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Comparative analysis of triacylglycerols from different *Stichococcus* strains by RP-HPLC/APCI-MS and chiral HPLC

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Abstract The main analytical benefit of this study is the development of methods enabling a rapid determination of triacylglycerols (TAGs) in different strains of the green alga *Stichococcus bacillaris* and detailed identification and quantification of a complex mixture of natural TAGs by reversed phase liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (RP-HPLC/APCI-MS) and chiral HPLC. Neutral ion scan can readily identify, both qualitatively and semiquantitatively, triacylglycerols containing 16:2 and 16:3 fatty acids (FAs) in the molecule. Since more detailed studies in this respect have been stymied by the shortage of 16:2 and 16:3 FAs, we decided to study the alga *Stichococcus* as a potential new biotechnological source of C16 polyunsaturated fatty acids (PUFAs). We conclude that the genus *Stichococcus* is a major producer of C16 PUFAs mostly contained in TAGs. Further, the ratio of regioisomers and enantiomers of TAGs depends on the content of fatty acids; with lowering temperature, the proportion of the achiral TAG increases, and so does the enantiomer ratio from 1:1. Dienoic FAs preferentially esterify position *sn*-3, whereas in trienoic acids, it is position *sn*-1.

Keywords Green alga · *Stichococcus bacillaris* · Triacylglycerols · APCI · Neutral loss scan · RP-HPLC/APCI-MS · Chiral LC

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Introduction

The genus *Stichococcus* (Chlorophyta) consists of small cylindrical cells which can be connected in short filaments and was originally classified within Ulothrichales (Ulvophyceae). However, according to currently accepted taxonomy, it belongs to the class Trebouxiophyceae (e.g., Karsten et al. 2005). It is a cosmopolitan genus with a wide ecological distribution, which can be found as terrestrial alga in alpine soils, cryobiontic one in polar regions where it causes a greenish tinge of snow, and in various freshwater, brackish, and marine habitats but also in warm springs (Van den Hoek et al. 1996). *Stichococcus* is highly variable; in culture, it is characterized by rapid growth. The most frequently cultured species is *Stichococcus bacillaris*, which represents a suitable experimental system for studies of physiological regulation and production of lipids and fatty acids.

Almost one third of algal strains in the Culture Collection of Algae at Göttingen University (SAG) contain 16:2ω-6 (7,10-hexadecadienoic) acid. Most of these strains belong to classes Chlorophyceae or Trebouxiophyceae. The highest proportions of 16:2ω-6 were found in algae of two orders, Chlamydomonadales or Microthamniales, respectively, especially in the genus *Chlamydomonas* and also in the genus *Stichococcus* in which this fatty acid (FA) was found in 17 out of 18 strains (Lang et al. 2011). A similar situation was with 7,10,13-hexadecatrienoic acid (16:3ω-3), the content of which sometimes even exceeds 20 % of total FAs; all the studied 18 strains contained this acid. Interestingly, a number of other studies (Chen et al. 2012; Olivieri et al. 2011, 2012) did not describe these polyunsaturated FA (PUFA) in the genus *Stichococcus*, although this green alga has been found to be an important producer of biotechnologically relevant PUFAs with 16 carbon atoms (Teoh et al. 2004; Lang et al. 2011).

Essential fatty acids (EFAs) are those fatty acids that are beneficial for animal and human health but have to be

replenished in the body via nutrition because the body cannot synthesize them (EFSA 2010, Simopoulos 1999). Linoleic acid, i.e., ω-6 FA (9,12–18:2), and α-linolenic acid, i.e., ω-3 FA (9,12,15–18:3), cannot be synthesized in mammals which lack the appropriate desaturases that introduce a double bond in fatty acids at carbon atoms farther than C_9.

Nearly 20 years ago, a study using isotope ratio mass spectrometry showed that rats fed on uniformly ^{13}C-labeled hexadecadienoate or hexadecatrienoate contained C18, C20, and C22 PUFAs. Hexadecatrienoate can cover about 5 % of the requirement for α-linolenate in the rat (Cunnane et al. 1995). The same holds also for tetradecadienoate (14:2ω-6) and tetradecatrienoate (14:3ω-3) but not for C12 PUFAs (Cunnane 2003). C14 PUFAs have so far not been found in sufficient amounts in nature, but C16 PUFAs are commonly present in low concentrations in green parts of plants, e.g., broccoli or spinach. A suitable non-animal source of C16 PUFAs could be represented by algae.

The effect of temperature on the composition of phospholipid membranes and content of FAs in algae has been intensively studied (Morgan-Kiss et al. 2006). Similar studies concerning lipids are much fewer (Chen et al. 2008), and to our mind, the effect of temperature on the ratio of regioisomers and enantiomers of triacylglycerols (TAGs) has not yet been studied at all.

We studied three strains of the green alga *S. bacillaris* that differed in their habitats. Strain CCALA 906 was isolated from soil collected on Svalbard, Norway; strain CCALA 493 was isolated from the Hafner See in Austria with mild climate; and strain NAP was isolated from a hot sulfur spring in Sicily, Italy. The purpose of our study was to determine how these strains respond to different temperatures in terms of production of TAGs, i.e., how, for instance, the strain from a cold habitat would respond to cultivation at 30 °C, a temperature suitable for the growth of a thermophilic strain.

The analysis of intact TAGs is usually performed by using two methods of analysis: either direct sample inlet to a mass spectrometer or a connection of a liquid chromatograph to a mass spectrometer (LC/MS). Using chiral LC on a polysaccharide-based chiral stationary phase, Iwasaki et al. (2001) separated enantiomers of TAGs, e.g., eicosapentaenoyl-dicapryoyl-glycerol (e.g., *sn*-ECC or *sn*-CCE). Nagai et al. (2011) and Gotoh et al. (2011) used chiral LC for the separation of dioleoyl-palmitoyl-glycerol (*sn*-OOP and/or *sn*-POO) from palm oil. Lisa and Holcapek (2013) separated, on two columns connected in series, TAG enantiomers containing from one to eight double bonds including both synthetic standards and TAGs from two natural mixtures—hazelnut oil and human plasma. In our preceding paper, we succeeded, by using chiral LC with the phase 3,5-dimethylphenyl carbamate-modified β-cyclodextrin, in separating TAGs containing palmitoleic acid from 11 different strains of yeast (Rezanka et al. 2013).

