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Enantiomeric separation of triacylglycerols containing fatty acids with a ring (cyclofatty acids)

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ABSTRACT

Triacylglycerols (TAGs) containing cyclofatty acids (cycloFAs) from two oilseeds of *Sterculia foetida* and *Hydnocarpus wightiana* were analysed using both reversed-phase (RP18) and chiral phase columns. TAGs were identified using high-resolution electrospray ionization mass spectrometry in the positive ion mode. Fifty-five molecular species of TAGs have been identified in sterculic oil, 27 of which contained at least one cyclopropenyl-FA (e.g., malvalic or sterculic acids). The structures of regioisomers and enantiomers were determined for five major TAGs with cyclopropenyl-FAs.

One hundred thirty-six TAGs were identified in chaulmoogra oil, 71 of which contained at least one cyclopentenyl-FA (e.g., gorlic, chaulmoogric, and hydnocarpic acids, etc.). Furthermore, in three molecular species, regioisomers and enantiomers were identified using HPLC on a chiral phase column. Eight molecular species of TAGs were prepared through organic synthesis to facilitate the identification of enantiomers. Retention times of fatty acid-containing triacylglycerols with one ring and one double bond are very similar to triacylglycerols with a dienoic fatty acid, but elution times are shorter. For example, dimalvaloylpalmitate elutes earlier than dilinoleylpalmitate.

The order of elution of TAGs on the chiral column differs. In TAGs with 2 degrees of unsaturation (ring and double bond, e.g. PStP-StPP-PPSt), the order of elution is symmetric-asymmetric-asymmetric TAGs. TAGs with 4 degrees of unsaturation (one ring and three double bonds or two rings and two double bonds) present a different pattern. When TAGs contain two rings and two double bonds, the order of elution TAGs is asymmetric-symmetric-asymmetric (StStP-StPSt-PStSt); when TAGs contain a ring and 3 double bonds, the elution order is symmetric-asymmetric-asymmetric TAGs (OStO-StOO-OOSt). In species with a higher degree of unsaturation (e.g., 5), the elution order of the TAGs is asymmetric-asymmetric-symmetric (e.g. CCO-OCC-COC).

1. Introduction

Vegetable oils are one of the main sources of energy for human nutrition. The most common sources include cultivated plants, such as canola, soybeans, sunflowers, and olive trees. Most of these vegetable oils contain four or five major fatty acids (FAs), i.e., palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) acid. Therefore, new and often non-traditional sources of vegetable oils [1] are still being sought. One potential source is cottonseed oil, but its dietary properties are poor [2]. An analysis of both FAs [3] and, less frequently, intact triacylglycerols (TAGs) [4] was performed for several vegetable oils. Gas chromatography (GC) is almost exclusively used to analyze FAs in oils containing cycloFAs, often in connection with mass spectrometry (GC–MS) [5,6]. Only a few articles describing the analysis of molecular species of TAGs containing cycloFAs [7,8] have been published.

Basically, two methods are used to analyse TAGs: a shotgun analysis, where the sample is directly introduced into the mass spectrometer [9], or a method in which the TAGs are separated using either GC or high-performance liquid chromatography (HPLC) to identify eluted compounds with soft ionization methods (atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI)) [10].

Natural triacylglycerols are always present in plant oil as the mixtures of many molecular species, including regioisomers or enantiomers. The number of TAGs is calculated to tens, hundreds or even thousands of molecular species depending on the number of FAs (see Table S1).

Abbreviations of fatty acids: A, arachidic; Be, behenic; C, chaulmoogric; dSt, dihydrosterculic; G, gorlic; H, hydnocarpic; Ho, hormelic; L, linoleic; Ln, α-linolenic; M, myristic; Ma, malvalic; O, oleic; On, oncobic; P, palmitic; Po, palmitoleic; S, stearic; St, sterculic; V, cis-vaccenic.

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The development of analytical techniques after 2000 year, has allowed the use of a liquid chromatograph connected to mass spectrometer, which uses soft ionization techniques such as APCI or ESI [11]. To explain how TAGs from vegetable oils are used in the human body, it is necessary to know their structure. It is known that in the gastrointestinal tract, pancreatic lipase cleaves FAs from the *sn*-1(3) positions to form *sn*-2-monoacylglycerols. For this reason, it is good to know the structure of the molecular species of TAGs, not only common vegetable oils but also less commonly occurring oils from plants, such as *Sterculia foetida* and *Hydnocarpus wightiana*. Both oils are characterized by the presence of cycloFAs, whether with a three- or five-membered rings [12].

Separation of regioisomers of TAGs of commonly occurring oils has been described, for example, in a review [13], unfortunately other oils, including dietary and pharmacologically important oils, have not yet been analyzed.

Non-aqueous reverse phase liquid chromatography (NARP-LC) [14] or silver-LC [15] are most commonly used for the separation of regioisomers of TAGs. Separation of conventional regioisomers has been described, although individual regioisomers have not always been identified. For example, an analysis of dozens of vegetable oils in Lisa et al. [13]. The identification of individual regioisomers can be performed on the basis of different abundances of ratios $[M+H-RiCOOH]^+$ ions as described Mottram [16]. In the case of enantiomers, mass spectra are identical and must be separated by chiral chromatography. Many chiral compounds have been used as a stationary phase, see, e.g., paper [17]. One of the most widely used phases was the phase with *tris*-(3,5-dimethyl-phenylcarbamate) covalently bound to β-cyclodextrin. The combination of both RP-LC/MS and chiral liquid chromatography/MS was used for the analysis of not very common vegetable oils.

Based on our previous experience with the analysis of regioisomers and enantiomers of TAGs, a serial coupling of two columns, either a reversed-phase (RP18) or chiral phase column based on *tris*-3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin, were used in the present study. TAGs were identified in all three oils, i.e., oils from *Sterculia foetida, Hydnocarpus wightiana* and *Dracunculus vulgaris*, in positive ESI mode using high-resolution MS.

2. Experimental methods

2.1. Standards

Common chemicals were purchased from Sigma–Aldrich (Prague, Czech Republic), the phospholipids, i.e., 1,2-dipalmitoyl-*sn*–glycero–3-phosphatidylcholine (16:0/16:0-PC), 1,2-dioleoyl-*sn*–glycero–3-phosphatidylcholine (18:1/18:1-PC), 1-oleoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine, (18:1-lyso-PC), and 1-palmitoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine (16:0-lyso-PC) were also purchased from Sigma. Sterculic acid (9,10-methylene-9-octadecenoic acid) was purchased from Cayman Chemical, Michigan, USA and chaulmoogric acid (13-(2-cyclopenten-1-yl)tridecanoic acid) was obtained from Coompo Research Chemicals, Wuhan (Hubei), China. Chaulmoogra oil (100% pure, natural & undiluted - 30 mL) was purchased from Allin Exporters, Noida, Uttar Pradesh, India and *Sterculia foetid*a (25 seed pack) was obtained from UrbanOrganic, Brooklyn, NY, USA.

