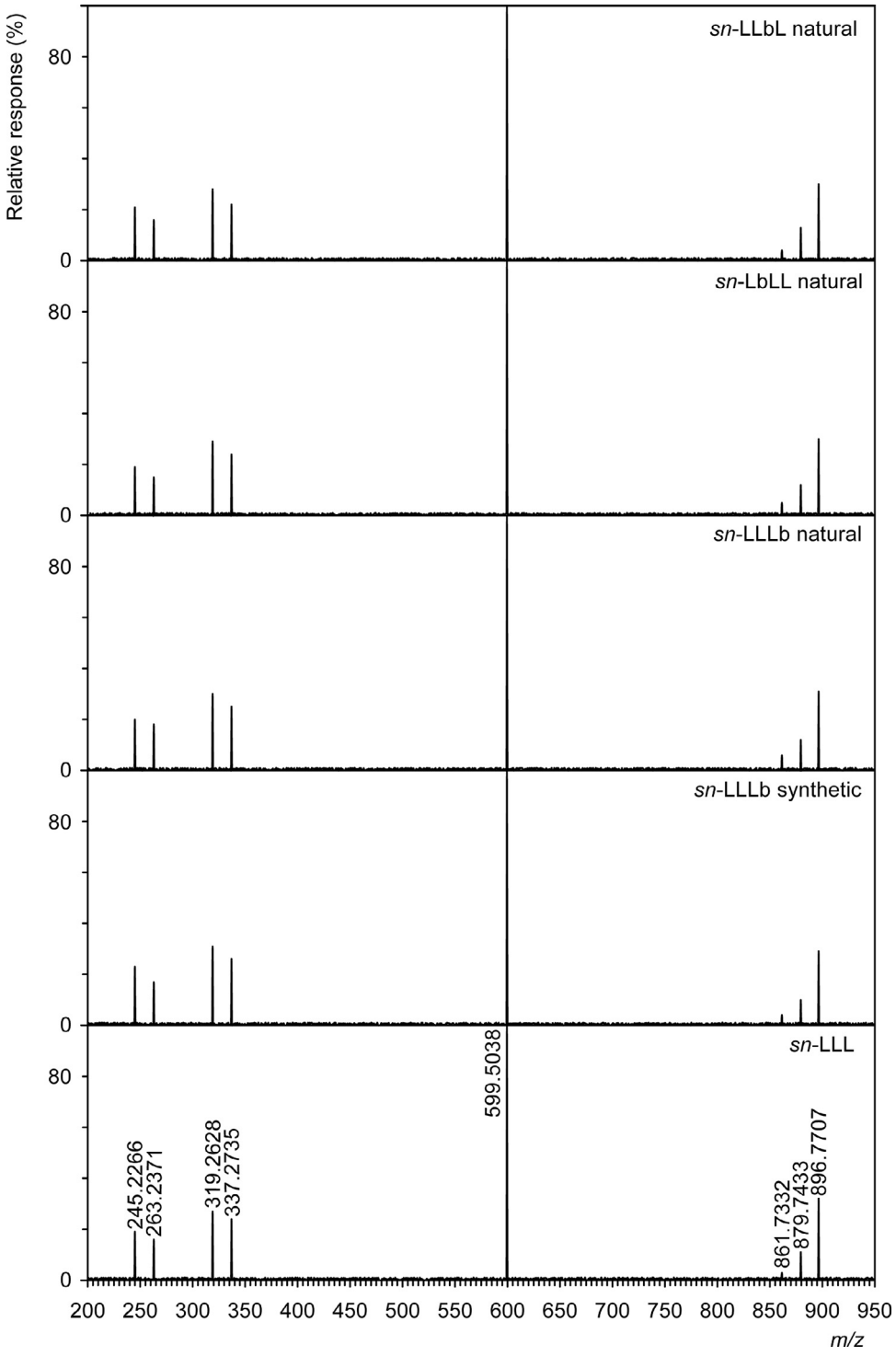

Fig. 3. Tandem mass spectra of the synthetic and natural regioisomer (*sn*-LLLb) and enantiomers (*sn*-LbLL or *sn*-LLbL), including standard (LLL). For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and discussion.

acteristic ion at *m/z* 150 resulting from β cleavage was identified. This mass spectrum was completely different when compared with the mass spectrum of methyl linoleate [13].

To fully identify and confirm the structures of stearolic, crepenynic and ximenynic acids, 3-pyridylcarbonyl esters (formerly called picolinyl esters) were prepared. The mass spectrum of the 3-pyridylcarbinol esters of octadec-9-ynoate (stearolate) showed peaks at *m/z* 92, 108, 151, and 164 and exhibited other diagnostic ions. In particular, a gap of 24 Da was present in the mass spectrum, between *m/z* 234 and *m/z* 258. Analogous to the 3-pyridylcarbinol esters of monoenoic acids, a gap of 38 Da ($-C{\equiv}C-CH_2-$) was identified between ions at *m/z* 234 and *m/z* 272. The ion at *m/z* 286 was abundant. 3-Pyridylcarbinyl crepenynate was also characterized. The $[M-1]^+$ type ion had nearly the