One of the first studies describing the determination of intact molecular species of TAGs from the alga *Phaeodactylum tricornutum* containing C16 PUFAs, e.g., PnPnPn or PnPoPn (Pn—7,10,13–16:3; Po—9–16:1), was published in 1993 (Yongmanitchai and Ward 1993). Rapid development of the analysis of intact algal TAGs came some 15 years later, spurred by the rapidly developing technique of mass spectrometry (Jones et al., 2012).

Algal TAGs containing C16 PUFAs were analyzed by means of the electrospray ionization mass spectrometry (ESI-MS), either as a direct inlet (Yu et al. 2009) or by ultra high performance liquid chromatography (UHPLC) coupled with positive ESI-MS and tandem MS (MS^2) modes (Samburova et al. 2013).

This study describes the identification of regioisomers and enantiomers of triacylglycerols of hexadecadienoic and hexadecatrineoic acids in three strains of the alga *S. bacillaris* cultivated at different temperatures using mass spectrometry with reversed and chiral phase liquid chromatography-mass spectrometry. The use of the two different phases contributes to ready identification, both qualitative and semiquantitative, of regioisomers and enantiomers of triacylglycerols containing 16:2 and 16:3 acids in the molecule. Our atmospheric pressure chemical ionization mass spectrometry (APCI-MS) method is well suited for direct determination of TAG profiles and for comparing the production of lipids after a change in microorganism cultivation conditions.

Materials and methods

Standards

Acetonitrile, 2-propanol, tetrahydrofurane (THF), hexane, chloroform, and methanol were purchased from Sigma-Aldrich (Prague, Czech Republic). Standards of *sn*-PnPlPn, *sn*-PlPnPn, *sn*-PlPnPl, and *sn*-PlPlPn were prepared by stereospecific esterification of 2,3-isopropylidene-*sn*-glycerol, removal of the protecting group, and another esterification yielding appropriate TAGs.

Cultivation of *Stichococcus* strains

The strains of the green alga *Stichococcus bacillaris* Nägeli were obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany ASCR, Třeboň, Czech Republic (CCALA 493 Hindák 1984/15 and CCALA 906 Elster 1998/28) and from the Algal Collection at University Federico II, Naples, Italy (ACUF 149/150 R. Taddei, abbreviated to NAP). The strains were isolated from contrasting ecological conditions: Hafner See in Austria with mild climate (CCALA 493); soil on a newly deglaciated moraine in Ny-

Ålesund, Svalbard (CCALA 906); and a sulfur spring, Mintina, San Cataldo, Sicily, Italy (NAP). The cultures were grown in liquid Bold's basal medium (BBM) (Bischoff and Bold 1963) at 10, 20, and 30 °C under continuous light provided by cool white fluorescent lights. After reaching stationary phase (determined by optical density at 750 nm), the cultures were harvested by centrifugation at 2,000 rpm for 15 min, and the samples were stored frozen in aluminum foil until solvent extraction. The yield of dry weight, total lipids, TAGs, and C16 PUFAs can be found in Table 1.

Isolation of lipids

The extraction procedure was based on the method of Bligh and Dyer (1959). Briefly, the sample is vortexed with $CHCl_3$/MeOH 1:2 (*v*/*v*), water and $CHCl_3$ are added at equal volumes, and $CHCl_3$ bottom phase is separated, except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases (Kates 1986). The alcohol-water mixture of the lyophilized cells was cooled, one part chloroform was added, and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts of 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure and weighed.

Total lipid extracts (see Table 1) were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropyl silica-based polar bonded phase), and neutral lipids with 40 mL of chloroform-methanol (7:3, *v*/*v*) and acidic lipids were eluted from the cartridge with 30 mL of chloroform-methanol-concentrated aqueous ammonia (70:30:2, *v*/*v*/*v*) containing 0.4 % (*w*/*v*) ammonium acetate. The eluate of neutral lipids was reduced in volume and mixed with 1 mL of hexane and the mixture was stirred for 15 min. The solution was filtered, hexane was evaporated and weighed, 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.

Analysis of fatty acid methyl esters

The total TAGs (~5 mg) were saponified overnight in 10 % KOH-MeOH at room temperature. A fatty acid fraction

Table 1 Yield of dry weight, total lipids, TAGs, C16 PUFAs, and fatty acid content (%) in TAGs from different strains of *S. bacillaris* cultivated at different temperatures

		Temperature							
		10 °C			20 °C			30 °C	
		906	493	NAP	906	493	NAP	493	NAP
Dry weight (g L^{-1})		2.25	1.98	2.13	2.59	2.41	2.67	1.76	1.83
Lipids (mg L^{-1})		429.8	318.8	470.7	644.9	706.1	643.5	242.9	333.1
Lipids in % of dry weight		19.1	16.1	22.1	24.9	29.3	24.1	13.8	18.2
TAGs in mg		232.5	208.2	275.4	399.2	516.9	362.9	128.3	166.9
TAGs in % of total lipids		54.1	65.3	58.5	61.9	73.2	56.4	52.8	50.1
mg Pl/1 L		19.76	15.20	19.28	24.75	31.53	21.05	6.54	6.18
mg Pn/1 L		42.08	29.56	31.40	45.11	50.66	20.69	7.95	8.01
Abbreviation	Shorthand designation								
M	14:0	0.1±0.1	0.4±0.1	0.9±0.2	0.1±0.1	0.7±0.2	2.5±0.6	4.3±0.3	3.7±0.6
P	16:0	0.3±0.1	1.1±0.2	4.8±0.8	3.4±0.6	10.3±2.1	21.7±2.8	35.9±4.2	42.4±3.7
Po	9–16:1	0.7±0.2	0.9±0.2	4.4±0.4	1.9±0.5	2.4±1.0	5.2±1.2	6.5±1.0	6.3±1.1
Pl	7,10–16:2	8.5±0.9	7.3±0.9	7.0±1.3	6.2±2.4	6.1±0.7	5.8±1.0	5.1±0.7	3.7±0.5
Pn	7,10,13–16:3	18.1±1.3	14.2±1.5	11.4±1.8	11.3±2.7	9.8±2.3	5.7±0.7	6.2±1.2	4.8±0.6
O	9–18:1	0.2±0.1	0.9±0.2	3.6±0.7	1.8±0.7	5.9±0.9	12.8±2.5	10.7±0.9	18.7±2.2
L	9,12–18:2	12.5±1.2	24.4±2.7	22.3±2.5	24.8±3.5	22.7±3.4	15.9±2.2	16.9±2.4	9.5±1.4
Ln	9,12,15–18:3	44.1±2.4	42.7±3.9	41.7±4.1	42.6±4.1	36.7±3.8	28.4±3.6	12.9±1.3	9.8±0.9
A	5,8,11,14–20:4	8.1±0.7	4.9±0.8	1.4±0.7	4.5±0.9	2.9±1.0	0.9±0.2	0.3±0.1	0.2±0.1
E	5,8,11,14,17–20:5	7.4±0.8	3.2±0.6	2.5±1.1	3.4±0.8	2.5±0.7	1.1±0.4	1.2±0.5	0.9±0.3
UI[a]		299	272	244	262	230	173	126	101

Arithmetic means from three analyses ± standard deviations

[a] Unsaturation index, which was calculated as the number of double bonds per 100 fatty acids (the percent abundance of each fatty acid × number of double bonds, summed up for all fatty acids)

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 FA fraction was methylated using BF_3/MeOH (Dembitsky et al. 1992, 1993).