2.2. Lipid extraction and isolation of TAGs

Seeds were powdered and extracted thoroughly with light petroleum ether to yield the oil. 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 (7:3). Solvents were evaporated and the oil samples were dissolved in an acetonitrile-2-propanol-hexane mixture (1:1:1, v/v/v), which was injected into the HPLC column [18] or transesterified, as described below.

2.3. Preparation of TAGs with a mixed FA composition

1,2-Dioleoyl- and 1,2-dipalmitoyl-*sn*–glycero–3-phosphatidylcholines were treated with phospholipase C (Type I from *C. perfringens* (*C. welchii*)), as described by Christie [19]. Similarly, both lysophosphatidylcholines (18:1-lyso-PC and 16:0-lyso-PC) were treated with phospholipase C. A detailed description of the preparation methods is provided in the Supplementary Information.

The esterification of glycerol derivatives with acids has been described previously [20,21]. Briefly, diacyl- and/or monoacylglycerol(s) were reacted with free acid(s) in the presence of 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) to yield the corresponding triacylglycerol(s); for details, see the Supplementary Information. The preparation of PPP13 (P is palmitic acid and P13 is 13-phenyl-tridecanoic acid) has been described previously [22].

2.4. Analysis of fatty acids

Fatty acid methyl esters (FAMEs) were prepared from neutral lipids using a standard procedure with sodium methoxide [23]. 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 1020 B instrument in the electron ionization (EI) mode and the esters were identified as previously described [24,25,26], see the Supplementary Information.

2.5. TAG analysis using RP–HPLC/MS–ESI

The LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and two s Hichrom HIRPB - 250A columns with a 250 × 2.1 mm ID and 5 μm particle size connected in series. This setup was used as a high efficiency column, approximately 105,000 plates/m (see manufacturer's literature), with a flow rate of 0.95 mL/min, an injection volume of 10 μL, and column temperature of 25 °C. TAGs were separated using a solvent gradient program with acetonitrile (ACN) containing 5 mM aqueous ammonium acetate, and 2-propanol (iPrOH) containing 5 mM aqueous ammonium acetate as follows: initial ACN/iPrOH (99:1, vol/vol), a linear change to 25:75 ACN/iPrOH (vol/vol) from 5 min to 100 min, and a hold for 10 min. The composition was returned to the initial conditions over 10 min. The performance was measured with triheptadecenoin as an internal standard under the conditions described above.

An LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany) high-resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. The ESI–MS analysis was performed in the FT positive ion mode. MS spectra were acquired with a target mass resolution of $R = 110{,}000$ at m/z 800. The electrospray voltage was set to 3.5 kV, and the scan range of the instruments was set to m/z 200–1500. Nitrogen was used as a nebulizer gas and set to 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. A CID normalization energy of 35% was used to fragment the precursor ions. The MS/MS product ions were detected in high-resolution FT mode. Further source conditions in the positive mode included a vaporizer temperature of 250 °C, capillary

temperature of 230 °C, capillary voltage of 50 V, and tube lens voltage of 170 V.

2.6. TAG analysis using chiral LC/MS–ESI

The LC system used for separation in the chiral mode was the same as the system used in the reversed-phase mode. TAGs (~1 mg/mL in mobile phase) were chromatographically separated on two Astec cyclobond™ I 2000 DMP (3,5-dimethyl-phenylcarbamate modified β-cyclodextrin) chiral LC columns (5 µm, 25 cm × 4.6 mm) (Sigma-Aldrich) connected in series. The mobile phase was a gradient from 95% A and 5% B at 0 min to 45% A and 55% B over 105 min, and then isocratically maintained for 10 min, where A is hexane and B is a hexane-2-propanol (97:3, v/v) mixture [27,28]. The flow rate, column temperature, and injection volume were 0.45 mL/min, 24 °C, and 10 µL, respectively. Other conditions were the same as those used for RP–LC, see above.

3. Results and discussion

3.1. Analysis of fatty acids

The GC–MS analysis of cycloFAs [29] is relatively straightforward. Using a relatively inert stationary phase and capillary column, including suitable (base-catalysed) derivatization, both cyclopropenyl (malvalic and sterculic acids) and cyclopentenyl (gorlic, chaulmoogric, and hydnocarpic acids, etc.) are easily analysed [6,7,30]. The representation of FAs from two natural oils, including their identification, is presented in Table S2a-b (Supplementary Information). Two mass spectra of 3-pyridylcarbinol esters (formerly called picolinyl esters) of sterculic and chaulmoogric acids are also shown in Figs. S1 and S2 (Supplementary Information).

3.1.1. Cyclopropenyl fatty acids

The mass spectrum of 3-pyridylcarbinyl sterculate (Fig. S1), in addition to the ions commonly present in these derivatives, exhibits primarily a diagnostic ion at *m/z* 293, which has the structure of acyl chain without 3-pyridylcarbinyl moieties [31]. Furthermore, two pairs of ions at *m/z* 234 and 272, and 220 and 286, resulting from α and β cleavage on both sides of the cyclopropene ring-containing hydrocarbon chain were identified. The mass spectrum of 3-pyridylcarbinyl malvalate is similar, except that the *m/z* values are shifted to lower values by 14 Da. The fatty acid content of *Sterculia foetida* is shown in Table S2a.

The presence of cyclopropenic FAs has been described in the lipids of seeds of plants from some families, particularly Malvaceae, Sterculiaceae, Bombaceae, Tiliaceae and Sapicidaceae [12,32]. The main representatives are sterculic (9,10-methylene-9-octadecenoic; St) and malvalic (8,9-methylene-8-heptadecenoic; Ma) acids. Sterculic acid (65.1%) [33] was present at high levels in *S. foetida* seeds. Cyclopropenyl-FAs are also found in cottonseed, kapok, and baobab oils, which are used as edible oils. The cyclopropenyl ring is responsible for physiological disorders in poultry, trout or rats [2].