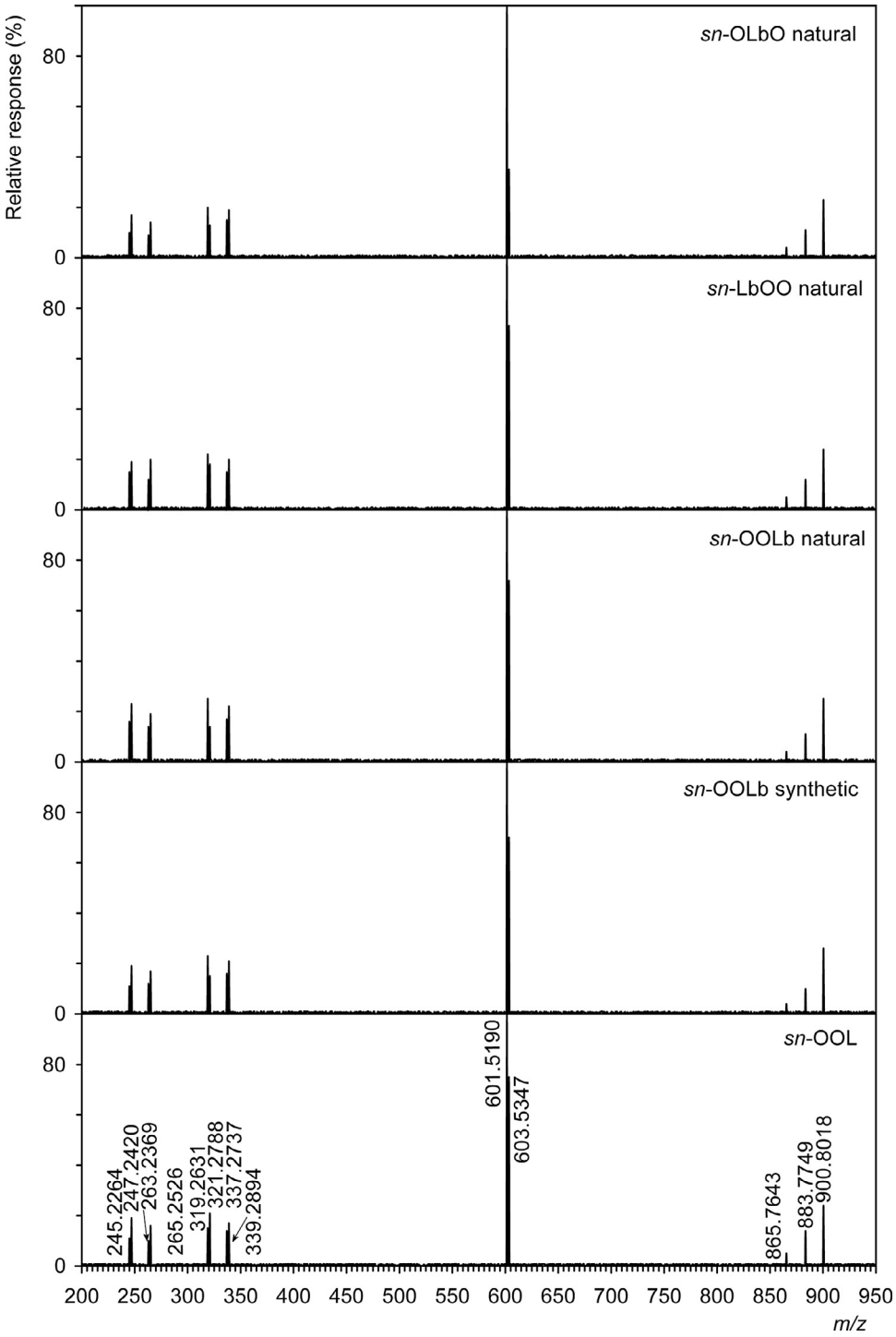

Fig. 4. Tandem mass spectra of the synthetic and natural regioisomer (*sn*-OOLb) and enantiomers (*sn*-LbOO or *sn*-OLbO), including standard (*sn*-OOL). For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and Discussion.

same abundance as the $[M]^+$ ion. In the mass spectrum, two gaps, one of 26 Da (double bond) between ions at *m/z* 234 and 260 and the other of 40 Da (*m/z* 234 to 274) were observed. These gaps are characteristic of triple bonds. An additional gap is present between ions at *m/z* 274 and at *m/z* 298; a gap of 38 Dawas observed from *m/z* 274 to 312 (Fig. 3S). A completely different mass spectrum is provided by the 3-pyridylcarbinol ester of ximenynic (santalbic), i.e., octadec-9-yn-11-trans-enoic acid, in which only diagnostic ions at *m/z* 220 and *m/z* 298 were identified (Fig. 4S).

The contents of fatty acids from the seeds of *Santalum* album and *Crepis foetida* are summarized in Tables 3S and 4S. These results are in agreement with published data [41], where acetylenic acids have been reported as the majority components.

3.6. Reversed-phase TAGs containing acetylenic fatty acids

The analysis of total TAGs from *Santalum album* seeds was performed on two RP-18 phase columns connected in series (Table 5S and Fig. 5). These results correspond with the order of elution of

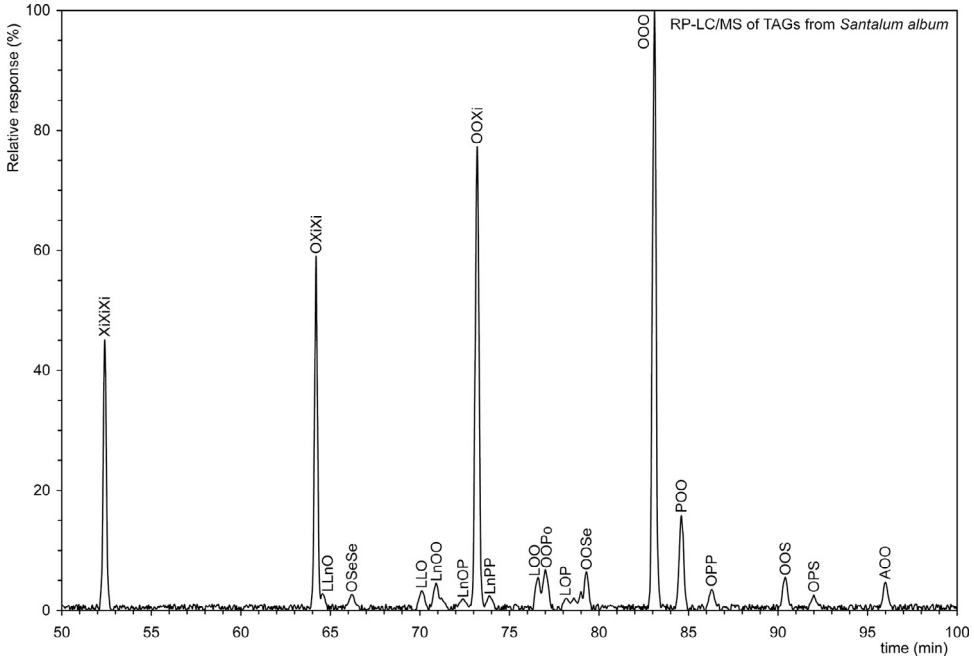

Fig. 5. Reverse phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP-LC/ESI–MS) chromatogram from 40 to 95 min of total ion current (TIC) of the TAGs from the *Santalum album* seeds. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e., ions resulting loss of neutral $RCOOH+NH_3$ from $[M+NH_4]^+$ in the interval from 480 to 680 Da. The $[DAG]^+$ type ion was always the base peak.