GC/MS of fatty acid methyl ester (FAME) was done on a Varian 450-GC, Varian 240-MS ion trap detector with electron impact ionization and CombiPal autosampler (CTC, USA). Splitless injection was at 100 °C, and a fused silica capillary column (Supelcowax 10; 60 m×0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C min^{-1} to 180 °C and at 2 °C min^{-1} to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm s^{-1}. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague).

Analysis of picolinyl esters

A solution of potassium *tert*-butoxide in tetrahydrofuran (0.5 mL, 1.0 M) was added to nicotinyl alcohol (1 mL). After mixing, the appropriate FAMEs (~0.5 mg) in dry dichloromethane (1 mL) were added, and the mixture was held at 40 °C for 30 min in a closed vial. After cooling to room temperature, water and hexane were added, and the organic phase was collected, dried over anhydrous sodium sulfate, and evaporated (Dembitsky et al. 1991).

A FA-picolinyl ester mixture was analyzed on the instrument described above. Injection temperature (splitless injection) was 100 °C, and an Ultra-1 capillary column was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C min^{-1} to 180 °C and at 2 °C min^{-1} to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm s^{-1}. All spectra were scanned within the range *m*/*z* 70–650.

Analysis of TAGs by RP-HPLC/MS-APCI

LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, USA), and two Hichrom columns HIRPB-250AM 250 × 2.1 mm i.d., 5 μm particle size, in series. This setup provided us with a high-efficiency column—approximately 26,000 plates/250 mm, a flow rate of 1 mL min^{-1}, an injection volume of 10 μL, and column temperature of 25 °C. The TAGs were separated using a gradient solvent program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, *v*/*v*); linear from 5 min to 110 min ACN/iPrOH 30:70 (*v*/*v*); held until 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.

The detector was an AB Sciex API 4000 mass spectrometer (AB Sciex, Canada) using APCI mass spectra. The ionization mode was positive, using the following settings: source temperature 420 °C, nebulizer gas (GS1) 5, nebulizer current 3 μA, curtain gas 10, collision gas high, and declustering potential 100 V. The optimal collision energy was dependent on the type of experiment and was set to +10 V (full scan mode) or +30 V (product ion mode). In all full scan runs, the spectra were obtained from *m*/*z* 200 to 1,100. To identify the exact composition of high *m*/*z* value TAG species, an enhanced product ion spectrum (optimal CE +35 V) was made of all ions with an *m*/*z* value over 650.

Collision-induced dissociation mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas as a collision target gas and applying collision energy of 50 eV in

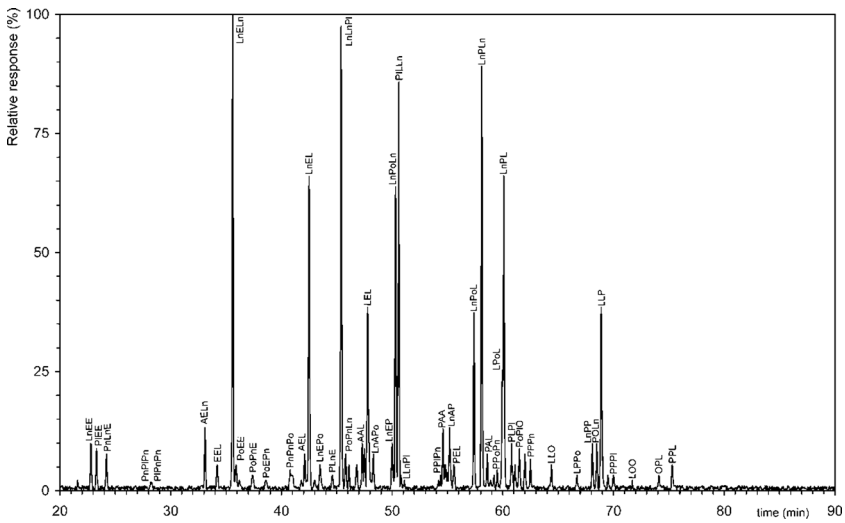

Fig. 1 RP-LC/APCI-MS chromatogram (TIC) of the TAGs from the alga of *S. bacillaris* CCALA 906, cultivated at 10 °C

Q2. Scanning range of Q3 was *m/z* 200–1,100 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3 % intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.2 software.

Analysis of TAGs 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 μg μL^{-1} in methanol) were chromatographed on two Astec CYCLOBOND™ I 2000 3,5-dimethylphenyl (DMP) carbamate-modified β-cyclodextrin chiral LC columns connected in series, 5 μm, 25 cm×4.6 mm from Sigma. Mobile phase was a gradient from 90 % of A and 10 % of B at 0 min to 50 % of A and 50 % during 120 min, where A is hexane and B is hexane-2-propanol (99:2, *v/v*) mixture (Rezanka et al. 2013). The flow rate, column temperature, and injection volume were 0.5 mL min^{-1}, 25 °C, and 10 μL, respectively. Other separation conditions used were as in the RP-LC (see above).