3.1.2. Cyclopentenyl fatty acids

For the 3-pyridylcarbinol ester of cyclopentenyl-FA (chaulmoogric acid) with a saturated hydrocarbon chain, a dominant gap between the molecular ion and ion M-67 (loss of cyclopentene ring) was observed, as shown in Fig. S2. When another double bond is present in the molecule, as in gorlic acid, a gap of 26 Da between ions at *m/z* 192 and 218 and a gap of 40 Da between ions at *m/z* 192 and 232 were observed. Both the systematic and trivial names of these and other FAs are listed in Table S2b.

Seeds of a large number of tropical shrubs and trees belonging to the family Flacourtiaceae (now Achariaceae) contain oils consisting mainly of triacylglycerols (TAGs), which, in addition to the straight even-numbered chains, also contain cyclopentenyl-FAs [12,32]. The most important cyclopentenyl-FAs are hydnocarpic (11-(2-cyclopenten-1-yl) undecanoic), chaulmoogric (13-(2-cyclopenten-1-yl) tridecanoic), and gorlic (13-(cyclopent-2-en-1-yl) tridec–6-enoic) acids. Both lower and higher molecular weight homologues are present in the oils, e.g., aleprylic, alepric and oncobic or hormelic acids [5]. For example, in *Hydnocarpus anthelmintica* oil, the amount of hormelic acid was 0.3% of the total FAs, and gorlic, hydnocarpic and chaulmoogric acid comprised 59.6, 3.4 and 26.0% of the total FAs [5]. In contrast, straight-chain FAs, such as palmitic, oleic, linoleic, and linolenic acids [34], have been identified as the major fatty acids of *H. odorata*. Seed-extracted oils, known as “chaulmoogra” or “gorli” oils, have been used for centuries to treat leprosy [35].

3.2. Synthesis of TAG standards

Eight standards were synthesized, as shown in Table 1. Commercially available lysophosphatidylcholines and phosphatidylcholines, 1-palmitoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine (16:0-LPC), 1-oleoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine (18:1-LPC), 1,2-dipalmitoyl-*sn*–glycero–3-phosphatidylcholine (16:0/16:0-PC), and 1,2-dioleoyl-*sn*–glycero–3-phosphatidylcholine (18:1/18:1-PC), were used for synthesis,

Table 1
The ratio of enantiomers synthetized from 1-palmitoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine (16:0-LPC), 1-oleoyl-2–hydroxy–*sn*–glycero–3-phosphatidylcholine (18:1-LPC), 1,2-dipalmitoyl-*sn*–glycero–3-phosphatidylcholine (16:0/16:0-PC), and 1,2-dioleoyl-*sn*–glycero–3-phosphatidylcholine (18:1/18:1-PC); retention time (tR, min), and two diagnostic ions of type $[AA]^+$ and $[AB]^+$ including their abundances (%). Bold letters indicate diagnostic ions of type $[AA]^+$.

Enantiomer	Optical purity of the major enantiomer (%)	Retention time (min)	*m/z* of the diagnostic ion type $[AA]^+$ (abundance)	*m/z* of the diagnostic ion type $[AB]^+$ (abundance)
18:1/18:1/19:2[a]	91	78.9	603.5347 (68)	615.5347 (100)
18:1/18:1/18:2[b]	93		603.5346 (65)	601.5190 (100)
16:0/16:0/19:2	95	77.2	551.5034 (69)	589.5191 (100)
16:0/16:0/18:2[b]	94	88.3	551.5034 (72)	575.5034 (100)
18:1/**19:2/19:2**	82	83.1	627.5347 (66)	615.5347 (100)
18:1/**18:2/18:2**	90	93.7	599.5034 (66)	601.5190 (100)
16:0/**19:2/19:2**	91	92.4	627.5347 (66)	589.5191 (100)
16:0/**18:2/18:2**	89		599.5034 (66)	575.5034 (100)

[a] sterculic acid.
[b] chaulmoogric acid.

see the Experimental Procedures. Their lipolysis with phospholipase C (Fig. S3) produced diacyl and monoacylglycerols, which provided the desired TAGs after the reaction of free fatty acids, i.e., commercially available sterculic and chaulmoogric acids. The synthesis of PPP13 from a triacylglycerol containing ω-phenyl-FA (13-phenyl-tridecanoic acid) in the molecule has been previously described [33].

3.3. Analysis of TAGs from natural oils on a reversed-phase column

3.3.1. RP–HPLC of TAGs containing cyclopropenyl-FAs

The results of the analysis of the molecular species of TAGs from sterculic oil are presented in Table 2 and Fig. 1. Fifty-five

Table 2
High-performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI–MS) of the TAGs from the sterculia seed oil, retention time (tR, min), acyl carbon number (ACN) number of double bond(s) (DB), equivalent carbon number (ECN), and relative quantities (%) of molecular species of TAGs.

tR (RP)	TAG	ACN	unsaturation	ECN	abund
48.0	LLL	54	6	42	1.46
48.5	PoLL	52	5	42	0.16
53.0	MaMaO	54	5	44	0.34
53.3	PoStSt	54	5	44	0.34
54.9	StStM	52	4	44	0.21
55.1	StStSt	57	6	45	0.47
55.4	MaMaP	52	4	44	4.11
57.4	LLO	54	5	44	3.70
59.5	LLP	52	4	44	4.99
60.5	OStPo	53	4	45	0.16
62.2	OStSt	56	5	46	3.00
62.5	PStPo	51	3	45	0.22
64.2	PStSt	54	4	46	15.66
64.4	MaOO	54	4	46	0.80
66.5	MaOP	52	3	46	1.08
66.6	LOO	54	4	46	2.62
68.2	LLS	54	4	46	1.65
68.4	LOP	52	3	46	3.53
68.8	MaPP	50	2	46	1.45
69.0	OOSt	55	4	47	3.78
71.0	LPP	50	2	46	4.76
72.0	OPSt	53	3	47	5.10
73.0	SStSt	56	4	48	1.87
73.4	PPSt	51	2	47	9.99
75.0	MaOS	54	3	48	0.36
75.6	OOO	54	3	48	1.86
76.8	ALL	56	4	48	0.57
77.3	SMaP	52	2	48	0.48
77.3	LOS	54	3	48	1.17
77.7	POO	52	2	48	2.50
79.5	SLP	52	2	48	1.58
79.6	SStO	55	3	49	1.69
80.1	PPO	50	1	48	3.38
81.5	AStSt	58	4	50	1.19
81.8	SStP	53	2	49	2.28
82.6	PPP	48	0	48	1.42
85.0	AMaP	54	2	50	0.17
85.1	MaSS	54	2	50	0.16
85.1	ALO	56	3	50	0.40
85.7	OOS	54	2	50	0.83
87.2	ALP	54	2	50	0.55
87.3	LSS	54	2	50	0.52
87.5	AStO	57	3	51	0.58
87.9	SPO	52	1	50	1.12
89.5	AStP	55	2	51	0.79
89.7	SSSt	55	2	51	0.76
91.0	SPP	50	0	50	1.51
93.4	AOO	56	2	52	0.29
94.7	ALS	56	2	52	0.18
95.6	AOP	54	1	52	0.39
96.1	OSS	54	1	52	0.37
97.1	AStS	57	2	53	0.26
98.7	APP	52	0	52	0.52
98.9	SSP	52	0	52	0.50
106.1	SSS	54	0	54	0.17