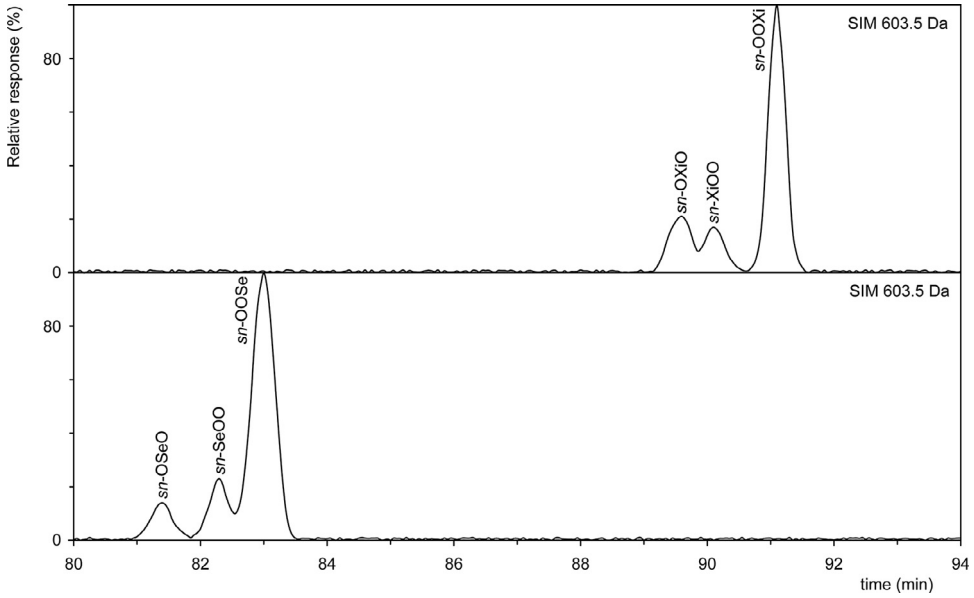

Fig. 6. Chiral-LC/MS selected-ion monitoring (SIM) chromatograms of the ion at *m/z* 603.5.

TAGs containing acetylenic FA molecules, e.g., OOL (tR = 76.6 min) versus OOSe (79.3 min). A similar tR shift was found in another pair containing one triple and one double bond, e.g., ximenynic acid with an unsaturation degree of three versus α-linolenic acid, namely, OOLn (tR = 70.9 min and OOXi (73.2 min). Molecule polarity is due to the uneven distribution of electrons. Alkynes are therefore more polar than alkenes due to the higher electron density (sp hybridization of –C≡) near the triple bond. The contribution of two double bonds, e.g., in linoleyl acyl, is greater than the contribution of one triple bond in stearolyl acyl (9a-18:2). A TAGs analysis of sandalwood seed oil containing ximenynic acid was described by Liu et al. [42]. Unfortunately, the authors only identified three TAGs (XiXiXi, XiXiO or XiOXi, and OOXi or OXiO); other peaks were not identified.

3.7. Chiral LC and tandem mass spectra of TAGs containing acetylenic fatty acids

To fully elucidate the structure of OOXi and OOSe, two fractions were collected. The first fraction was collected in the interval of 72–75 min and the second in the range of 77.5–80.5 min. Both fractions were analyzed on a chiral column (Fig. 6). From the analysis, it is evident that identification by selected ion monitoring (SIM) at 603.5 Da (ion having the structure