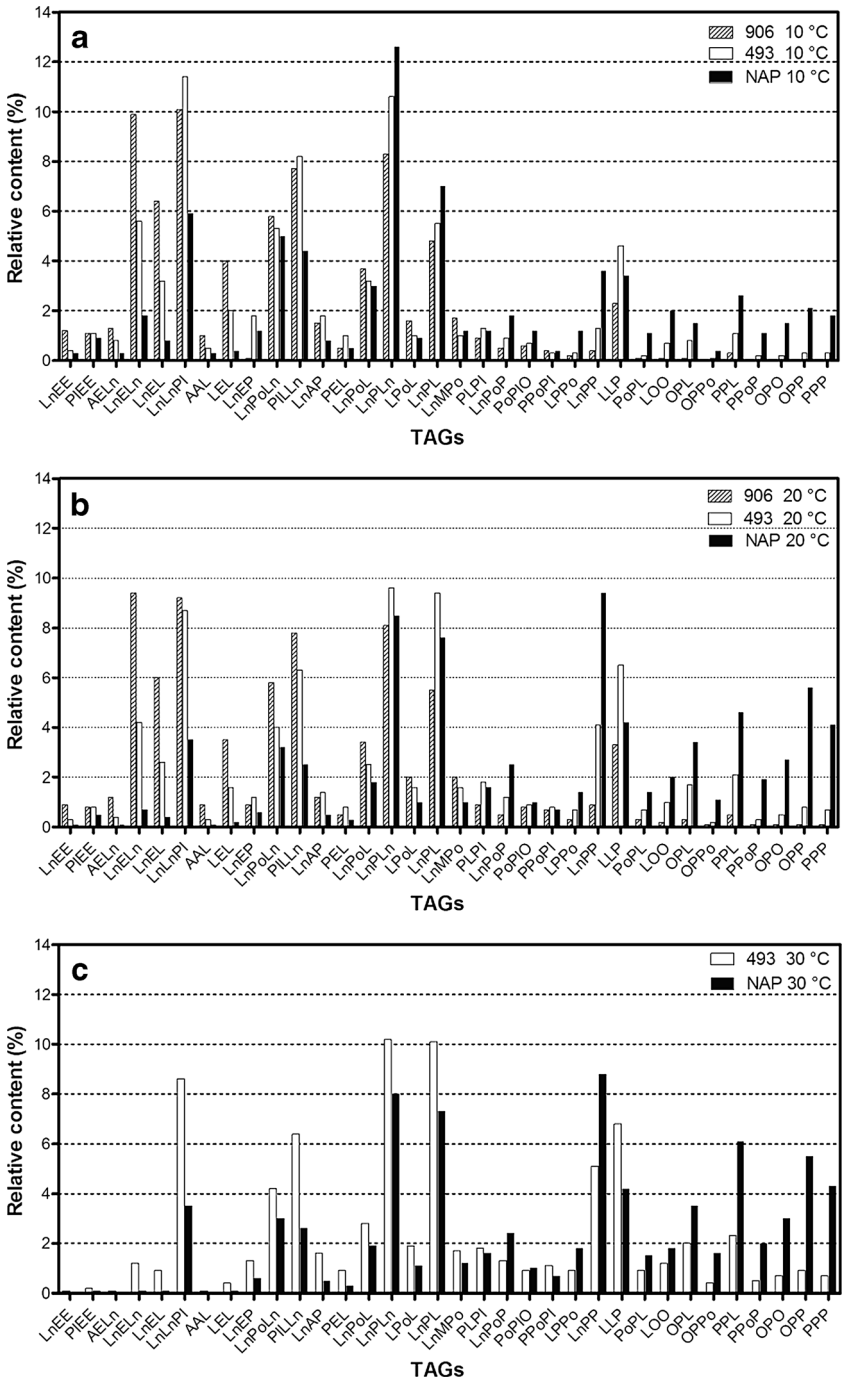

Fig. 2 Relative quantities (%) of molecular species of TAGs identified in the three strains of the alga *S. bacillaris* at 10 °C (**a**), 20 °C (**b**), and 30 °C (**c**). Only TAGs with relative quantity ≥1 % in either of the samples are displayed; for their complete list, see Table 1S

Statistical analysis

To perform multivariate statistical analyses, we used the program CANOCO 5 (Microcomputer Power, USA). Since the gradient lengths in the data were short, we used a principal component analysis (PCA) to visualize the variability within the dataset.

Results and discussion

Identification of fatty acids

Methyl esters obtained after hydrolysis of TAGs were esterified and then analyzed by GC/MS (Table 1). Eight out of ten peaks were identified by using retention characteristics, commercially obtained standards, and mass spectra of FAMEs. Peaks with retention times 16.93 min (equivalent chain lengths, i.e., ECL=16.67) and 19.31 min (ECL=17.32) were not identified. The methyl esters of PUFAs on polar capillary column were eluted in the order of ω-6 and ω-3 (Santercole et al. 2012), and it was possible to determine at least tentative positional isomers. We assumed, on the basis of retention times, that these peaks contain methyl esters of 16:2ω-6 (7,10–16:2) and 16:3ω-3 (7,10,13–16:3) FAs, respectively. However, despite all advances in the analysis of FAMEs, i.e., determination of the positions of double bonds based on diagnostically important ions such as ω ions, i.e., ion at *m/z* 150 characteristic for a ω-6 terminal group and one at *m/z* 108 characteristic for a ω-3 terminal group (Fellenberg et al. 1987), the only reliable method is still the analysis of nitrogen derivatives (Christie 1989).

We used picolinyl esters for the complete identification because these esters permit the location of the double bonds. Different positional isomers were found to give different mass spectra. Picolinyl esters of 16:2ω-6 and 16:3ω-3 acids have been measured many times (http://lipidlibrary.aocs.org/), and it is possible to distinguish between ω-3 and ω-6 positional isomers. Mass spectra of picolinyl esters of both acids are given in Figs. 1S and 2S (Supplements). Picolinyl ester of 16:2ω-6 acid showed a gap of 26 Da between *m/z* 206 and 232, which locates the double bond in position 7, while the gap between *m/z* 246 and 272 locates a double bond in position 10 (see Fig. 1S). It is however easier to spot gaps of 40 Da for the double bond and an associated methylene group, i.e., gaps from *m/z* 192 to 232 and gaps from *m/z* 232 to 272. The spectra of our compound (picolinyl ester of 16:2ω-6) and the same substance shown on the website (http://lipidlibrary.aocs.org/) are very similar. This clearly implies the structure of 16:2ω-6, i.e., the earlier eluted peak.

