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Enantiomeric separation of triacylglycerols containing polyunsaturated fatty acids with 18 carbon atoms

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ABSTRACT

Regioisomers and enantiomers of triacylglycerols (TAGs) containing any combination of stearidonic (18:4n-3) and octadecapentaenoic (18:5n-3) acids were prepared by organic synthesis. Gradient polar organic liquid chromatography/high resolution atmospheric pressure chemical ionization-tandem mass spectrometry (NARP-LC/HRMS^2-APCI) and chiral liquid chromatography were used for the separation and identification of molecular species of these TAGs. Further, NARP-LC and chiral LC were used to separate natural mixtures of TAGs obtained from the haptophyte alga *Coccolithophora* sp. cultivated in a salinity range from 7.5 to 60‰. The ratio of regioisomers and enantiomers was found to change with increasing salinity of the culture medium. This can be explained by variable activity of acyltransferases in cells exposed to salt stress.

1. Introduction

Essential fatty acids are needed for the proper functioning of the body. Humans and most mammals have to take in these compounds in the diet because they cannot be biosynthesized. Essential fatty acids are not only used as an energy source, but also required for a number of biological processes in the body. They are necessary substrates for the synthesis of prostaglandins, thromboxanes, prostacyclins, etc. For mammals, including humans, only two fatty acids (FAs) are primarily essential – linoleic acid (18:2ω-6) and α-linolenic acid (18:3ω-3). Other fatty acids, which are referred to as "conditionally essential", include most polyunsaturated fatty acids (PUFAs). According to their chemical composition, several groups of PUFAs are distinguished. The prominent acids of ω-3 group are α-linolenic, stearidonic, in some cases called moroctic acid (St, 18:4ω-3), and octadecapentaenoic acid (Op, 18:5ω-3) [1].

The natural sources of stearidonic acid are vegetable oils, e.g. seeds of some species of hemp, borage, *Lithospermum arvense* or blackcurrant [2]. The richest sources are however photosynthetic microorganisms, particularly green algae (*Ankistrodesmus* or *Monoraphidium*), wherein the content of the acid is up to tens of percent of total FAs. The major producers of octadecapentaenoic acid are especially algal genera from the classes Dinophyceae and Haptophyceae (*Ceratium, Coccolithophora, Cystodinium, Chromulina, Isochrysis, Lingulodinium*, or *Prymnesium*) with up to 12.0% of total fatty acids [3].

Triacylglycerols (TAGs) are chemical compounds that are major components of vegetable oils and animal fats. They are formed by one molecule of glycerol esterified by fatty acid molecules in all three OH groups. The chemical formula of triacylglycerols is RCOO—CH2-CH(-OOCR')CH2OOCR" wherein R, R', and R" are long alkyl chains. The length of the fatty acid chains in naturally occurring TAGs may vary, but the most common are chains having 16, 18, 20, or 22 carbon atoms. The three fatty acids RCOOH, R'COOH and R"COOH may be completely different, or two of them or all of them may be identical.

When the two primary hydroxyl groups are esterified with different fatty acids, the resulting triacylglycerol can be asymmetric and thus can display 'optical activity', although this is usually too low to be measured. Thus, e.g., 1-oleoyl-2,3-dipalmitoyl-sn-glycerol showed [α]D20 = 0.00 (c 7.05, CHCl3) [4]. Instead of the usual D/L or R/S nomenclature, a special nomenclature is used for the stereochemistry of TAGs in which the primary hydroxyl groups are often termed α- and α' and the secondary β, or the "stere-

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ospecific numbering" (sn) system recommended by the IUPAC-IUB commission [5].

The number of theoretically possible molecular species increases with the growing number of fatty acids (FAs) forming a mixture of TAGs, see Table 1S.

The number of FAs and thus also of the TAGs is dependent on the natural source. The most common vegetable oils contain up to 10 FAs and the number of identified TAG reaches dozens [6]. Oils of marine organisms, e.g. fish oils or oils from krill or other zooplankton contain tens of FAs. For instance, over 500 TAGs have been identified in cod liver oil [7], not including regioisomers and enantiomers. The examples of important chiral separations of TAGs are shown in Table 2S (Supplements), including references, column type, mobile phase; temperature/infusion solvent/matrix ion source analyzer, mass range (*m*/*z*), and sample matrix.One of the biggest problems in the analysis of regioisomers and enantiomers is very poor availability of commercially available TAG standards. One can exceptionally buy some regioisomers from specialized companies but enantiomers cannot be bought at all. The first step is therefore the need to prepare appropriate standards by organic synthesis. Researchers with sufficient experience can separate and identify regioisomers using NARP-LC/MS based on the well-known rule that the fatty acid in the *sn*-2 position exhibits a higher abundance of the diacylglycerol (DAG) fragment ion than when it is in position 1 or 3 [8,9]. 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 elution order [10]. We describe here a method for separating and identifying regioisomers and enantiomers of TAGs that enabled as to identify molecular species containing stearidonic and octadecapentaenoic acids.

2. Experimental

2.1. Standards and instrumentation

Common chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic), including stearidonic acid ((all*Z*)-6,9,12,15-octadecatetraenoic acid), (S)-(+)-1,2-isopropylideneglycerol ((S)-(+)-2,2-dimethyl-1,3-dioxolane-4-methanol) and (S)-(+)-1,2-isopropylideneglycerol ((R)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol). The 3,6,9,12,15-octadecapentaenoic acid was purchased from Foot Chemical Co. Limited, Tianjin, China.

2.2. Cultivation of alga

The SAG 912-1 strain of *Coccolithophora* sp. was provided by the Culture Collection of Algae at the University of Göttingen, Germany. The cultures were grown in liquid artificial seawater medium with vitamins (ASM + V) (www.epsag.uni-goettingen.de) and bubbled by air. Apart from standard ASM + V medium with salinity close to the average value of seawater (30‰), four modifications of the ASM medium were used (salinity 7.5‰, 15‰, 45‰ and 60‰). The light was provided continuously by cool white fluorescent tube (Standard 8W, Sylvania, USA) and the cultivation temperature was 22 °C. After reaching stationary phase, the biomass was harvested by centrifugation at 1800 rpm for 15 min, and the samples were stored frozen at −20 °C until analysis.