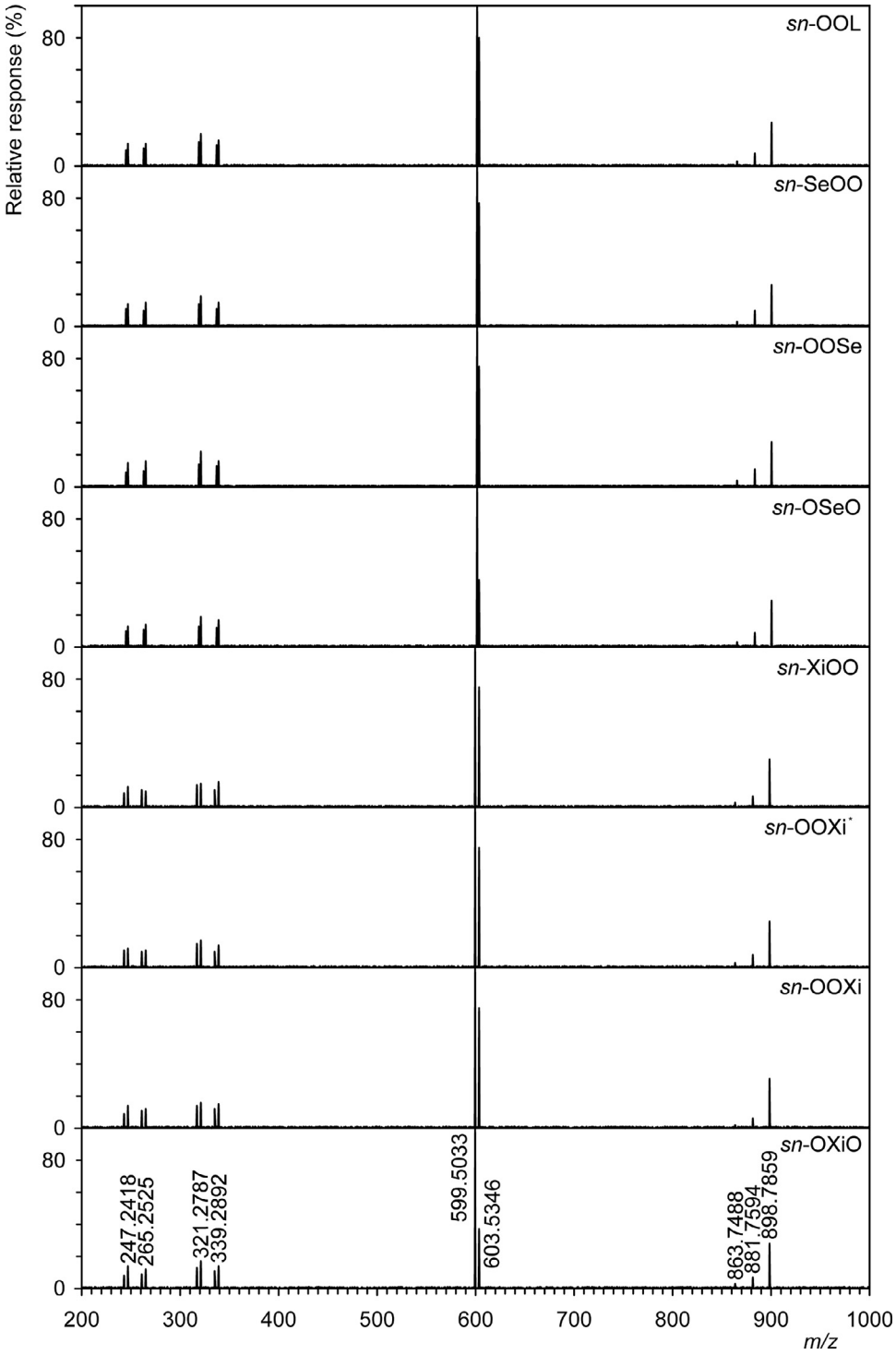

Fig. 7. Tandem mass spectra of the synthetic (*sn*-OOXi) and natural regioisomer and enantiomers. For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and discussion.

$[M+NH_4-NH_3-RCOOH]^+$, also called the $[DAG]^+$ ion), the TAG of OOXi was split into three molecular species, i.e., *sn*-OOXi, *sn*-OXiO and *sn*-XiOO at a ratio of approximately 2:3:14 (Fig. 6). The mass spectra of these three TAGs, including the mass spectrum of *sn*-OOLn, had nearly identical abundances for all ions except for the $[DAG]^+$ ions. For symmetrical TAGs, e.g., the *sn*-OXiO ion, the abundance of these ions varied, i.e., $[OXi]^+$ at *m/z* 599.5 and $[OO]^+$ at *m/z* 603.5, respectively. They were separated on a chiral column of TAGs containing another FA with triple bonds, i.e., stearolic acid. Three isomers (*sn*-OOSe, *sn*-SeOO, and *sn*-OSeO) were separated. Their tandem mass spectra were nearly identical ((Fig. 7) except for the $[DAG]^+$ ions. These mass spectra confirmed that the Mottram rule [1,2], " regioisomers of TAGs can be distinguished by the lower relative occurrence of the $[M+NH_4-NH_3-RCOOH]^+$ fragment ion, which results in a neutral loss of FA from the *sn*-2 position", applies to these less natural TAGs. Regarding the tR of the individual regioisomers and enantiomers, the results published by Lisa and Holčapek [3] for TAGs having identical molecular mass, i.e.,

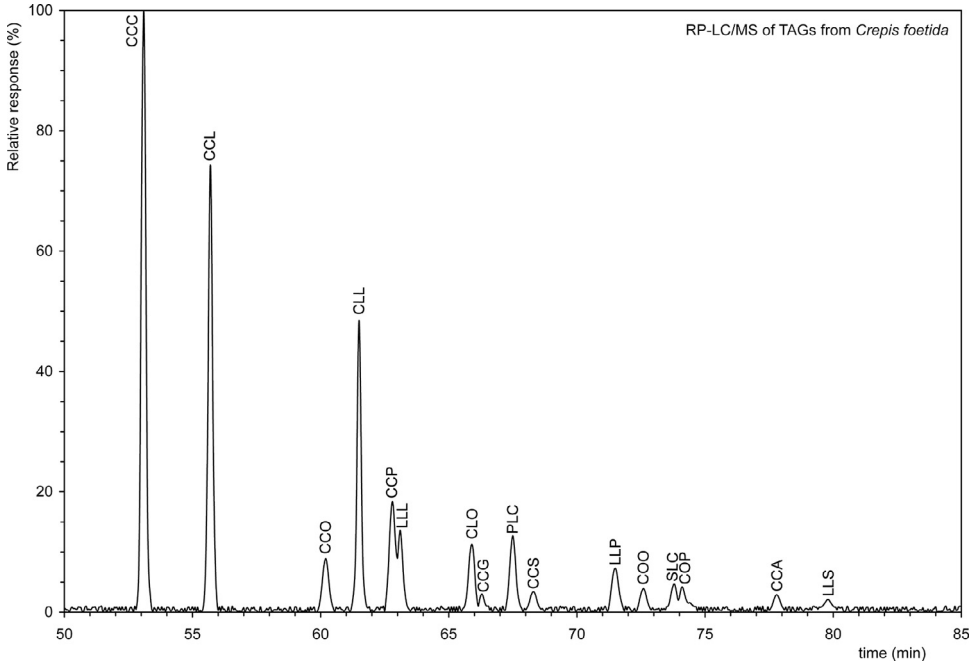

Fig. 8. Reverse phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP-LC/ESI–MS) chromatogram from 50 to 85 min of total ion current (TIC) of the TAGs from the *Crepis foetida* seeds. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e., ions resulting loss of neutral $RCOOH+NH_3$ from $[M+NH_4]^+$ in the interval from 480 to 680 Da. The $[DAG]^+$ type ion was always the base peak.