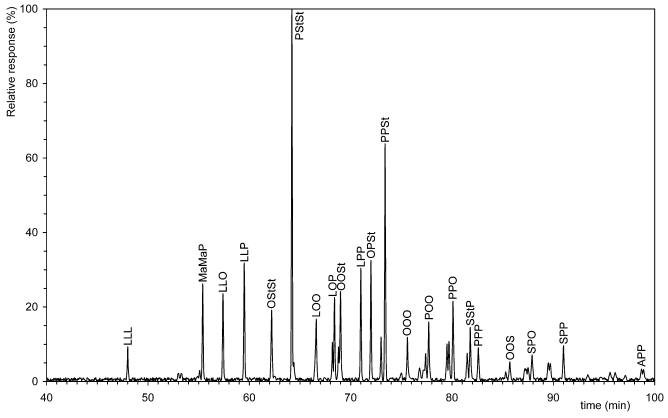

Fig. 1. Reverse phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP–LC/ESI–MS) chromatogram from 40 to 100 min of total ion current (TIC) of the TAGs from the sterculia oil. Identification of the peaks, see Table 2. TIC was filtered and indicates the type of ions $[DAG]^+$, 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.

TAGs were separated by the two columns connected in series with the RP-18 phase. Some of the major TAGs were collected and further identified as described below. The chromatographic behaviour of TAGs with cyclopropene rings is similar but not the same as TAGs with dienoic acids. The tandem mass spectrum distinguished symmetric, e.g., PStP, and asymmetric (PPSt and/or StPP) triacylglycerols, as shown in Fig. 2, but separation was done by chiral chromatography. These regioisomers were identified based on the well-known rule formulated by Mottram et al. [16,36] stating that "the regioisomers of TAGs can be distinguished by the lower relative occurrence of the $[M+H-RCOOH]^+$ fragment ion, which results in a neutral loss of FA from the *sn*-2 position".

It is generally known that tR increases with the length of the hydrocarbon chain, which is universally true for every type of lipid analyzed using non-aqueous RP-U/HPLC. This phenomenon has been confirmed for TAGs not having the usual structures, see below. The retention time (tR) increases as the chain length of TAGs containing two cyclopropenyl-FAs (e.g., two sterculic acids) increases: a series of four TAGs - MStSt (tR = 54.9 min), PStSt (64.2), SStSt (73.0), and AStSt (81.5). See Tables S2a–b for definitions of the abbreviations for FAs. Furthermore, TAGs with cyclopropenyl-FAs were eluted from the column before the corresponding TAG in which the cyclopropenyl-FA was replaced with dienoic acid. Examples include the comparisons of the TAGs MaMaO (53.0 min) with LLO (57.4 min), MaMaP (55.4) - LLP (59.5), MaOO (64.4) - LOO (66.6), etc. The so-called critical pairs of TAGs, i.e., pairs that have the same equivalent carbon number (ECN), such as OOSt (69.0) - OPSt (72.0), were also separated.

Aichholz et al. [37] identified approximately 20 TAGs in native oil from the *Chorisia speciosa* (now *Ceiba speciosa*) tree, including TAGs containing cyclopropenyl-FAs, such as POSt, OOSt, or OLSt. After the hydrogenation of the oil and high-temperature gas chromatography (HTGC), many TAGs with cyclopropenyl-FAs were present at low levels in the native oil and were not identified. These TAGs included PSdMa (dMA - dihydromalvalic acid) and SdStdSt (dSt - dihydrosterculic acid), and therefore ten additional TAGs containing cyclopropenyl-FAs were identified. In essence, the only drawback of the published article is the use of HTGC, which is not the most suitable method for vegetable oils, as mentioned by Rezanka and Mares [38]. This deficiency was manifested in the ratio of identified TAGs with cyclopropenyl-FAs from native and hydrogenated oils.

A report by Yeboah et al. [39] showed that edible seeds of *Pachira insignis* (asiato), i.e. a tree or shrub that is distributed

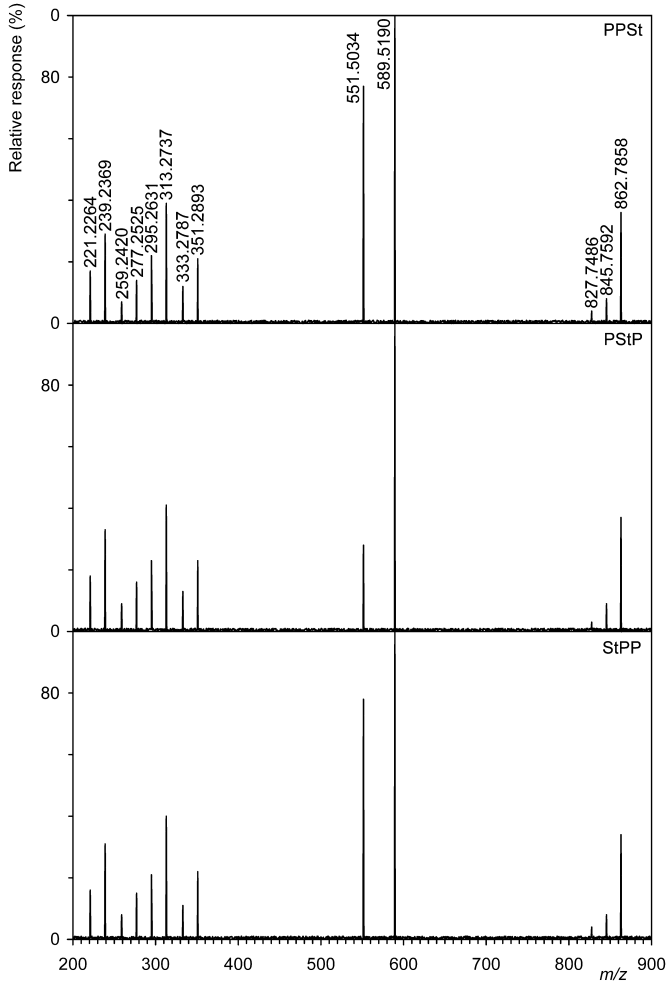

Fig. 2. Tandem mass spectra of the synthetic and natural regioisomer (*sn*-PStP) and enantiomers (*sn*-PPSt or *sn*-StPP). For experimental details, see Experimental conditions and explanation of chromatographic behavior, in all figures, including mass spectra, see Results and discussion. .