2.3. Preparation of standards

All mixtures of enantiomers and regioisomers of TAG were prepared from two TAGs (StStSt and OpOpOp) according to Lisa and Holcapek [11], see Fig. 1. Briefly, one μmole of StStSt and one micromole of OpOpOp and 1.5 μmol of sodium methoxide in 1 mL of hexane were refluxed for 30 min. Reaction mixture was washed with water, dried and analyzed by LC, see 2.6 and 2.7.

Two enantiomers (sn-OpStSt and sn-OpOpSt) were synthetized according to Lisa and Holcapek [11], see Fig. 2. Briefly, 1 μmol of 1,2- or 2,3-isopropylidene-sn-glycerol, 1.2 μmol of appropriate fatty acid, 1.2 μmol of 4-dimethylaminopyridine and 1.5 μmol of dicyclohexylcarbodiimide was stirred at 20 °C for 2 h. A 30-min hydrolysis according to [12] in the mixture of AcOH/H_2O (4:1, v/v) at 50 °C was used. The two free groups of monoacyl glycerol were esterifies by the method described for TAG synthesis (see above).

Two TAGs (StStSt or OpOpOp) were prepared by reaction of free glycerol with free fatty acid using a modification of a previously used method [13]. Briefly, 1 μmol of glycerol, 3.3 μmol of free fatty acid, 3.6 μmol of 4-dimethylaminopyridine and 4.5 μmol of dicyclohexylcarbodiimide was stirred at 20 °C for 2 h. The resulting reaction mixture was processed (see above) and the TAG produced in the reaction was used either for analysis or for further reaction (randomization).

2.4. Preparation and analysis of fatty acid methyl esters (FAMEs)

The total TAGs (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A fatty acid fraction obtained from the saponification was partitioned between alkali solution (pH 9) and diethyl-ether to remove basic and neutral components. The aqueous phase containing fatty acids was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using BF_3/MeOH (14% solution of BF_3 from Sigma-Aldrich). Gas chromatography-mass spectrometry of FAMEs was done on a GC–MS system consisting of Varian 450-GC (Varian BV, Middelburg, The Netherlands), Varian 240-MS ion trap detector with electron ionization (EI), and CombiPal autosampler (Chromtech – Analytical Instruments, Idstein, Germany) equipped with split/splitless injector. A DB-5MS column was used for separation (60 m, 0.25 mm ID, 0.25 μm film thickness). The temperature program started at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 160 °C at a rate of 25 °C min^{-1}. The second temperature ramp was up to 280 °C at a rate of 2.5 °C min^{-1}, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 3 scans s^{-1} under electron ionization at 70 eV, mass range *m*/*z* 50–450. FAMEs were identified according to their mass spectra [14,15] and using a mixture of chemical standards obtained from Sigma-Aldrich.

2.5. Lipid extraction and isolation of TAGs

Briefly, the biomass of *Coccolithophora* sp. cultivated at different salinity was extracted according Bligh and Dyer [16] and extracts of total lipids were applied to Sep-Pak Cartridge Vac 35 cc (Waters, Prague, Czech Republic; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted neutral lipids 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 on the HPLC column [17].

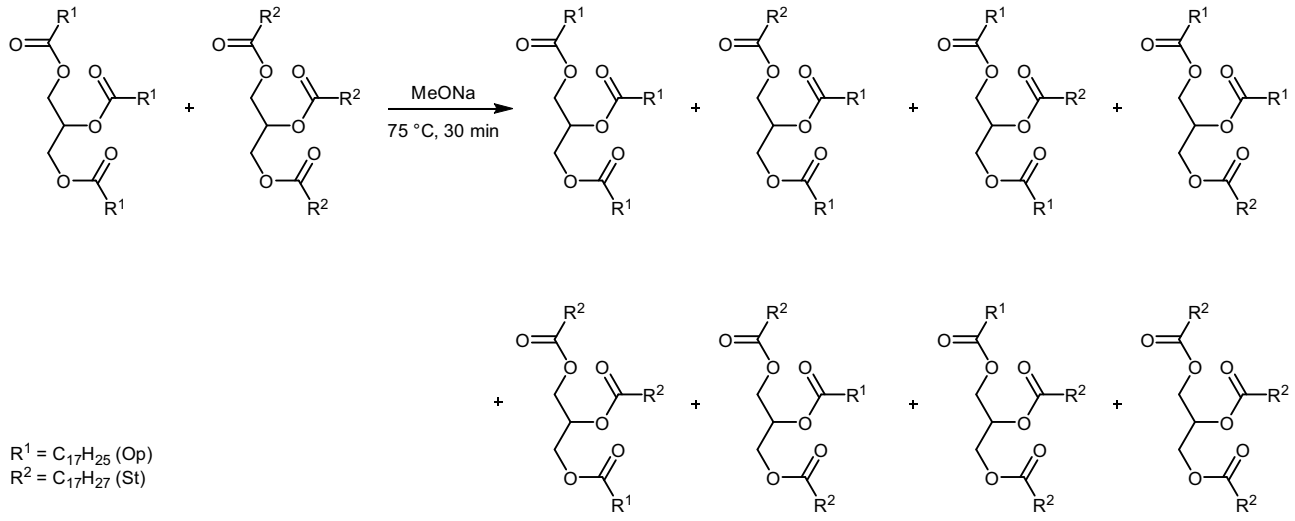

Fig. 1. Synthesis of regioisomers of TAG by randomization. MeONa, sodium methoxide; R^1 hydrocarbon radical $C_{17}H_{25}$ (substituent group) of octadecapentaenoic acid; R^2 hydrocarbon radical $C_{17}H_{27}$ (substituent group) of stearidonic acid.