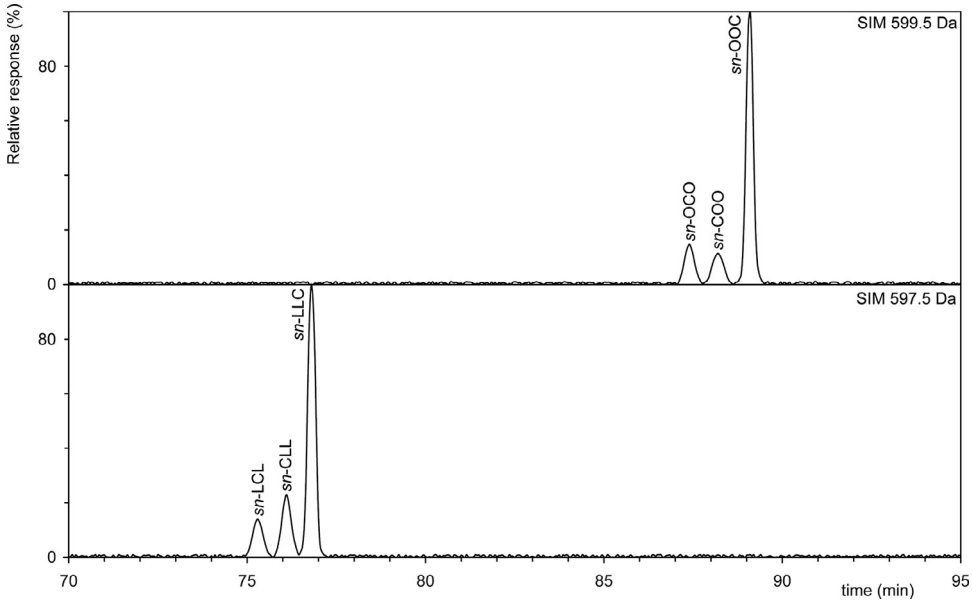

Fig. 9. Chiral separation of natural TAG, i.e., enantiomers and a regioisomers of TAGs from *Crepis foetida* seeds. The *sn*-LLC and *sn*-OOC were also synthesized. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e., ions resulting loss of neutral $RCOOH+NH_3$ from $[M+NH_4]^+$ in the interval from 480 to 680 Da. The $[DAG]^+$ type ion was always the base peak.

OOL, which is isomeric with OOSe, OOLn, and OOXi, have the same order of elution from the chiral column. This means that TAGs containing an acyl carbon number (ACN) of 54 with 4 double bonds (DB) elute in order of symmetric-asymmetric-asymmetric, which grows for both conventional (oleic, linoleic, and α-linolenic fatty acids) and less common fatty acids (e.g., stearolic and ximenynic).

The only previous work that has described a stereospecific analysis of TAGs from *Santalum album* seed oil is Lie Ken Jie et al. [43]. Total TAGs were separated by means of silica gel-coated TLC. FAs in individual fractions were analyzed by gas liquid chromatography after lipolysis by pancreatic lipase. In fraction A (Rf = 0.6), more oleic acid was identified in the β position than in the α position and in total fraction A. In fraction B (Rf = 0.5), most of the ximenynic acid was identified in the α position (60.2%), then the β position (56.1%); in the total fraction, B content was 58.8%. Therefore, it is apparent from the results that both O and Xi were nearly equally represented on both the primary and secondary hydroxyls of the glycerol backbone. Our results (Figs. 6 and 7) indicated that the *sn*-3 position was preferred for ximenynic acid. Similarly, in stearolic acid, the situation is similar, i.e., the *sn*-3 position is