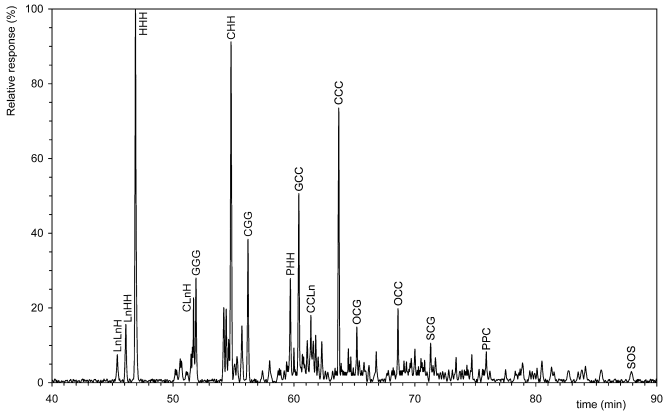

Fig. 3. Reverse phase liquid chromatography/electrospray positive ionization-mass spectrometry (RP–LC/ESI–MS) chromatogram from 40 to 90 min of total ion current (TIC) of the TAGs from the chaulmoogric oil. Identification of the peaks, see Table 3. TIC was filtered and indicates the type of ions $[DAG]^+$, 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.

in a tropical area, contain mainly palmitic and sterculic (dihydrosterculic) acids. Its TAGs were analysed using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry in the positive ion mode. Eighteen TAGs were identified, of which 6 had an odd number of acyl groups (number of acyl groups:unsaturations), e.g., 49:2, 51:2, 53:4, etc. From the overview and based on the FAME analysis, TAG 51:2 contains sterculic acid (19:2) and two palmitic acids ($2 \times 16:0$), i.e., ($19:2 + 16:0 + 16:0 = 51:2$). Unfortunately, the authors did not perform tandem MS, and thus they were only able to speculate about the content of molecular species or regioisomers present in the oils.

Howarth and Vlahov [40] reported application of ^{13}C NMR for stereospecific analysis, but only oleoyl acyl was identified in the *sn*-2 position. Although in their Scheme 1, the *sn*-2 position of the major TAG (dipalmitoylsterculate) was esterified with sterculic acid. Similarly, TAGs containing sterculic and dihydrosterculic acids (LStP, LLSt, LdStP, OStP, etc.) were identified in cottonseed oil using ESI–MS [7].

3.3.2. RP–HPLC of TAGs containing cyclopentenyl-FAs

An analysis of TAGs from seed oil of *Hydnocarpus wightiana* yielded some interesting results. It was observed, that tR increased of the TAG triad CPP (75.9 min), CPS (80.5 min), and CSS (84.1 min). However, completely different results were obtained for molecular species having the analogous characteristics (number of acyl moieties:unsaturations = 54:5), such as a pair of LLO versus CCO triacylglycerols with tRs of 68.0 min and 68.6 min, respectively. Although formally, equating the loss of two hydrogens due to cyclization to a double bond (which has Π-bonding). It was found that two hydrogens lost due to cyclization do not equate to a double bond and therefore differ tR LLO versus CCO TAGs, see below. TAGs containing FAs with a cyclopentenoic ring in the molecule eluted with a later tR than TAGs with straight-chain FAs, as shown in Table 3 and Fig. 3. The same trend was observed in other pairs, including LnLnS-GGS, LnLnO-GGO, LLLn-LLG, LPP-CPP, etc., as shown in Table 3. Unlike cyclopropenyl-FAs TAGs, this change in elution order, i.e., TAGs with straight-chain(s) elute first followed by cyclopentenyl-FAs TAGs, is explained by the location of the cyclopropene ring of the fatty acid in the middle of the chain, whereas the cyclopentene ring is located at the end of the hydrocarbon chain. Based on this spatial arrangement, the cyclopropene group disrupts the van der Waals interactions in the middle of the chain, while the cyclopentene-containing chains contain well-functioning chains with a stationary phase for most of the chain length. Therefore, the order of elution of TAGs with cyclopropenyl-FAs is reversed relative to cyclopentenyl TAGs. The hydrocarbon chain(s) is presumed to interact much more strongly with the stationary phase via the Van der Waals forces than the cyclopentene ring and/or cyclopropene ring due to its spatial arrangement. This can be explained by the fact that the majority of the chain behaves like FA having two fewer sites of unsaturation (dienoic FAs are compared with FAs having one ring and one double bond). This fact causing stronger retention, and the cyclopentene ring on the end of the molecule has much less effect on the retention than the long chain of carbons. In addition, as shown in Fig. S4, the spatial arrangements of oleic, linoleic, malvalic and chaulmoogric acids differ.

Astonishingly, this oil that has been used for several centuries to treat leprosy has only rarely been analysed. Although the FAME analysis has been described many times, including the references cited in the database Seed Oil Fatty Acids (SOFA) [41] or in other papers [42,43,44], the triacylglycerols have not been extensively analysed. To the best of our knowledge, only two articles describing the analysis of intact TAGs have been published. In the first article [8], which was published more than 30 years ago, i.e., at the time when the HPLC separation of TAGs was not nearly as sensitive as it is today, the authors analysed TAGs from *Caloncoba echinata* and *Hydnocarpus anthelmintica* using two C18-linked

Table 3
High-performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI–MS) of the TAGs from the chaulmoogra seed oil, retention time (tR, min), acyl carbon number (ACN) number of double bond(s) (DB), effective carbon number (ECN), and relative quantities (%) of molecular species of TAGs.