Similar principles were applied to the second eluted peak, i.e., 16:3ω-3 picolinyl ester (Fig. 2S). The mass spectra show differences between the two picolinyl esters, i.e., different values of the 26-Da and/or 40-Da gaps. The gap of 26 Da between *m/z* 206 and 232 locates the double bond in position 7, while the gap between *m/z* 246 and 272 locates a double bond in position 10 and the 26-Da gap between *m/z* 286 and 312 in position 13. Again, it is easier to spot gaps of 40 Da for the double bond and a neighboring methylene group, i.e., only three gaps from *m/z* 192 to 232 and from 272 to 312. In agreement with the literature, the position of the first double bond was detected only with difficulty (http://lipidlibrary.aocs.org/). Yet on the basis of our values (retention time and mass spectra of both picolinyl esters), we identified these compounds as 16:2ω-6 and 16:3ω-3 acids. These compounds are found in the genus *Stichococcus* (Lang et al. 2011). For instance, Olivieri et al. (2011) identified only five out of eight peaks and found no C16 unsaturated acid. In another study, Olivieri et al. (2012) identified only three C18 unsaturated acids (palmitic acid only in one case out of ten). Further, Teoh et al. (2004) identified 16:2 and 16:3 but did not

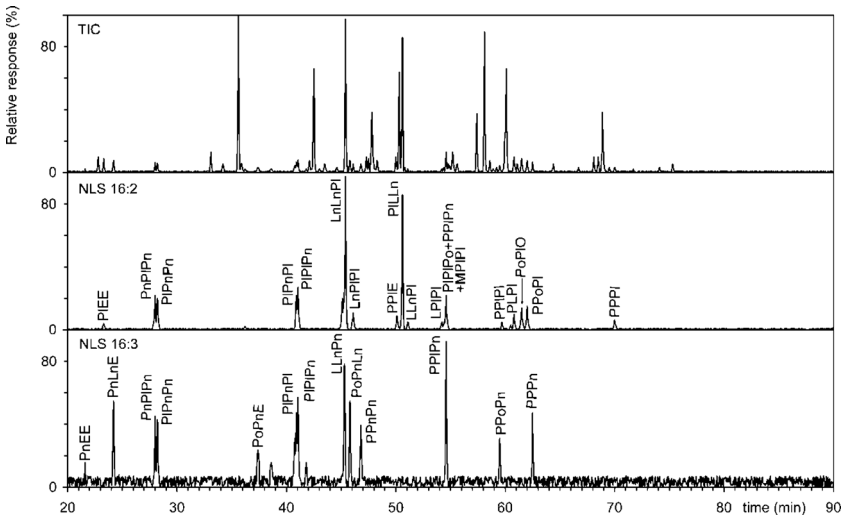

Fig. 3 TAGs containing hexadecadienoic and hexadecatrienoic acids (16:2 and 16:3, respectively) as revealed by the neutral loss scan for $[M + H]^+$—252 and $[M + H]^+$—250 Da, respectively, during APCI of total TAGs from *S. bacillaris* CCALA 906, cultivated at 10 °C

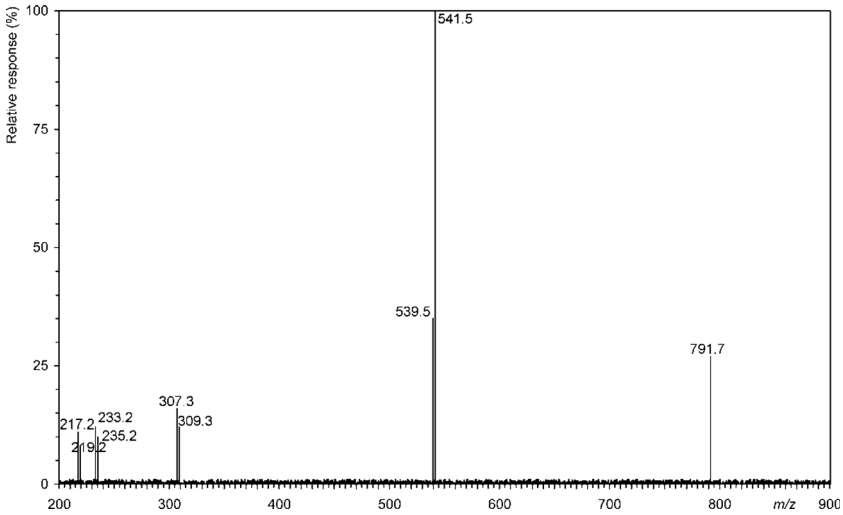

Fig. 4 Tandem mass spectrum of natural PnPlPn TAG

determine the positions of the double bonds. The only results suitable for comparison are thus those of Lang et al. (2011), in which the positions of double bonds were determined and, to our mind, correct.

Identification of triacylglycerols

Preliminary identification of TAGs, which was later confirmed by tandem MS, was based on the elution order, which best expresses the equivalent carbon number (ECN) (ECN = ACN − 2×DB (ACN = acyl carbon number; DB = number of double bond(s)) (Mu and Hoy 2000; Momchilova et al. 2004). TAGs having the same ECN value can be separated on a high-efficiency column such as a combination of two 25 cm columns in series (Rezanka et al. 2011), which provides an efficiency of about 52,000 theoretical plates. The combination effectively separated the TAGs and, in addition, also regioisomers of TAGs, as illustrated in Table 1S and Figs. 1 and 2.

MS analysis produces a large number of scans. To simplify the identification, we used the neutral loss scan (NLS) method. Its principle is in the first and third quadrupoles being scanned together, but with a constant mass offset, which permits the selective identification of all ions fragmenting in the second quadrupole and leads to the loss of neutral fragment (in our case, 16:2 and 16:3 acids). This method greatly simplifies the analysis of complex mixtures such as algal TAGs. Figure 3 depicts total ion current (TIC) and two NLS of the hexadecadienoic and hexadecatrienoic acids, i.e., ions $[M + H\text{-}C_{15}H_{27}COOH]^+$ and $[M + H\text{-}C_{15}H_{25}COOH]^+$. Corresponding neutral ion fragments were identified in the MS/MS spectra; based on their abundances, it was possible to determine the relative proportions of the corresponding molecular species of TAGs given in Table 1S.