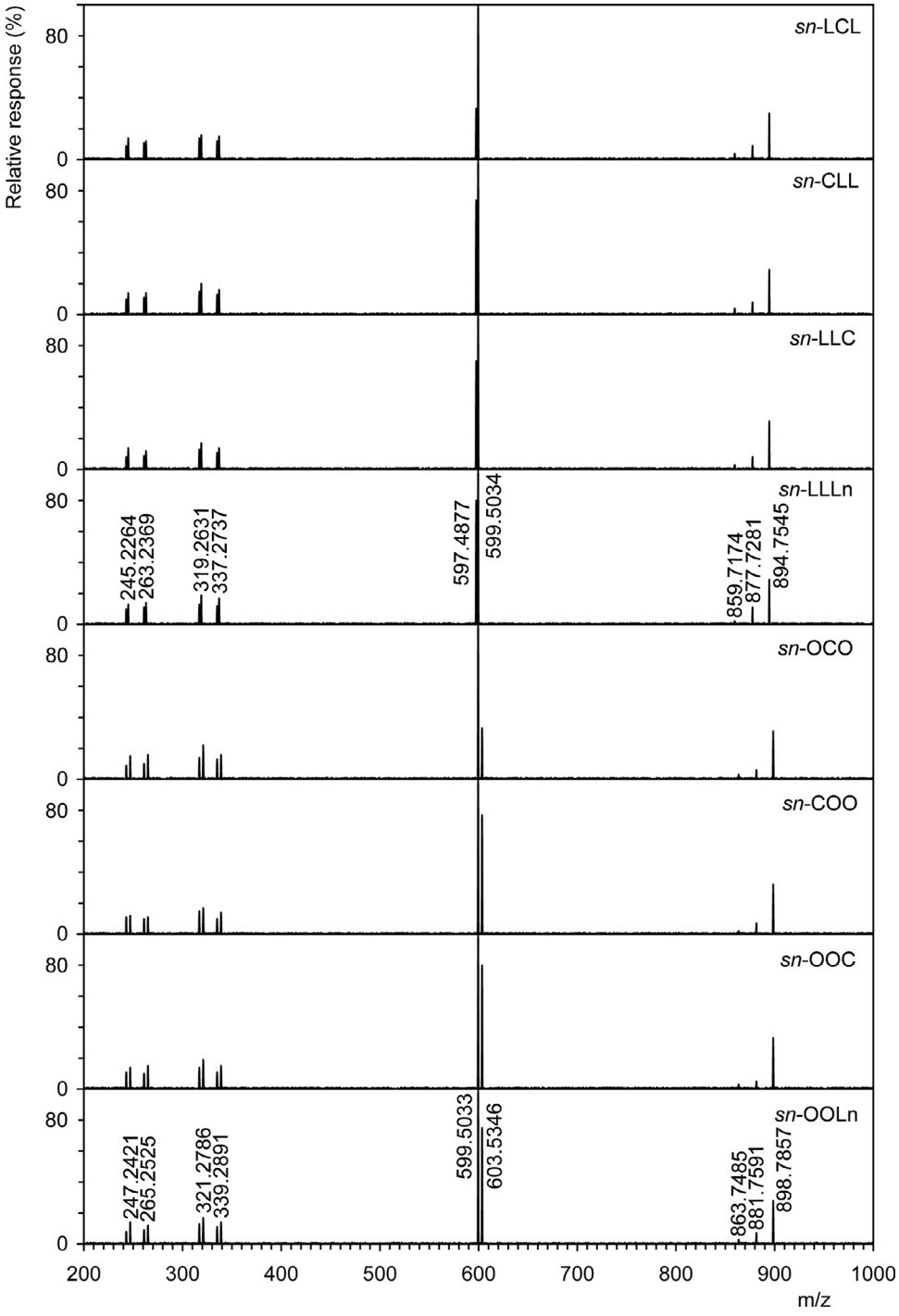

Fig. 10. Tandem mass spectra of the synthetic (*sn*-OOC and *sn*-LLC) and natural regioisomer and enantiomers, including standard (*sn*-OOLn). For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and discussion.

preferred. This is the first report to the best of our knowledge on the stereospecificity of TAGs from genus *Santalum*.

The third analyzed vegetable oil was an oil from *Crepis foetida* subsp. *foetida*. An analysis of *Crepis* seed oils has been published; crepenynic and linoleic acids were predominant FAs [38]. Palmitic, stearic and oleic acids have been identified as minor FAs. In the analysis of native TAGs by Ag^{+}-HPLC, these acids were separated according to double bonds, and eleven peaks were identified. The majority of peaks were CLL, CCO, CCL (two isomers) and CCC [44].

In another paper, TAGs from *Crepis alpin*a were separated on RP-HPLC, but detection was performed with a flame ionization detector (FID). Unfortunately, only two of seven peaks (CCC and CCL) were fully identified. The other peaks contained one or two acyls of the other FAs (palmitic, stearic, oleic, and linoleic acids) [45].

In a third and most recent paper, atmospheric pressure chemical ionization (APCI) detection was used. This method of analysis made it possible to identify 17 molecular species of TAGs. Again, CCC and CCL were found as the majority, but TAGs containing myristic, palmitic, stearic, oleic, linoleic, arachidic and eicosenoic

acids were also detected. Although two isomers of CCL were identified in the study [44], CCL was unfortunately not separated into two peaks by LC-MS/APCI on the reversed-phase column. APCI allows the identification of regioisomers [1,2] based on $[DAG]^+$ ion abundance. From the mass spectra shown in Fig. 3b and Table 1 of the paper by Neff and Byrdwell [46], it is assumed that a given peak, i.e., CCL at tR 16.29 min, is a mixture of two regioisomers (CLC versus CCL/LCC). To determine the proportion of regioisomers and enantiomers, *Crepis* oil was analyzed on a reversed-phase column and chiral column. Table 6S and Fig. 8 show the results from the reversed-phase column. The retention times of TAGs containing crepenynic acid were lower than those of TAGs containing linolenic acid (18 carbon atoms and unsaturation degree of 3). An example is the comparison of CCL and LnLnL (tR = 61.5 min and 51.0 min, respectively). Similar retention characteristics had a pair of TAGs, i.e., COO and LnOO (tR = 72.6 versus tR = 58.3). To fully elucidate the molecular species of some TAGs, two fractions were collected, i.e., peaks eluting from RP-column at intervals of 60.5–62.0 min and 72.0–73.0 min, respectively. These fractions were further separated on a chiral phase column (Fig. 9). The most represented molecular species of the three TAGs (*sn*-OOC, *sn*-COO and *sn*-OCO) is the *sn*-OOC isomer. The same method was applied to the other TAG isomer, e.g., LLC. Here, the major molecular species was *sn*-LLC. Identification of TAGs containing one or two crepenynoyl acyls by ESI^+ has been successful [46]. Unfortunately, two TAGs of the same molecular mass (LnLnLn and CCC) have very similar mass spectra. Fig. 10 shows the tandem mass spectra of four TAGs, i.e., namely, *sn*-CLL, *sn*-LLC, *sn*-LCL, and for comparison *sn*-LLLn. The only differences in ion abundance were observed for $[DAG]^+$, i.e., at *m/z* 597.5 and at *m/z* 599.5. Thus, the rule published in Mottram et al. [1,2] also applied to acetylenic type TAGs.

4. Conclusion

The combination of two techniques, i.e. separation by reversed-phase column and analysis by chiral chromatography tandem mass spectrometry, was used to analyze TAGs containing less common FAs with allenic or acetylenic bonds. Three vegetable oils from the seeds of *Crepis foetida, Leucas aspera*, and *Santalum album* were analyzed; these oils contained the three most common acids, i.e., allenic (laballenic acid) and acetylenic acids (ximenynic and crepenynic acids). As previously described [47,48], one of the limiting factors is the inability to obtain TAG enantiomers. Therefore, six TAGs were prepared from 1,2-diolein and 1,2-dilinolein, which allowed for the identification of regioisomers and enantiomers. Reversed-phase chromatography revealed that TAGs with allenic and acetylenic acids always had higher tR than the corresponding isomeric TAGs containing FAs with the same unsaturation (e.g., 9,12-octadecadienoic versus 5,6-octadecadienoic or 9,12,15-octadecatrienoic versus octadec-11-en-9-ynoic). For TAGs containing allenic FAs and TAGs containing acetylenic FAs, TAGs eluted from the chiral column in the order symmetric-asymmetric-asymmetric. The different elution times, unlike those previously reported [3], may be explained by the presence of allenic and acetylenic bonds.