tR (RP)	TAG	ACN	Unsaturation	ECN	Abundance
45.4	HLnLn	52	8	36	0.59
46.1	HHLn	50	7	36	1.41
46.9	HHH	48	6	36	10.17
50.2	LnLnLn	54	9	36	0.20
50.3	LnLnPo	52	7	38	0.17
50.6	HLLn	52	7	38	0.48
50.7	GLnLn	54	9	36	0.41
51.6	GGL	54	8	38	0.80
51.7	CHLn	52	7	38	2.14
51.9	GGG	54	9	36	2.68
54.2	HHL	50	6	38	1.87
54.4	HHPo	48	5	38	1.84
54.5	LLnLn	54	8	38	0.17
54.6	HHM	46	4	38	1.01
54.8	CHH	50	6	38	9.10
55.1	GLLn	54	8	38	0.34
55.3	CLnLn	54	8	38	0.54
55.7	CGLn	54	8	38	1.38
56.2	CGG	54	8	38	3.74
57.4	LLnPo	52	6	40	0.15
58.0	CLnPo	52	6	40	0.43
58.7	LLLn	54	7	40	0.15
58.8	LnLnP	52	6	40	0.22
58.9	LnOPo	52	5	42	0.17
59.2	LnPoPo	50	5	40	0.15
59.5	GLL	54	7	40	0.28
59.6	HPoPo	48	4	40	0.38
59.7	HHP	48	4	40	2.66
59.8	LnLnO	54	7	40	0.20
59.9	LLPo	52	5	42	0.05
60.2	LnMPo	48	4	40	0.12
60.3	GLnO	54	7	40	0.40
60.4	CCG	54	7	40	4.97
60.6	HMPo	46	3	40	0.24
60.7	CLnP	52	5	42	0.60
60.8	HLP	50	4	42	0.53
60.9	HMP	46	2	42	0.32
61.1	GGO	54	7	40	0.97
61.2	LnPPo	50	4	42	0.19
61.3	GLnP	52	6	40	0.45
61.4	CHL	52	6	40	1.66
61.5	HOPo	50	4	42	0.47
61.6	CCLn	54	7	40	0.96
61.7	CPoPo	50	4	42	0.11
61.8	GGP	52	6	40	1.12
62.0	HPPo	48	3	42	0.53
62.3	HHO	50	5	40	10.58
62.6	LPoPo	50	4	42	0.14
62.8	LMPo	48	3	42	0.11
63.2	LLL	54	6	42	0.14
63.4	CMPo	48	3	42	0.22
63.5	LLPo	52	5	42	0.14
63.7	CCC	54	6	42	7.31
63.8	PoPoPo	48	3	42	0.14
63.9	CLPo	52	5	42	0.36
64.0	LLnO	54	6	42	0.17
64.1	MLnP	48	3	42	0.14
64.2	MPoPo	46	2	42	0.11
64.3	LLM	50	4	42	0.11
64.6	GLO	54	6	42	0.33
64.7	CLnO	54	6	42	0.52
64.8	LLOn	56	7	42	0.09
64.9	CLM	50	4	42	0.23
65.0	CLO	54	5	44	0.50
65.1	LLnP	52	5	42	0.19
65.2	CGO	54	6	42	1.34
65.4	CCOn	56	7	42	0.43
65.6	LnLnS	54	6	42	0.16
65.8	GLP	52	5	42	0.37
65.9	OPoPo	50	3	44	0.15
66.2	GLnS	54	6	42	0.29
66.8	GGS	54	6	42	0.67
67.8	LPPo	50	3	44	0.16
68.1	LLO	54	5	44	0.15
68.1	LnPP	50	3	44	0.23
68.6	CCO	54	5	44	1.85
68.7	LnOO	54	5	44	0.20
68.8	LOPo	52	4	44	0.15
68.9	PoPoO	50	3	44	0.15
69.0	LLP	52	4	44	0.16
69.1	COPo	52	4	44	0.42
69.2	HoLL	56	6	44	0.09
69.3	GOO	54	5	44	0.39
69.4	MLO	50	3	44	0.12
69.5	LnOP	52	4	44	0.20
69.7	CLP	52	4	44	0.49
69.9	LLnS	54	5	44	0.14
70.0	HPP	48	2	44	0.74
70.3	CMO	50	3	44	0.26
70.4	PPoPo	48	2	44	0.16
70.5	CPPo	50	3	44	0.49
70.6	CLnS	54	5	44	0.37
70.7	GLS	54	5	44	0.24
70.8	GOP	52	4	44	0.44
71.0	LMP	48	2	44	0.12
71.3	CGS	54	5	44	0.91
71.4	MPPo	46	1	44	0.12
71.5	CMP	48	2	44	0.30
71.7	GPP	50	3	44	0.51
72.1	MMP	44	0	44	0.10
72.5	LOOn	56	6	44	0.10
72.8	LOO	54	4	46	0.17
73.1	PoOO	52	3	46	0.17
73.4	COO	54	4	46	0.52
73.7	LLS	54	4	46	0.13
73.9	LPO	52	3	46	0.18
74.1	LOP	52	3	46	0.18
74.2	MOO	50	2	46	0.13
74.3	CLS	54	4	46	0.31
74.4	OPPo	50	2	46	0.18
74.6	LnSO	54	4	46	0.16
74.7	COP	52	3	46	0.59
75.3	LPP	50	2	46	0.20
75.6	PPPo	48	1	46	0.20
75.7	LnPS	52	3	46	0.17
75.8	MOP	48	1	46	0.14
75.9	CPP	50	2	46	0.67
76.2	MPP	46	0	46	0.14
77.5	OOO	54	3	48	0.19
78.3	LOS	54	3	48	0.14
78.4	POO	52	2	48	0.07
78.6	OOOn	56	5	46	0.11
78.8	OPoS	52	2	48	0.14
78.9	COS	54	3	48	0.37
79.5	PLS	52	2	48	0.15
79.7	LnSS	54	3	48	0.13
79.9	OPP	50	1	48	0.08
80.5	CPS	52	2	48	0.41
81.3	PPP	48	0	48	0.25
81.5	MPS	48	0	48	0.12
82.7	OOS	54	2	50	0.15
83.5	LSS	54	2	50	0.12
83.8	POS	52	1	50	0.17
84.1	CSS	54	2	50	0.27
85.4	PPS	50	0	50	0.18
87.9	SSO	54	1	52	0.13

reversed-phase columns. Unfortunately, the authors [8] did not detect C20 cyclopentenyl-FAs or straight-chain fatty acids because of their low oil content. We detected both types of acids, although only five TAGs contained C20 cyclopentenyl-FAs. Numerous TAGs containing straight-chains have been identified, as listed in Table 3. Furthermore, the authors [8] state that "a comparison of triglycerides containing cyclopentenyl fatty acids with those of straight-chain fatty acids show that CCC is equal to OOO, PCC is equal to POO and PPC is equal to PPO, respectively". In contrast to their

results, these so-called critical pairs were separated in the present study; for example, PPL had a tR of 75.3 min, while PPC had a tR of 75.9 min. Similarly, PLL had a tR of 69.0 min, while PLC had a tR 69.7 min, etc. The effect of the later elution of cyclopentenyl-FA-containing TAGs was observed (see above).