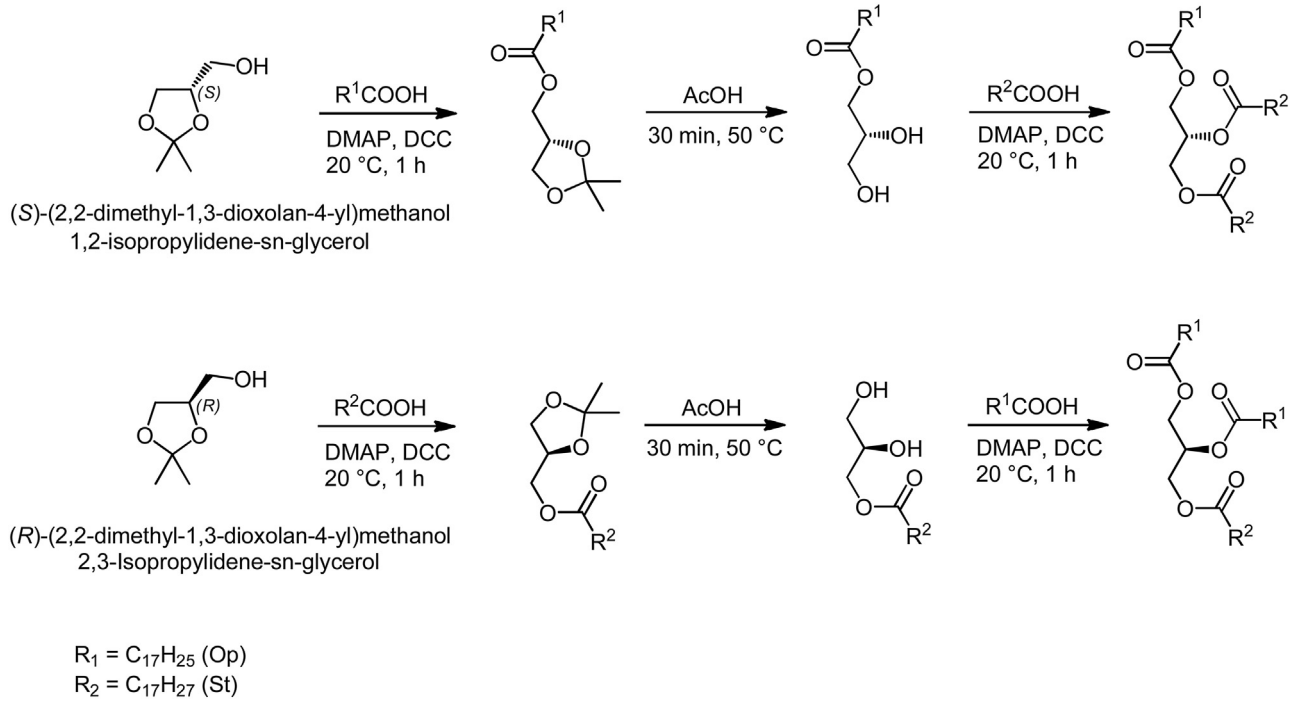

Fig. 2. Synthesis of enantiomers by stereospecific esterification of 1,2- and 2,3-isopropylidene-sn-glycerols. MeONa, sodium methoxide; DMAP, 4-dimethylaminopyridine; DCC, dicyclohexylcarbodiimide; AcOH, acetic acid; R^1 hydrocarbon radical $C_{17}H_{25}$ (substituent group) of octadecapentaenoic acid; R^2 hydrocarbon radical $C_{17}H_{27}$ (substituent group) of stearidonic acid.

2.6. TAGs analysis by RP-HPLC/MS-APCI

LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and two columns Hichrom HIRPB – 250A 250 × 2.1 mm ID, 5 μm particle size, in series. This setup was used as a high efficiency column – approximately 104000 plates/m, with a flow rate of 1 mL/min and an injection volume of 10 μL, and column temperature of 25 °C. The TAGs were separated using a solvent gradient program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 5 min to 110 min of ACN/iPrOH 30:70 (vol/vol); held for 10 min. The composition was returned to the initial conditions over 10 min. The performance was measured by triheptadecenoin as an internal standard under the conditions given above.

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface (HESI) was used. ESI–MS analysis was performed in the FT positive ion mode. MS spectra were acquired with target mass resolution of R = 105,000 at *m*/*z* 800. The ion spray voltage was set at 3.5 kV and the scan range of the instruments was set at *m*/*z* 200–1500. Nitrogen was used as a nebulizer gas – set at 18 arbitrary units (sheath gas) and 7 arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product

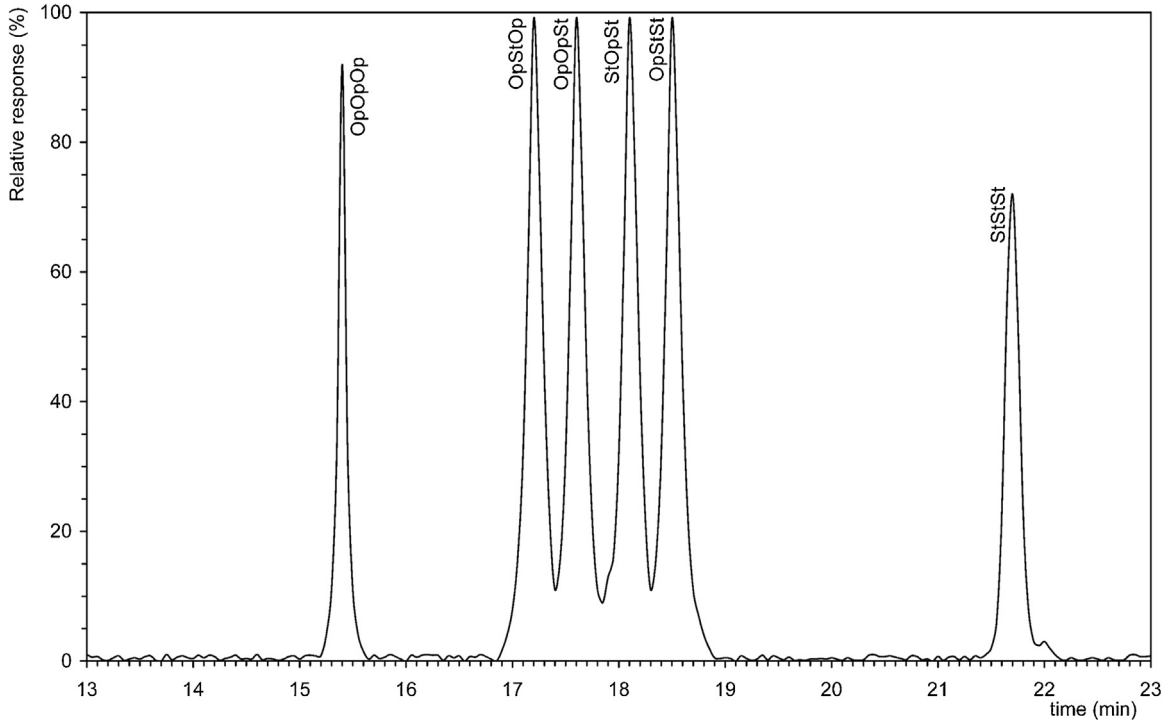

Fig. 3. The mixture of regioisomers prepared by randomization of OpOpOp and StStSt and separated and identified by NARP-LC/MS-APCI (Op – octadecapentaenoic acid, St – stearidonic acid). TAGs were separated on two Hichrom HIRPB columns using a solvent gradient program with acetonitrile and 2-propanol, connected with high resolution mass spectrometer (LTQ Orbitrap Velos) operated in positive electrospray mode. Tandem mass spectra were obtained on the same instrument- for details see subsection 2.6 TAGs analysis by RP-HLPCHPLC/MS-APCI.

ions were detected by the high resolution FT mode. Further source conditions in the positive mode: vaporizer temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; tube lens voltage, 170 V.