For separations, especially on chiral columns, at least one enantiomer is required because both enantiomers have identical mass spectra. The regioisomers were separated and identified by tandem mass spectrometry. For their identification, the ratio of $[DAG]^+$ ions was used. The rule formulated by Mottram et al. [1,2] can be applied to allenic and acetylenic TAGs. The analysis of TAGs containing allenic and acetylenic FAs can also be used for other natural sources, i.e., acetylenes from marine origin, such as algae, sponges, invertebrates [49] and mosses [50].

CRediT authorship contribution statement

-Andrea Palyzová performed the analyses.

-Tomáš Řezanka performed the analyses and (together with Andrea Palyzová) drafted the article and approved the version to be submitted.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgments

The research was supported by the Ministry of Education, Youth and Sports of the Czech Republic (LO1509).

References

[1] R.H. Mottram, Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques, W.C Byrdwell (Ed.), AOCS Press, Champaign, 2005.
[2] R.H. Mottram, E.S. Woodbury, P.R. 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.
[3] M. Lísa, M. Holčapek, Characterization of triacylglycerol enantiomers using chiral HPLC/APCI-MS and synthesis of enantiomeric triacylglycerols, Anal. Chem. 85 (2013) 1852–1859, doi:10.1021/ac303237a.
[4] 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, doi:10.1021/jf901189u.
[5] T. Řezanka, M. Vítová, 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, doi:10.1007/s11745-015-4042-8.
[6] T. Řezanka, I. Kolouchová, L. Nedbalová, K. Sigler, Enantiomeric separation of triacylglycerols containing very long chain fatty acids, J. Chromatogr. A. 6 (2018) 9–19, doi:10.1016/j.chroma.2018.04.064.
[7] A. Palyzová, T. Řezanka, Enantiomeric separation of triacylglycerols containing fatty acids with a ring (cyclo-fatty acids), J. Chromatogr. A, in peer review. 2020. In press, DOI: doi.org/10.1016/j.chroma.2020.461103.
[8] 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, doi:10.1016/j.phytochem.2014.01.017.
[9] T. Řezanka, L. Nedbalová, K. Sigler, Enantiomeric separation of triacylglycerols containing polyunsaturated fatty acids with 18 carbon atoms, J. Chromatogr. A 1467 (2016) 261–269, doi:10.1016/j.chroma.2016.07.006.
[10] M. Kalpio, M. Nylund, K.M. Linderborg, B. Yang, B. Kristinsson, G.G. Haraldsson, H. Kallio, Enantioselective chromatography in analysis of triacylglycerols common in edible fats and oils, Food Chem. 172 (2015) 718–724, doi:10.1016/j.foodchem.2014.09.135.
[11] T. Nagai, H. Mizobe, I. Otake, K. Ichioka, K. Kojima, Y. Matsumoto, N. Gotoh, I. Kuroda, S. Wada, Enantiomeric separation of asymmetric triacylglycerol by recycle high-performance liquid chromatography with chiral column, J. Chromatogr. A 1218 (2011) 2880–2886, doi:10.1016/j.chroma.2011.02.067.
[12] T. Řezanka, Identification of very long polyenoic acids as picolinyl esters by Ag^+ ion exchange high-performance liquid-chromatography, reversed-phase high-performance liquid-chromatography and gas-chromatography mass-spectrometry, J. Chromatogr. A 513 (1990) 344–348.
[13] W.W. Christie, Gas Chromatography and Lipids: A Practical Guide, The Oily Press, Ayr., Scotland, 1989.
[14] F.E. Ziegler, G.D. Berger, A mild method for the esterification of fatty-acids, Synth. Commun. 9 (1979) 539–543.
[15] A. Halldorsson, C.D. Magnusson, G.G. Haraldsson, Chemoenzymatic synthesis of structured triacylglycerols by highly regioselective acylation, Tetrahedron 59 (2003) 9101–9109.
[16] B. Kristinsson, K.M. Linderborg, H. Kallio, G.G. Haraldsson, Synthesis of enantiopure structured triacylglycerols, Tetrahedron 25 (2014) 125–132, doi:10.1016/j.tetasy.2013.11.015.
[17] T. Řezanka, L. Nedbalová, K. Sigler, Enantiomeric separation of triacylglycerols containing polyunsaturated fatty acids with 18 carbon atoms,, J. Chromatogr. A 1467 (2016) 261–269, doi:10.1016/j.chroma.2016.07.006.
[18] S. Hamilton, R.J. Hamilton, P.A. Sewell, Lipid analysis: a practical approach, in: R.J. Hamilton, S. Hamilton (Eds.), IRL Press at Oxford University Press, Oxford, 1992.
[19] T. Řezanka, V.M. Dembitsky, Novel brominated lipidic compounds from lichens of Central Asia, Phytochemistry 51 (1999) 963–968.
[20] A. Vančura, T. Řezanka, J. Maršálek, K. Melzoch, G. Basařová, V. Křištan, Metabolism of L-threonine and fatty-acids and tylosin biosynthesis in *Streptomyces fradiae*, FEMS Microbiol. Lett. 49 (1988) 411–415.