According to Waktola et al. [45], TAGs from *Carpotroche brasiliensis* were analysed. The authors used high-temperature gas chromatography-electron impact mass spectrometry (HTGC–EIMS) and identified 21 TAGs containing straight-chain FA. Six peaks were separated; unfortunately, despite the use of a 17.5 m long column, each peak was a mixture of several molecular species. For instance, the most abundant peak contained four TAGs, i.e., OHH, LPoH, CHH, and GHH. The authors did not mention the identification of regioisomers, e.g., OHH versus HOH. Of course, enantiomers were not separated. As mentioned above, the majority of TAGs mainly contained three cyclopentenyl FAs, i.e., chaulmoogric, hydnocarpic, and gorlic acids.

3.4. Chiral chromatography of TAGs

Mottram [16] and Cottle [35] reported that symmetrical and asymmetrical TAGs, e.g., PStP and PPSt/StPP, respectively can be distinguished based on their mass spectra. Conversely, both enantiomers have identical mass spectra [27], as shown in Fig. 1. A combination of MS data and the retention time of at least one enantiomer must be used to identify the enantiomers (Fig. 2). However, their separation, i.e., the order of enantiomer elution, is very difficult to predict. Possible mechanisms of the separation on chiral phase are based on the interaction of Π electrons of the stationary phase with double bond of acyl(s). It is also the possibility of the interaction Van der Waals forces between two methyls (stationary phase) and CH_2 acyl groups.

In 2013, Lisa et al. [46] described elution of commonly occurring TAGs present in vegetable oils from a chiral column. TAGs with two double bonds, such as dipalmitoyllinolein, eluted in the following order from the chiral column: the symmetric TAG eluted first, followed by the asymmetric TAG, i.e., PLP-LPP/PPL. In the present study, TAGs with two degrees of unsaturation (one ring and one double bond) eluted in the order of symmetric-asymmetric-asymmetric TAGs. For example, the elution of the regioisomers and enantiomers is described in Table 4. TAGs containing two palmitic and one sterculic acid, as well as two palmitic and one chaulmoogric acid, are eluted from the chiral column in the order of symmetric-asymmetric-asymmetric TAGs (PStP-StPP-PPSt, respectively PCP-CPP-PPC), as shown in Fig. 4.

The elution profile of TAGs with four degrees of unsaturation (one ring and three double bonds or two rings and two double bonds) is more complicated. TAGs containing two rings and two double bonds elute in the order of asymmetric-symmetric-asymmetric TAGs (e.g., StStP-StPSt-PStSt). TAGs with one ring and 3 double bonds elute in the order of symmetric-asymmetric-asymmetric TAG. Again, Lisa et al. [46] described the elution profile of TAGs with four degrees of unsaturation in the order of symmetric-asymmetric-asymmetric TAGs, e.g., OLO-LOO-OOL. Elution in the symmetric-asymmetric-asymmetric order was observed for TAGs with five degrees of unsaturation, such as triacylglycerols with one oleic acid and two acids containing one ring and one double bond. An example is the elution of TAGs in the order of StStO-OStSt-StOSt or CCO-OCC-COC. This order of elution is identical to a previous report [46], where the order of elution of TAGs with five degrees of unsaturation, such as dilinoleylolein, was LLO-OLL-LOL.

Furthermore, consistent with the published data [46], enantiomers with a higher degree of unsaturation in the *sn*-1 position eluted earlier than the enantiomer with a lower degree of unsaturation, as shown in Table 4. This profile was observed for two TAGs, for which no standards were synthesized. An example is the order of elution of asymmetric TAGs, e.g., MaMaP-PMaMa or HHP-PHH.

Table 4
Chiral LC/ESI$^+$–MS of the TAGs from the sterculia, chaulmoogra and *Dracunculus* seed oils, retention time (tR, min), equivalent carbon number (ECN), total acyl carbon number (ACN), sum of ring and double bond(s) (unsaturation), and relative quantities (%) of molecular species (including enantiomers) of TAGs. (bold – synthesized enantiomers).

tR (chi)	TAG	Abund (%)	ACN	Unsaturation	ECN
76.7	PStP	67	51	2	47
77.0	StPP	25	51	2	47
77.2	**PPSt**	9	51	2	47
79.1	OStO	62	55	4	47
79.6	StOO	26	55	4	47
79.9	**OOSt**	12	55	4	47
82.4	StStO	35	56	5	46
83.1	**OStSt**	21	56	5	46
83.6	StOSt	44	56	5	46
87.6	PCP	4	50	2	46
88.0	CPP	69	50	2	46
88.3	**PPC**	27	50	2	46
92.4	StStP	26	54	4	46
92.7	StPSt	5	54	4	46
92.9	**PStSt**	69	54	4	46
93.3	CCO	11	54	5	44
93.8	**OCC**	80	54	5	44
94.1	COC	9	54	5	44
94.4	MaMaP	7	52	4	44
94.7	MaPMa	82	52	4	44
94.9	PMaMa	11	52	4	44
95,1	P13PP	10	51	4	43
95,3	**PPP13**	82	51	4	43
95,7	PP13P	8	51	4	43
97.1	HHP	74	48	4	40
97.4	HPH	7	48	4	40
97.6	PHH	19	48	4	40

A report by Howarth and Vlahov [40] performed a stereospecific analysis of sterculic oil using ^{13}C NMR and TAGs were identified following subsequent pancreatic lipase lipolysis. When ^{13}C NMR was used, the inner acyl group was sterculate, whereas malvalate was not preferred in any position. In another article [44] describing the analysis of TAG derivatives after lipolysis, the *sn*-2 position was preferred for triacylglycerols containing one sterculic acid, but sterculic acid was present at *sn*-1/3 in approximately ~33% of the TAGs with an *sn*-2 preference. When two sterculic acids were present in the TAG molecule, the major sterculate was located at the *sn*-2 position, although this FA was located at *sn*-1/3 in half of the molecules. In contrast, malvalic acid was preferably esterified at *sn*-1/3. For other FAs, i.e., acids that do not contain a cyclopropene ring, such as palmitic, oleic or linoleic acids, their distributions on the glycerol backbone differ. Palmitic acid is preferentially bound to the *sn*-2 position, whereas oleoyl and linoleoyl acyls equally esterify all glycerol hydroxyls.