The same apparatus was used for negative ion mode. MS spectra were acquired with target mass resolution of R = 30,000 at *m*/*z* 400. The ion spray voltage was set at −2500 V and the scan range of the instruments was set at *m*/*z* 200–2000. Nitrogen was used as a nebulizer gas – set at 18 arbitrary units (sheath gas) and 7 arbitrary units (aux gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the parent ions fragmentation. The MS/MS product ions were detected by the high resolution FT mode. The H-ESI temperature was set to 250 °C.

2.7. TAGs analysis by chiral LC/MS-APCI

The LC system used for separation in the chiral mode was the same as that used in the reversed-phase mode. TAGs (~1 mg/mL in methanol) were chromatographed 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) connected in series, from Sigma-Aldrich. Mobile phase was a gradient from 90% of A and 10% of B at 0 min to 50% of A and 50% of B during 180 min, and then isocratically for 30 min, where A is hexane and B is hexane-2-propanol (99:2, v/v) mixture [17,18]. The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 μL, respectively. Other separation conditions were as used in the RP-LC, see above.

3. Results and discussion

Lipidomic analysis or shotgun analysis, i.e. analysis without separation by HPLC, cannot determine regioisomers. Only when a calibration mixture is made from the synthetic standards, it is possible to determine the ratio of regioisomers, but never enantiomers, whose mass spectra are identical.

3.1. Preparation of enantiomer standards

Three methods may be used for the synthesis of standards. The first involves the use of phospholipase C to obtain one of the enantiomers of DAG from commercially available phosphatidylcholine (PC) or another phospholipid. The DAG is further esterified to a corresponding TAG. Depending on commercially available diacyl phospholipids it is then possible to obtain, for example in the case of 20:4/20:4-PC, *sn*-20:4-20:4-DAG and further esterification then provides *sn*-20:4-20:4-FA TAG, wherein FA is any available fatty acid [19].

The second method, which has been successfully used and described, consists in reacting the free acid with 1,2 (2,3)-isopropylidene-*sn*-glycerol, and then deprotecting and esterifying the two remaining free hydroxyls (at the *sn*-1 and *sn*-2, or *sn*-2 and *sn*-3 positions) with the corresponding FA [12,20,21], see Fig. 1.

The third method involves transesterification (randomization) of two TAGs [11], each with a different acyl, see Fig. 2. The racemization includes an intra- and internal transesterification resulting in a mixture of both the starting two TAG, and a mixture of regioisomers and enantiomers, in our case of randomization of OpOpOp and StStSt this yield a total of 6 or 8 TAGs, see Figs. 3 and 4. In the first case, i.e. using tandem mass spectra can be used to identify all four regioisomers, see Fig. 5. Enantiomers, i.e. the pairs of molecular species *sn*-StOpOp and *sn*-OpOpSt, and *sn*-StStOp and *sn*-OpStSt cannot be of course separated on a reverse phase. Using a chiral phase it was possible to separate both regioisomers as well as enantiomers. However, as described above, the mass spectra of peaks, i.e. both pairs of enantiomers, with retention times – 177.5 and 177.9, or 186.4 and 186.7 min were identical, see Fig. 6. There-

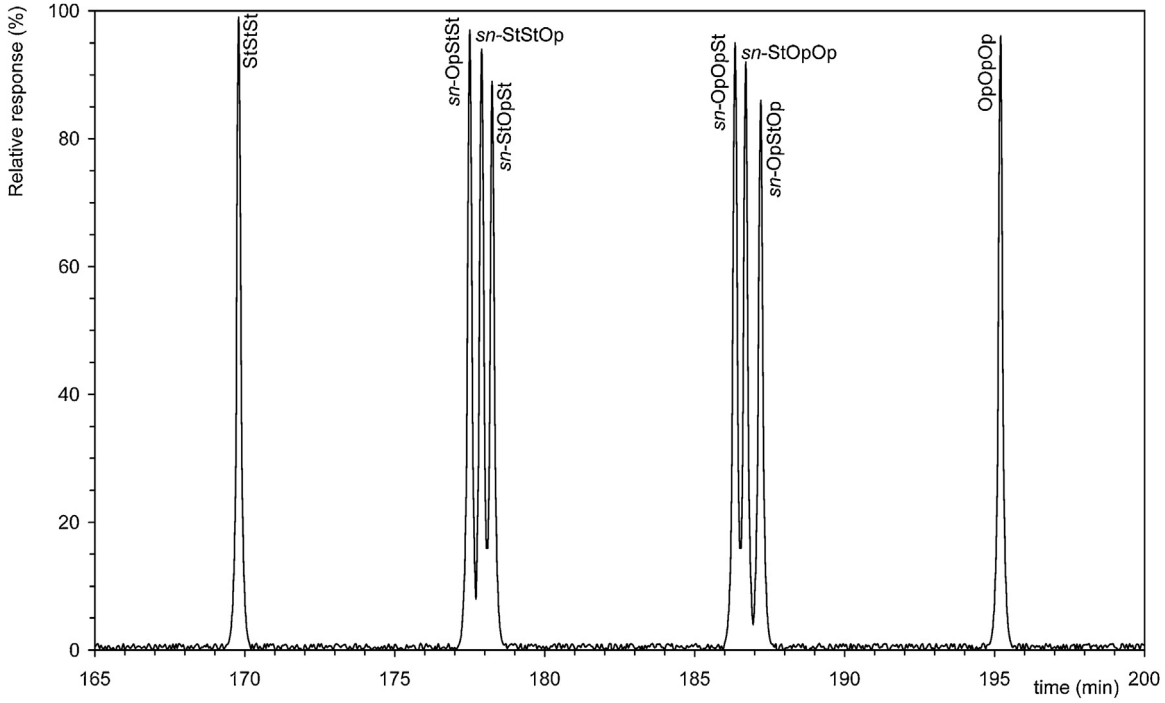

Fig. 4. The mixture of enantiomers prepared by randomization of OpOpOp and StStSt and separated and identified by chiral-LC/MS-APCI (Op – octadecapentaenoic acid, St – stearidonic acid). Astec cyclobondT I 2000 DMP chiral LC columns was connected with mass spectrometer, exact conditions are given in subsection 2.7 TAGs analysis by chiral LC/MS-APCI.