[21] T. Řezanka, V.M. Dembitsky, gamma-Lactones from the soft corals *Sarcophyton trocheliophorum* and *Lithophyton arboreum*, Tetrahedron 57 (2001) 8743–8749.
[22] T. Řezanka, J. Lukavsky, K. Sigler, L. Nedbalova, M. Vitova, Temperature dependence of production of structured triacylglycerols in the alga *Trachydiscus minutus*, Phytochemistry 110 (2015) 37–45.
[23] 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.
[24] V.M. Dembitsky, T. Maoka, Allenic and cumulenic lipids, Prog. Lipid Res. 46 (2007) 328–375.
[25] J.M. Hagemann, F.R. Earle, I.A. Wolff, A.S. Barclay, Search for new industrial oils. XIV, Seed oils of labiatae, Lipids 2 (1967) 371–380.
[26] A.K. Choudhary, P. Sunojkumar, G. Mishra, Fatty acid profiling and multivariate analysis in the genus *Leucas* reveals its nutritional, pharmaceutical and chemotaxonomic signifikance, Phytochemistry 143 (2017) 72–80, doi:10.1016/j.phytochem.2017.07.007.
[27] R.C. Badami, K.B. Patil, Structure and occurrence of unusual fatty acids in minor seed oils, Prog. Lipid Res. 19 (1980) 119–153, doi:10.1016/0163-7827(80)90002-8.
[28] W.W. Christie, E.Y. Brechany, M.S.F. Lie Ken Jie, C.F. Wong, Mass spectrometry of derivatives of isomeric allenic fatty acids, Biol. Mass Spectrom. 21 (1992) 267–270.
[29] F. Nasirullah, S.M. Osman, *Leucas urticaefolia* seed oil - a rich source of labal-lenic acid, Fette Seif. Ans. 85 (1983) 314–315, doi:10.1002/lipi.19830850806.
[30] F.D. Gunstone, M. Lie Ken Jie, Fatty acids, part 25 thin layer and gas liquid chromatographic properties of some diunsaturated C_{18}-esters, Chem. Phys. Lipids 4 (1970) 131–138.
[31] A. Seher, H.J. Fiebig, Stereospezifische analyse von triacylglycerinen mit selten vorkommenden fettsäuren, Fette Seif. Ans. 85 (1983) 333–338, doi:10.1002/lipi.19830850901.
[32] V.I. Deineka, L.A. Deineka, A.M. Ermakov, M.Y. Tretýakov, HPLC investigation of triglycerides from plants of the Lamiaceae family, Chem. Nat. Compd. 40 (2004) 413–416, doi:10.1007/s10600-005-0001-6.
[33] T.V. Khomova, D.T. Asilbekova, S.D. Gusakova, A.U. Umarov, A study of the structure of plant triacylglycerols by the method of stereospecific analysis, Chem. Nat. Compd. 19 (1983) 266–269, doi:10.1007/BF00579755.
[34] F. Ianni, F. Blasi, D. Giusepponi, A. Coletti, F. Galli, B. Chankvetadze, R. Galarini, R. Sardella, Liquid chromatography separation of α- and γ-linolenic acid positional isomers with a stationary phase based on covalently immobilized cellulose tris(3,5-dichlorophenylcarbamate), J. Chromatogr. A 1609 (2020) 460461, doi:10.1016/j.chroma.2019.460461.
[35] C.R. Smith Jr, Occurrence of unusual fatty acids in plants, Progr. Chem. Fats Lipids 11 (1971) 139–177, doi:10.1016/0079-6832(71)90005-X.
[36] C.Y. Hopkins, M.J. Chisholm, W.J. Cody, Fatty acid components of some Santalaceae seed oils, Phytochemistry 8 (1969) 161–165, doi:10.1016/S0031-9422(00)85808-2.
[37] S. Thomaeus, A.S. Carlsson, S. Stymne, Distribution of fatty acids in polar and neutral lipids during seed development in *Arabidopsis thaliana* genetically engineered to produce acetylenic, epoxy and hydroxy fatty acids, Plant Sci. 161 (2001) 997–1003, doi:10.1016/S0168-9452(01)00500-3.
[38] F.R. Earle, A.S. Barclay, I.A. Wolff, Compositional variation in seed oils of the *Crepis* genus, Lipids 1 (1966) 325–327, doi:10.1007/BF02532675.
[39] G. Satoto, A.S. Fernandes, N. Saraiva, F. Santos, N. Neng, J.M. Nogueira, T. Santos de Almeida, M.E.M. Araújo, An overview on the properties of *Ximenia* oil used as cosmetic in Angola, Biomolecules 10 (2019) 1–11, doi:10.3390/biom10010018.
[40] K. Aitzetmüller, Santalbic acid in the plant kingdom, Plant Syst. Evol. 298 (2012) 1609–1617, doi:10.1007/s00606-012-0678-5.
[41] B. Matthäus, The database seed oil fatty acids, 2011. https//sofa.mri.bund.de.
[42] Y.D. Liu, R.B. Longmore, M.R. Boddy, J.E.D. Fox, Separation and identification of triximenynin from *Santalum spicatum* R. Br, J. Am. Oil Chem. Soc. 74 (1997) 1269–1272.
[43] M.S. Lie Ken Jie, M.K. Pasha, F. Ahmad, Ultrasound-assisted synthesis of santalbic acid and a study of triacylglycerol species in *Santalum album* (Linn.) seed oil, Lipids 31 (1996) 1083–1089.
[44] W.E. Neff, R.O. Adlof, M. El-Agaimy, Silver ion high-performance liquid chromatography of the triacylglycerols of *Crepis alpina* seed oil, J. Am. Oil Chem. Soc. 71 (1994) 853–855, doi:10.1007/BF02540461.
[45] W.E. Neff, R.O. Adlof, H. Konishi, D. Weisleder, High-performance liquid chromatography of the triacylglycerols of *Vernonia galamensis* and *Crepis alpina* seed oils, J. Am. Oil Chem. Soc. 70 (1993) 449–455, doi:10.1007/BF02542574.
[46] W.E. Neff, W.C. Byrdwell, Triacylglycerol analysis by high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry: *Crepis alpina* and *Vernonia galamensis* seed oils, J. Liq. Chromatogr. 18 (1995) 4165–4181.
[47] 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. Amer. Oil Chem. Soc. 90 (2013) 1327–1342.
[48] 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 tricomutum*, Phytochemistry 80 (2012) 17–27.
[49] Z.F. Zhou, M. Menna, Y.S. Cai, Y.W. Guo, Polyacetylenes of marine origin: chemistry and bioactivity, Chem. Rev. 115 (2015) 1543–1596, doi:10.1021/cr4006507.
[50] R.E. Minto, B.J. Blacklock, Biosynthesis and function of polyacetylenes and allied natural products, Prog. Lipid Res. 47 (2008) 233–306, doi:10.1016/j.plipres.2008.02.002.