Positive ion APCI mass spectra of TAGs typically exhibited $[M + H]^+$ ion and fragment ions of type $[M + H\text{-}RCOOH]^+$ ($[DAG]^+$), resulting from the loss of an acyl chain from TAG. Monoacyl glycerol ($[MAG]^+$) is equivalent to $[RCO+74]^+$

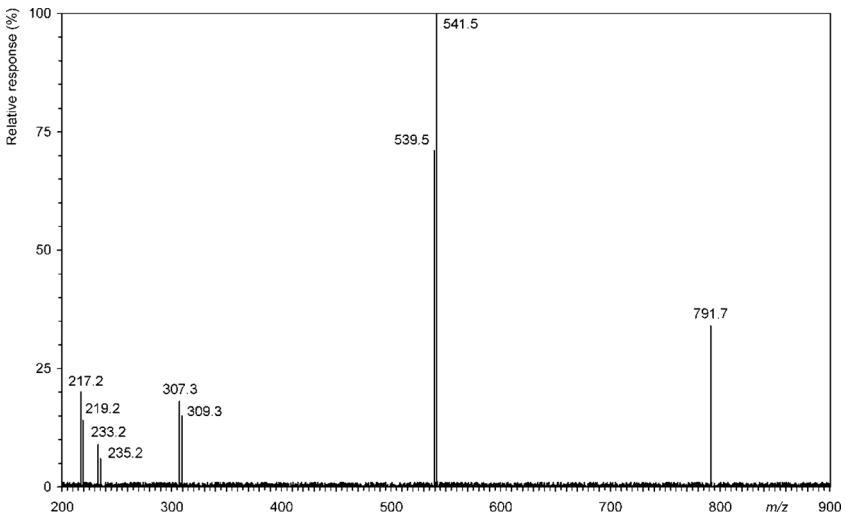

Fig. 5 Tandem mass spectrum of natural PlPnPn TAG

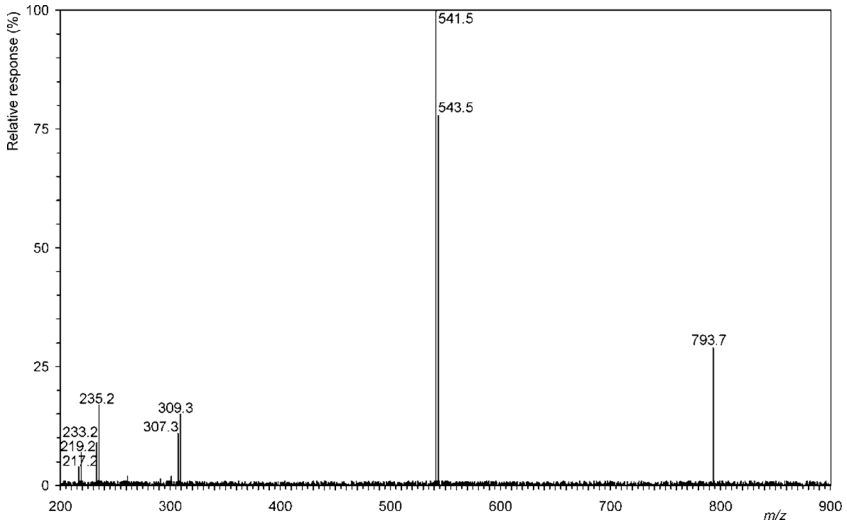

Fig. 6 Tandem mass spectrum of natural PlPlPn TAG

ion. The positive ion APCI mass spectrum (PlPnPn, for abbreviations of FAs, see Table 1), i.e., the peak at tR 28.2 min, shows $[M + H]^+$ ion at *m/z* 791.7 and the fragment ions $[M + H\text{-RCOOH}]^+$ at *m/z* 539.5 $[PnPn]^+$ and at *m/z* 541.5 $[PnPl]^+$. Further ions at *m/z* 309.3 $[Pl + 74]^+$ and at *m/z* 307.3 $[Pn + 74]^+$ or $[RCO]^+$ at *m/z* 235.2 [palmitolinoleyl] and at *m/z* 233.2 [palmitolinolenyl] and also $[RCO\text{-}18]^+$ (16:2) at 219.2 Da and $[RCO\text{-}18]^+$ (16:3) at 217.2 Da were also present. These ions were observed with sufficient intensity to analyze the mass spectrum.

Further TAGs were identified in a similar manner; the identification was often complicated by the coelution of several TAGs. For instance, two TAGs, PPlPn and MPlPl, were coeluted to form the peak at retention time 54.6 min. They, as well as other coeluted peaks, were identified by tandem MS, which clearly identified peaks of the $[DAG]^+$ type, i.e., $[PPl]^+$ at *m/z* 547.5, $[PPn]^+$ at *m/z* 545.5, and $[PlPn]^+$ at *m/z* 541.5, or $[MPl]^+$ and $[PlPl]^+$ at *m/z* 519.5 and *m/z* 543.5. TAGs often occur as regioisomers, i.e., positional isomers on the glycerol backbone (Mottram et al. 2001).

Achiral TAGs of the POP type were eluted before the corresponding chiral TAGs such as PPO (Lin et al. 1997; Momchilova et al. 2004). The identification of regioisomers is based on the fact that the loss of FA in the secondary hydroxyl is assumed to be energetically less favorable than the loss of FA from the primary hydroxyls (Mottram et al. 2001; Byrdwell 2005).

In the present study, we examined two pairs of regioisomers of the most abundant TAGs having C16 PUFAs, i.e., *sn*-PnPlPn (Fig. 4), *sn*-PlPnPn (Fig. 5), *sn*-PlPnPl (Fig. 6), and *sn*-PlPlPn (Fig. 7, see also Table 1S). The least abundant $[M\text{-RCOO}]^+$ ion ($[DAG]^+$ ion, i.e., $[PnPn]^+$ at *m/z* 539.5) is formed by the loss of palmitolinoleate from the *sn*-2 position. The more abundant $[DAG]^+$ ion is due to loss of palmitolinolenate from *sn*-1 or *sn*-3 position giving $[PlPn]^+$ ion at *m/z* 541.5. The $[PnPn]^+/[PlPn]^+$ ratio observed