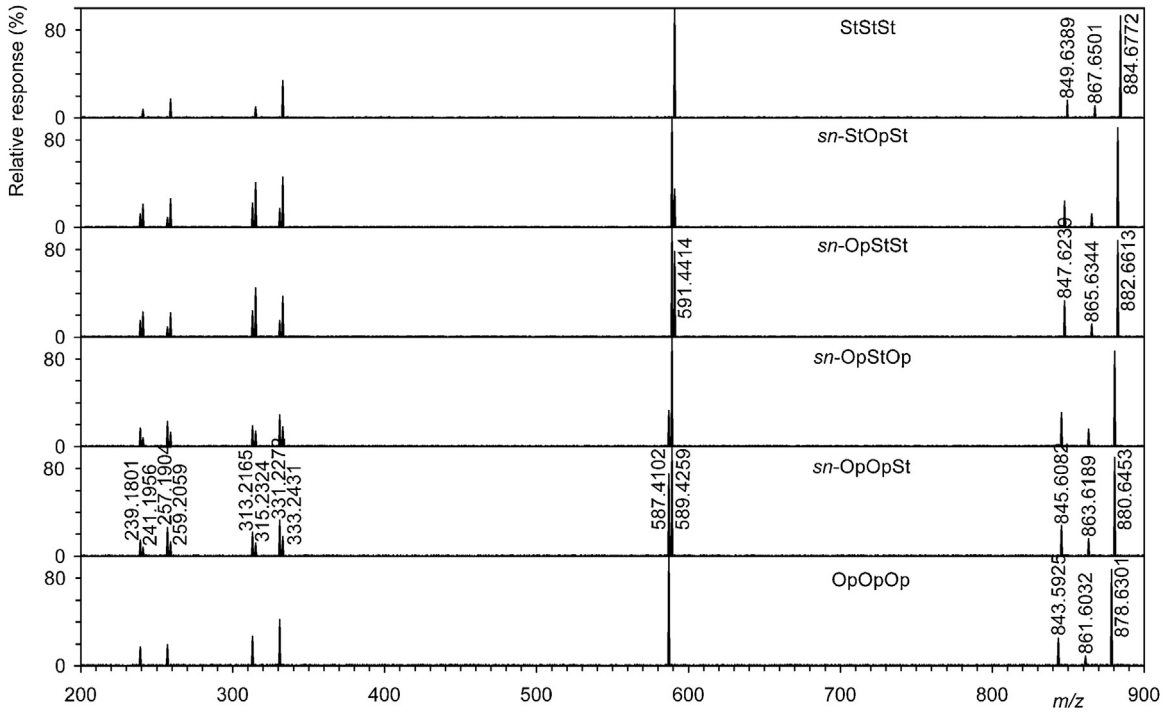

Fig. 5. Tandem mass spectra of all four regioisomers and two TAG before randomization. For experimental details, see subsection 2.6.

fore, at least one of the two enantiomers had to be prepared by organic synthesis.

The best appears to be the method shown in Fig. 1. This is essentially a three-step organic synthesis that has already been described many times [12,20,21], in which commercially available R(S)-dioxolanes are used to prepare an ester of appropriate fatty acid, which is then is hydrolyzed to obtain the monoacylglycerol (MAG). This hydrolysis is the critical point of the entire sequence of reactions because, when using inappropriate reaction conditions, intra- and inter-transesterification takes place producing a mixture of both enantiomers and regioisomers. On the basis of literature data [12,20,21] we chose as the best method that using acetic acid at 50 °C. The MAG is then acylated to the corresponding fatty acid to give the corresponding enantiomer. Fig. 7 shows separation of

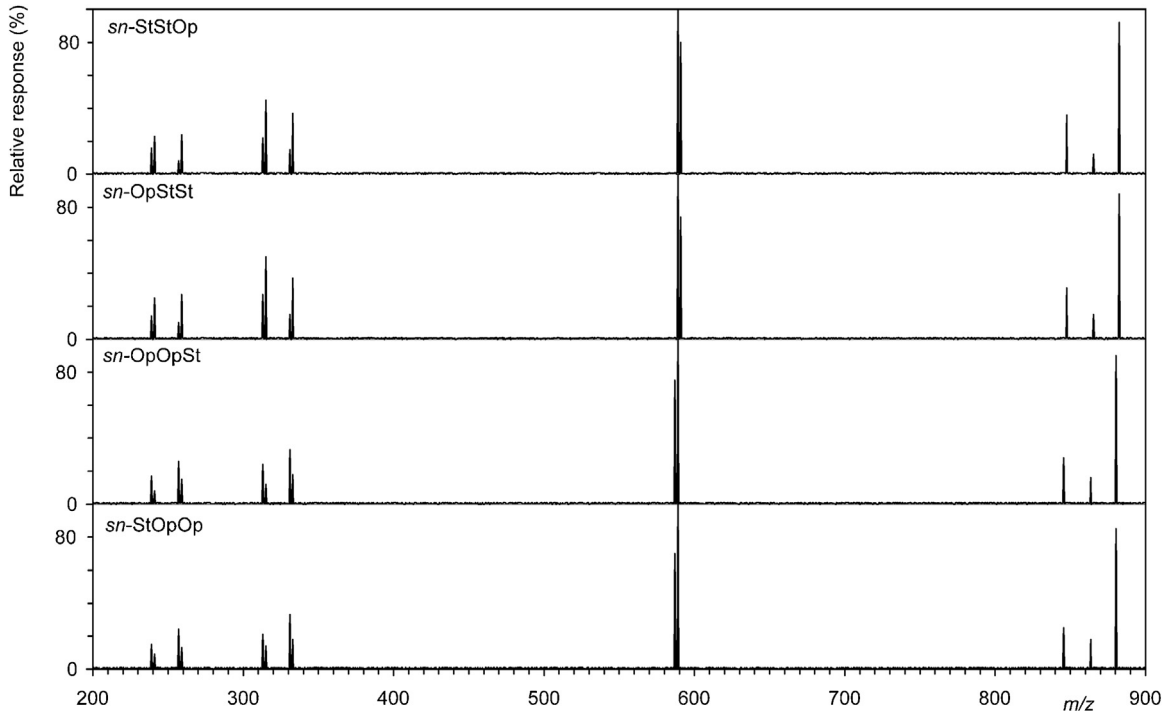

Fig. 6. Tandem mass spectra of the two pairs of enantiomers (StStOp, OpStSt and OpOpSt, StOpOp), (Op – octadecapentaenoic acid, St – stearidonic acid). For experimental details, see subsection 2.7.