Schuch et al. [47] described TAGs from *Bombax munguba* seed oil. Palmitic acid is always the major species at *sn*-1/3, whereas sterculic acid was preferred at *sn*-2.

According to Mani and Lakshminarayana [48], approximately 50 years ago, the molecular species of TAGs from *H. wightiana* seeds were simulated using a computer program. Total TAGs were subjected to both alkaline hydrolysis and treatment with pancreatic lipase, and the molecular species of TAGs were predicted from the identified FAs. The authors predicted HHH and CHH as the main triacylglycerol species, consistent with our results. Furthermore, using pancreatic lipase, the authors identified the predominance of hydnocarpic acid in the *sn*-2 position, and approximately 50% of the total oleic acid was located at the same position. Unfortunately, other results obtained by random, and restricted random methods, among others, were far less successful. The necessity to abandon the theory of predicting molecular species of TAGs has been

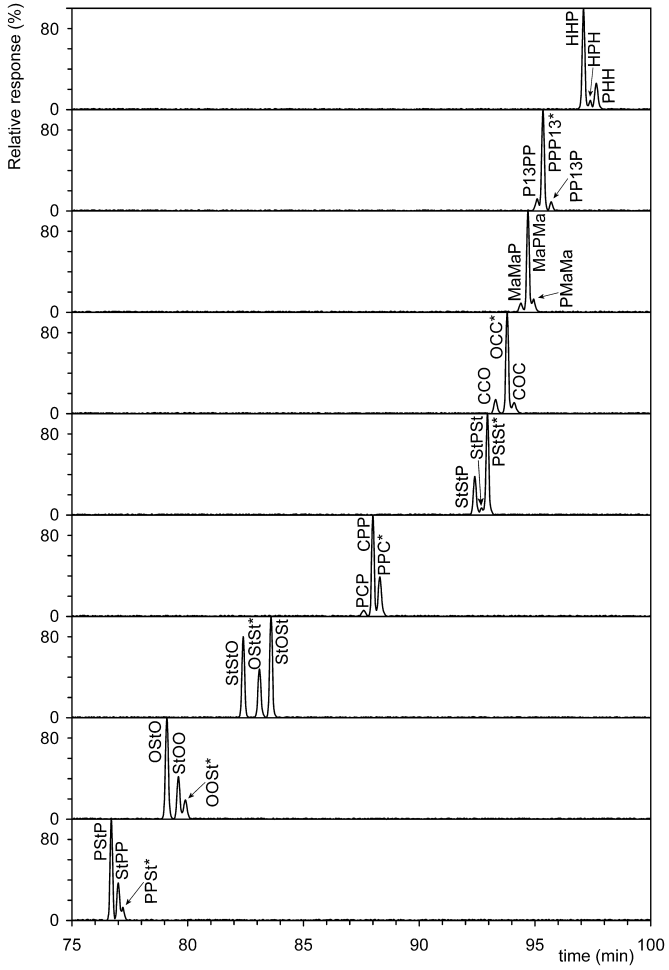

Fig. 4. Chiral separation of natural TAG, i.e. enantiomers and a regioisomers, see Table 1. The synthesized enantiomers are marked by an asterisk. TIC was filtered and indicates the type of ions $[DAG]^+$, i.e. ions resulting loss of neutral $RCOOH+NH_3$ from $[M+NH_4]^+$ in the interval from 480 to 680 Da. The $[DAG]^+$ type ion was always the base peak.

mentioned by Christie, who stated that "molecular distribution theories are the best forgotten" [49].

The stereospecificity of TAGs containing ω-phenylalkanoic fatty acids from different plant seeds has not yet been analysed. TAGs containing two palmitic acids and one 13-phenyltridecanoic acid, i.e., TAGs with the structure of *sn*-PPP13, were identified as the major component of three molecular species in *Dracunculus* oil, but a hypothesis about preferential acylation of the glycerol backbone has not been proposed [22].

4. Conclusions

The survey described above provides some important insights into the chromatographic behaviour of TAGs containing at least one fatty acid with a ring. Separation of both regioisomers and enantiomers is possible, similar to TAGs that contain only straight-chain saturated and/or unsaturated FAs. Unfortunately, as described in previous studies [2,50,51] the limiting factor is not chromatographic separation, but commercially available standards. When separating TAGs with cycloFAs on a reversed-phase column, standards are necessary for correct identification, as evidenced by the differences in the order of elution of TAGs containing cyclopropenyl-FAs and TAGs with cyclopentenyl-FAs. Again, regioisomers were separated and identified based on the mass spectra. The ion abundance ratio $[M-RCOO]^+$, i.e., $[DAG]^+$ ions, is the best approach to identify these species. As expected, enantiomers have the same mass spectra. Therefore, the tR of the synthetic standard and the real natural mixture must be compared to identify the enantiomers. Eight TAGs were prepared through stereospecific synthesis using phospholipase C from the appropriate molecular species of phosphatidylcholines (lysophosphatidylcholines) and subsequent reactions with cycloFAs. Both commercially available and prepared oils, i.e., sterculic and chaulmoogric oils, contain numerous TAGs, approximately half of which are TAGs containing at least one cycloFA. Based on the synthesized standards, the order of elution of TAGs from the chiral column was determined. Chiral LC enables the identification of TAGs, including regioisomers and enantiomers, thus helping to elucidate the biosynthetic and metabolic processes in living cells. Analytical methods (separation and identification of regioisomers and/or enantiomers) are, of course, not limited to plants, but may be used in the future for other organisms, including algae, fungi and other microorganisms that biosynthesize cycloFAs. Examples include *E.* coli [52] containing lactobacillic acid, or ω-cyclohexyl- or ω-cycloheptyl fatty acids from thermophilic bacteria [53,54].

Declaration of Competing Interest

The authors declare no competing financial interest.

CRediT authorship contribution statement

Andrea Palyzová: Data curation, Methodology, Writing - original draft. **Tomáš Řezanka:** Conceptualization, Software, Writing - review & editing, Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461103.

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