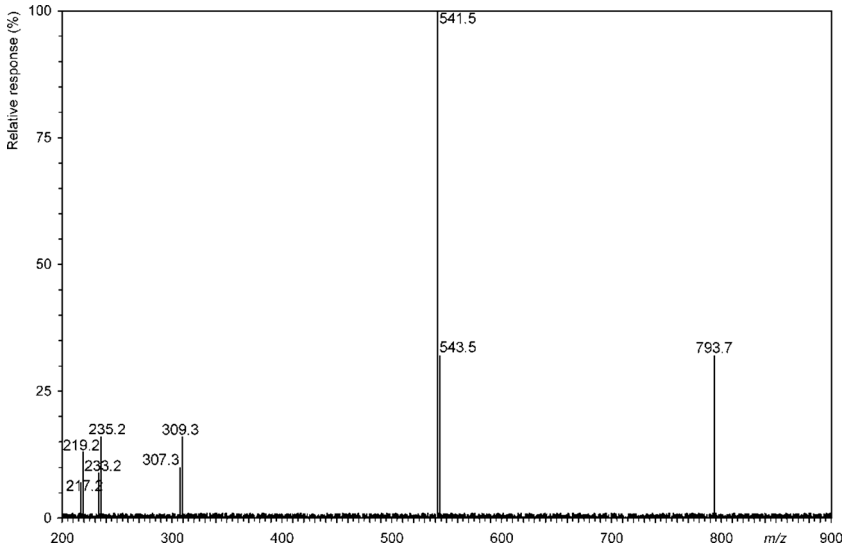

Fig. 7 Tandem mass spectrum of natural PlPnPl TAG

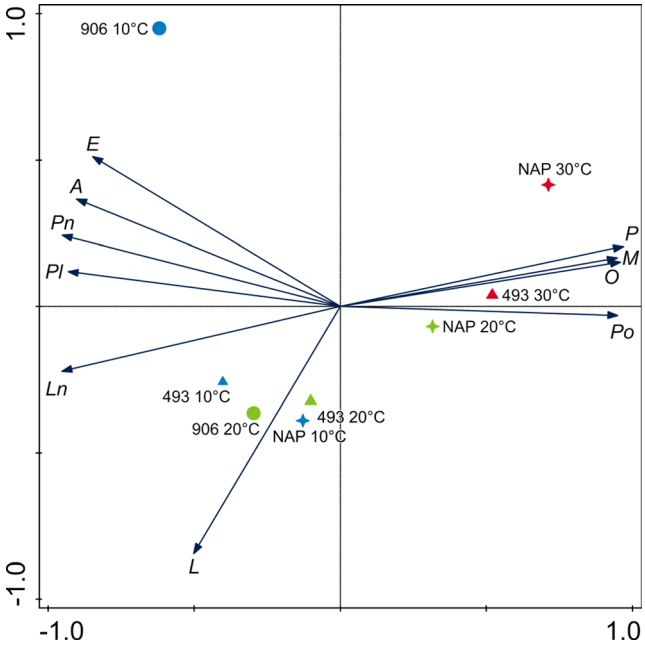

Fig. 8 Principal component analysis (PCA) of fatty acid composition in the three strains of *S. bacillaris* cultivated at 10, 20, and 30 °C. The first two canonical axes are plotted; they explained 94.5 % of the variability. For FA abbreviations, see Table 1

for PnPlPn is 35:100. Consequently, the two enantiomers show mass spectra differing only in intensities of $[DAG]^+$ ions.

A similar situation was found in another pair of regioisomers. Two compounds, i.e., PlPnPl and PlPlPn, were separated with elution times 40.9 and 41.0 min, respectively. The mass spectra also demonstrate the structure of separated TAGs. Different abundances of $[PlPl]^+$ and $[PlPn]^+$ ions were again found, their ratio for PlPnPl being 36:100 and for PlPlPn 78:100.

Composition of fatty acids

The fatty acid composition in green algae (Chlorophyta) is very diverse and dependent on environmental conditions (Wright et al. 1980; Ahlgren et al. 1992). The three strains of *Stichococcus* showed a considerable proportion of PUFAs (especially C16 and C18), which was reflected in relatively high unsaturation indexes (Table 1). In another member of the family Trebouxiophyceae, *Chlorella vulgaris*, Maazouzi et al. (2012) also reported an unsaturation index >200. In contrast to *Stichococcus*, it was significantly influenced by an increased content of C20 and C22 PUFAs.

A clear pattern was observed with respect to cultivation temperature. We cultivated the strains of *S. bacillaris* at 10, 20, and 30 °C. Only two strains grew at 30 °C, while the strain CCALA 906 isolated from a cold habitat (Svalbard) failed to grow at 30 °C. Teoh et al. (2004) stated that their strain did not grow even at 20 °C. Significant differences in temperature optima of growth between *Stichococcus* strains originating from cold and temperate localities were observed by Kviderova and Lukavsky (2005). The content of FAs in the eight cultivations performed in this study is given in Table 1, which shows the trend of increasing content of PUFAs and decreasing content of saturated FAs with decreasing cultivation temperature. A principal component analysis confirmed this pattern, separating the cultivations at contrasting temperatures along the first canonical axis (Fig. 8). The general response of the strains to different temperature conditions was similar. However, the fatty acid composition of the particular strains differed; the highest proportion of PUFAs was recorded in the strain CCALA 906 (Table 1, Fig. 8). As mentioned above, the comparison of the proportions of FAs in our cultivations with previous data on genus *Stichococcus* is complicated by the incomplete analysis of FAs. Thus, Chen et al. (2012) did not identify C16 PUFAs, i.e., 16:2 and 16:3 acids. Even so, some comparison can be done. The significantly higher proportion of PUFAs with decreasing temperature indicates that fatty acid unsaturation is apparently an important mechanism of cold adaptation in *Stichococcus* (Morgan-Kiss et al. 2006). However, Chen et al. (2012) found that the amount of 18:3 acid in *S. bacillaris* NJ-10 increased in the interval of 4–15 °C and then dropped in algae cultured at 25 °C, whereas in strain FACHB753, the production of this acid merely decreased with increasing cultivation temperature. An increase in the content of 18:3, with a maximum at 9 °C, and a subsequent drop with increasing cultivation temperature were also described by Teoh et al. (2004).

The content of the requisite 16:2 and 16:3 acids varied between 3.7 and 18.1 % of total FAs. It depended on two

Table 2 Relative quantities (%) of regioisomers and enantiomers of TAGs from *S. bacillaris*

	10 °C 906	20 °C 906	10 °C 493	20 °C 493	30 °C 493	10 °C NAP	20 °C NAP	30 °C NAP
PnPnPl	2.0	1.5	1.0	0.7	0.6	0.6	0.5	0.4
PlPnPn	0.7	0.6	0.3	0.6	0.5	0.5	0.4	0.3
PnPlPn	2.6	2.4	1.4	1.3	0.7	0.9	0.8	0.5
PlPlPn	0.8	0.7	0.3	0.2	0.2	0.2	0.1	0.1
PnPlPl	1.9	1.3	0.8	0.4	0.5	0.4	0.5	0.4
PlPnPl	2.6	2.2	1.3	1.0	0.7	0.6	0.9	0.6