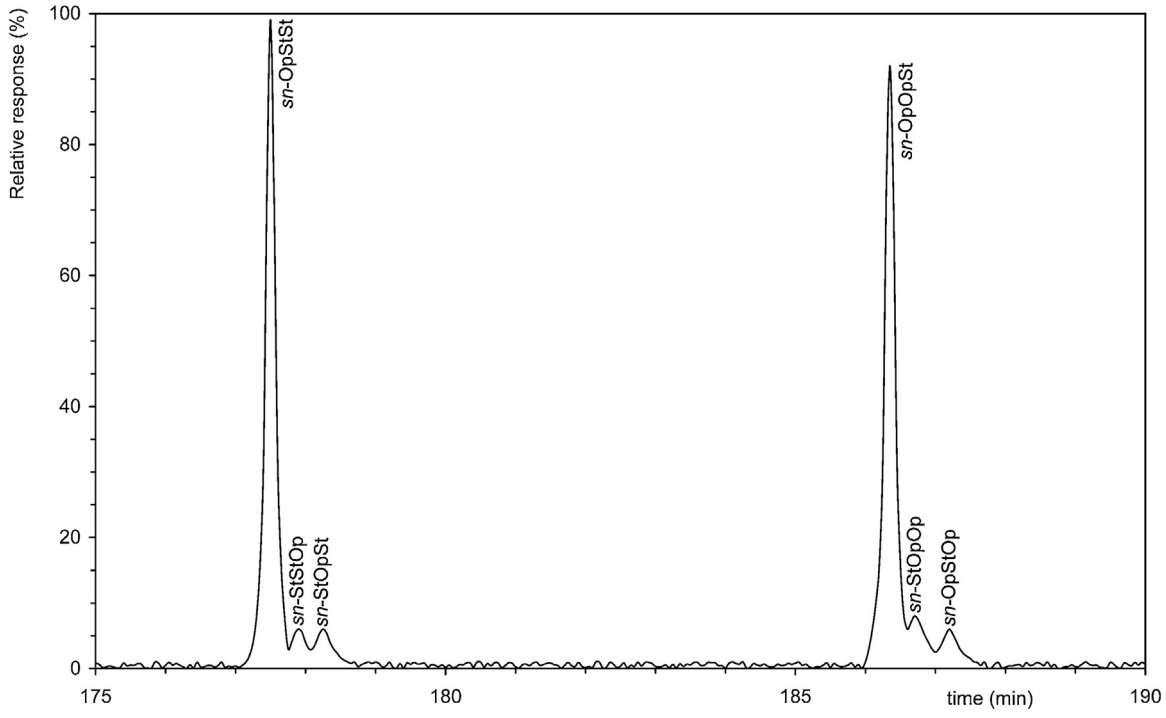

Fig. 7. The separation of both synthetic standards of enantiomers, i.e. reaction mixture after stereospecific synthesis. For experimental details, see subsection 2.7.

the two synthetic standards, i.e. *sn*-OpStSt and *sn*-OpOpSt, respectively. Optical purity was calculated based on peak area integration of the individual regioisomers (enantiomers). All ions in the range 200–1000 Da (total ion current) were used. Optical purity of the enantiomers was 92–94%, see Table 1 and also Fig. 7. Using randomization of two starting TAGs (OpOpOp and StStSt) and two stereospecific syntheses all the 6 or 8 peaks in Figs. 3 and 4 were identified, and the method be could therefore used for the natural mixture.

3.2. Separation of TAGs containing stearidonic and octadecapentaenoic acid

The natural mixture containing both stearidonic and octadecapentaenoic acid in this study was represented by the lipids from

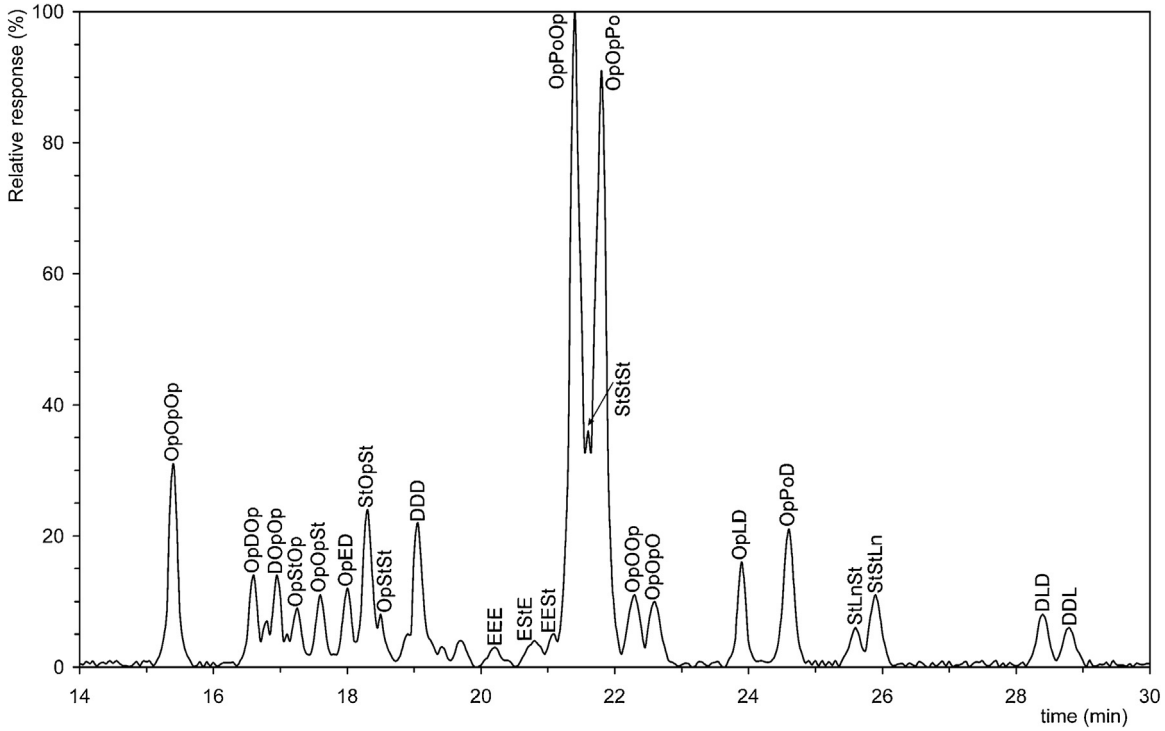

Fig. 8. The separation of regioisomers in natural mixtures of TAGs obtained from the alga *Coccolithophora* sp. Abbreviations of FAs: Po, 16:1n-7; O, 18:1n-9; L, 18:2n-6; Ln, 18:3n-3; E, 20:5n-3; D, 22:6n-3. For experimental details, see subsection 2.6.

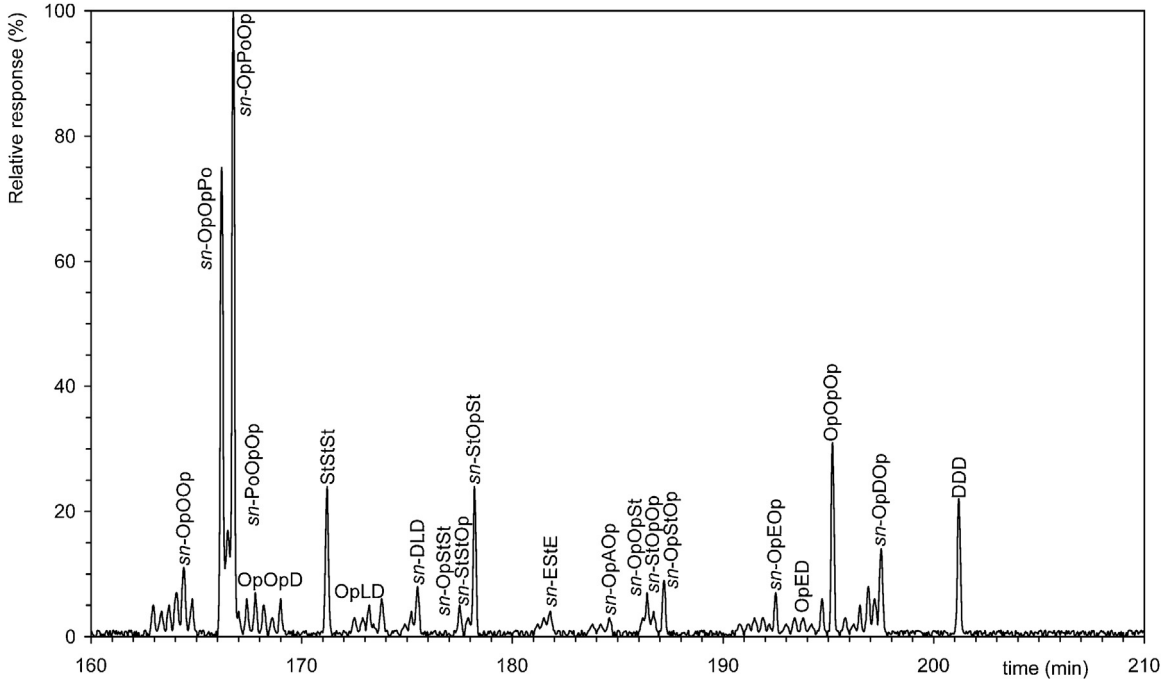

Fig. 9. The separation of enantiomers in natural mixtures of TAGs obtained from the alga *Coccolithophora* sp. For FAs abbreviations, see Fig. 6. For experimental details, see subsection 2.7.

the planktonic marine microalga *Coccolithophora* sp. (Haptophyta), cultivated in a broad salinity range. Haptophytes are found in large numbers in the open water of the Worldís Ocean and they play an important role in the global biogeochemical cycles and climate. The genus *Coccolithophora* belongs to the group of haptophytes that is characterised by special calcium carbonate scales called coccoliths; their massive development is indicated by milky coloration of sea water (white tide). Figs. 8 and 9 show the cutout of the entire chromatogram obtained by using both NARP-LC and chiral column. Optimization of separation has been developed based on our previous results, which are listed in Table 2S.

Separation of the four synthesized positional isomers (*sn*-OpOpSt, *sn*-OpAtOp, and *sn*-StStOp, *sn*-StOpSt) was performed. In agreement with literature data all 4 positional isomers were sep-

Table 1
The ratio of enantiomers and regioisomers synthetized from stearidonic (St) and octadecapentaenoic (Op) fatty acids; retention time (tR, min), acyl carbon number (ACN), double bonds (DB), and equivalent carbon number (ECN).

tR chiral	mol spec	ACN	DB	ECN	enantiomer (%)
177.5	sn-OpStSt	54	13	28	93
177.9	sn-StStOp	54	13	28	3
178.2	sn-StOpSt	54	13	28	4
186.4	sn-OpOpSt	54	14	26	91
186.7	sn-StOpOp	54	14	26	4
187.2	sn-OpStOp	54	14	26	5

arated in less than 200 min. In all studies published so far on the subject [22–24] the symmetrical positional isomer always had a shorter retention time than its asymmetric counterpart, which was confirmed also in this study. This fact corresponds also to the observation of our previously published papers [17,18] describing the separation of *sn*-APP and *sn*-PPA, or *sn*-ALL and *sn*-LLA, and formulated the above rule by stating that the isomer with the unsaturated acyl residue in either 1- or 3-position was retained more strongly than the respective 2-isomer. The authors [22–24] explained this phenomenon as being 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.

3.3. Identification of regioisomeric TAGs by mass spectrometry

To determine if the fatty acid is in *sn*-2 position one can use the well-known fact that fatty acid in this position exhibits a higher abundance of the DAG fragment ion than when it is in position 1 or 3 [8,9]; however, the intensity of this ion can be affected by many factors, the most important being the number and positions of the bond(s) [11]. The mass spectra show the differences between two regioisomeric doublets, i.e. *sn*-OpOpSt, *sn*-OpStOp, and *sn*-StStOp, *sn*-StOpSt, respectively. All four TAGs, see above, showed the following DAGs ratios: the ratio of intensities of DAGs for *sn*-OpOpSt, i.e. $[OpOp]^+/[OpSt]^+$, was 75:100, the $[OpOp]^+/[OpSt]^+$ ratio for *sn*-OpStOp was 33:100, $[PP]^+/[EP]^+$ ratio for *sn*-StStOp was 78:100 and the $[StSt]^+/[OpSt]^+$ ratio for *sn*-StOpSt was 35:100. Lisa et al. [25] gave for LLnLn TAG the DAG ($[LnLn]^+/[LLn]^+$) the ratio of 73:100 and for LnLLn TAG the DAG ($[LnLn]^+/[LLn]^+$) the ratio of 44:100. The above results led to the conclusion which is in accord with the data published for non-PUFA TAGs [8,9], i.e. that the ratio of intensities of $[AA]^+/[AB]^+$ in AAB type TAGs (asymmetrical TAGs) is 2/3:1, whereas in ABA (symmetrical TAGs) the ratio of intensities of DAG ions $[AA]^+/[AB]^+$ is 1/3:1.

3.4. Separation and identification of enantiomeric TAGs

In accordance with previously published data [11] TAGs having a more highly unsaturated FA in the sn-1 position are eluted earlier, see e.g. sn-OpStSt versus sn-StStOp (tR = 177.5 and 177.9 min, respectively). As described above, the TAGs containing stearidonic acid were several times identified in various vegetable oils (borage, black currant, hemp) [6] but their regioisomers and/or enantiomers were not determined. Only Kallio et al. [26] determined the proportions of regioisomers in some TAGs containing stearidonic acid. Based on the ratio of $[M\text{-}H\text{-}RiCOOH\text{-}100]^-$ ions it has been found that in both currants (wild northern red currant and wild alpine currant) stearidonic acid in StLL is bound predominantly to the primary hydroxyls of glycerol, i.e. sn-1 or sn-3. The TAGs from black currant oil were analyzed by Kallio et al. [27] and it was found that the 18:4n-3 acid was mainly located in the primary positions (sn-1 or sn-3).

Table 2
The representation (%) of individual regioisomers and enantiomers of TAGs containing both stearidonic and octadecapentaenoic acids in *Coccolithophora* sp.: dependence on salinity.

TAG	7.5‰	15.0‰	30.0‰	45.0‰	60.0‰
sn-StOpOp	30	20	15	10	5
sn-OpOpSt	60	55	45	40	35
Sum of enantiomers	*90*	*75*	*60*	*50*	*40*
sn-OpStOp	*10*	*25*	*40*	*50*	*60*
sn-OpStSt	20	15	7	4	1
sn-StStOp	40	33	18	11	4
Sum of enantiomers	*60*	*48*	*25*	*15*	*5*
sn-StOpSt	*40*	*52*	*75*	*85*	*95*

Chiral chromatography was also used for the separation of enantiomers in natural mixtures of TAGs obtained from the alga *Coccolithophora* sp. Fig. 8shows a NARP LC-chromatogram of the TAGs at 30‰ salinity, Fig. 9 shows the same sample separated by chiral LC. As mentioned above with the standards, the FA which is in the sn-2 position and those in sn-1(3) positions were identified based on the tandem mass spectrum. Noteworthy is the different elution order of the regioisomers. While in the case of NARP-LC the sequence is OpPoOp-OpOpPo, i.e. first symmetric and then asymmetric TAG, in the case of chiral LC it is OpOpPo-OpPoOp-PoOpOp (asymmetric – symmetric – asymmetric). In addition, the retention time of StStSt is shifted; while in the NARP StStSt coelutes with OpOpPo, in chiral LC it forms a separate peak. The number of peaks identified by chiral LC is obviously higher since certain regioisomers have been separated to enantiomers, even if the exact structure of all molecular species was not determined because of the lack of standards, particularly those of minor TAGs containing 3 different FA. However, the higher number of identified TAG is paid for by a very long time of analysis, basically 3.5 h.

Table 2 shows abundant TAGs, regioisomers and enantiomers from cells cultured at different salinity. To calculate the ratio of the individual isomers, both regioisomers and enantiomers, the abundance of $[DAG]^+$ type ions was used. For instance, in the case of OpOpSt it was the ratio of $[OpOp]^+$ to $[OpSt]^+$. In many cases, all molecular species were not identified due to the great complexity of natural mixtures, particularly in case of TAGs containing three different FAs and therefore six TAG isomers.

One possibility of how to solve this situation is to substantially increase the efficiency of the column, e.g. by developing a new stationary phase. An alternative approach, viz. the combination of multiple columns in series, is not suitable since it leads to increased retention time of eluted TAG.

As mentioned in the description of the analysis of standards, the ratio of relevant/DAG/+ determines the ratio of individual molecular species. Table 2 shows that the salinity at which the alga is grown has a significant influence on the composition of TAGs. As an example, we chose TAGs comprising three C18 PUFAs (Table 3S), which constitute more than 40% of the total fatty acids of triacylglycerols (Table 2).

The preferential biosynthesis of a particular isomer at a given salinity can be explained by the selectivity of the three acyltransferases required for the biosynthesis of TAGs. It should be borne in mind that the marine alga *Coccolithophora* sp. has a growth optimum close to 30‰; the two extreme salt concentrations (7.5‰ to 60‰) produce stress to which the cells respond by synthesizing appropriate metabolites.

4. Conclusion

Two different methods of organic synthesis were used to prepare regioisomers and enantiomers of TAGs containing any combination of stearidonic and octadecapentaenoic acids. Fur-

thermore, we described for the first time the separation and identification of molecular species of the eight above-mentioned TAGs. Chiral LC was used to separate natural mixtures of TAG obtained under various culture conditions. The alga *Coccolithophora* sp. was cultivated in a salinity range from 7.5 to 60‰, and the ratio of regioisomers and enantiomers was found to change with increasing salinity of the culture medium. This can be explained by variable activity of acyltransferases in cells exposed to salt stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.07.006.

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