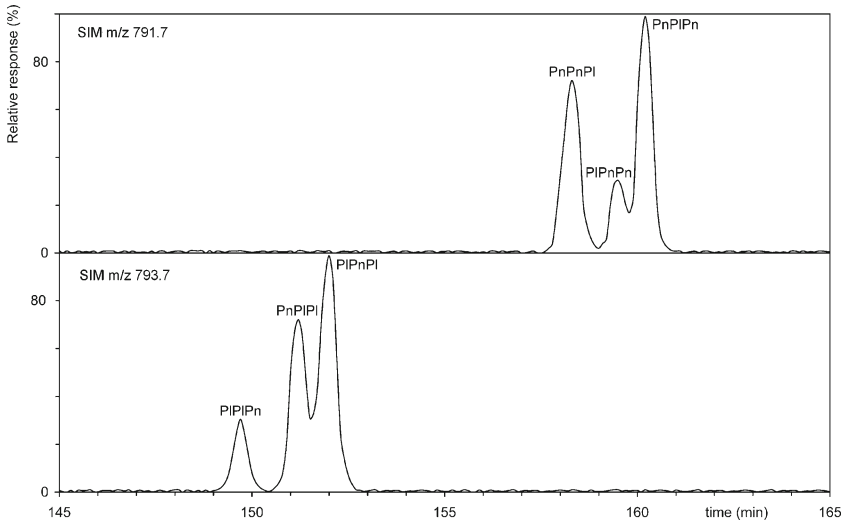

Fig. 9 Chiral LC of TAGs from *S. bacillaris* CCALA 906 cultivated at 10 °C

factors: the algal strain used for fatty acid production and the temperature of cultivation. However, the production of both C16 PUFAs was affected by cultivation temperature much less than that of, e.g., oleic or palmitic acids. We can conclude that the strain CCALA 906 from Svalbard cultivated at 10 °C provides the highest proportion of 16:2 and 16:3 acids. However, the most convenient in terms of production of these acids referred to 1 L cultivation medium is the strain CCALA 493 from the Hafner See (see Table 1), which shows a low percent content of both C16 PUFAs at 20 °C but produces higher amounts of biomass.

Composition of triacylglycerols

Natural TAGs are always a mixture of different molecular species, which differ in chain length and number of double bond(s). Unfortunately, the vast majority of all research in this area has been directed toward TAGs which could be used in the food industry (for example, for the production of chocolate), without any marked effort being devoted to understanding the physiological and biochemical importance of oil bodies in plants, especially in algae (Craven and Lencki 2013). A major adaptation of metabolic functions influencing growth and photosynthesis at low temperatures is the maintenance of membrane fluidity. Yet the studies always focused on the effect of temperature on the FA composition and membrane fluidity (Morgan-Kiss et al. 2006) and completely neglected the chiral or achiral structure of TAGs on the living cell.

Based on two preceding studies (Rezanka et al. 2012, 2013) concerning the separation of chiral TAGs, we now separated TAGs by RP-HPLC and also by chiral LC separation of appropriate fractions of polyenoic TAGs, i.e., TAGs containing some C16 and C18 PUFAs. The considerable complexity of the samples prevented a direct chiral separation similar to that performed by Rezanka et al. (2013). A detailed description of separation conditions is given in the "Materials and methods" section. It should be noted, however, that in our study, the quantification was performed by tandem MS with APCI, unfortunately without the use of standards, which are not commercially available. As already published, the length of the acyl chains of lipids including TAGs strongly affects the MS response (Lisa et al. 2007). The ratios of regioisomers of TAGs are given in Table 2 and they are likely influenced by the temperature of alga cultivation.

Chiral LC/MS-APCI revealed the relative proportions of individual TAGs. Table 1S and Fig. 8 give their chiral separations performed by tandem MS. Achiral TAGs are seen to be

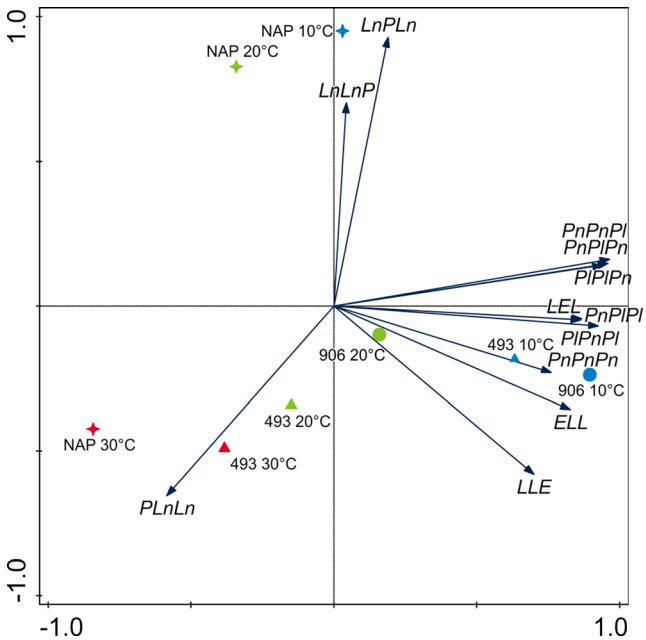

Fig. 10 Principal component analysis (PCA) of the relative proportions of regioisomers and enantiomers of TAGs in the *S. bacillaris* strains cultivated at 10, 20, and 30 °C. Three trios of TAGs were analyzed. The first two canonical axes are plotted; they explained 80.0 % of the variability. For FA and TAG abbreviations, see Table 1S

major in the two trios of TAGs mentioned above as well as in two trios of other TAGs, i.e., *sn*-LEL, *sn*-LLE, and *sn*-ELL and also *sn*-LnPLn, *sn*-LnLnP, and *sn*-PLnLn. This holds for all three strains at all temperatures. The ratio of enantiomers depends above all on the content of fatty acids. In general, with lowering temperature, the proportion of the achiral TAG increases, and so does the enantiomer ratio from 1:1. The effect of temperature on the relative proportions of regioisomers and enantiomers is demonstrated using a principal component analysis, where the first axis scores of the samples are clearly correlated with the temperature of cultivation (Fig. 8). PCA analysis of the 30 most abundant TAGs is shown in Fig. 4S, even if the image is not so illustrative as compared with Fig. 9.

Dienoic FAs preferentially esterify position *sn*-3, whereas in trienoic acids, it is position *sn*-1. This situation, which occurs irrespective of the chain length but is recorded always in 9,12- and 9,12,15-PUFAs, is best illustrated in Fig. 3S in Supplements. By contrast, this preference does not hold in the case of 20:5, i.e., 5,8,11,14,17-eicosapentaenoic acid, and also in saturated FAs such as palmitic acid. We attribute this phenomenon to the different affinities of the enzymes *sn*-glycerol-3-phosphate acyltransferase and diglyceride *O*-acyltransferase for the substrate, i.e., for methylene interrupted unsaturated chains of PUFAs. Our literature search did not reveal any such case, and this observation thus requires further measurements in order to establish a valid hypothesis about the biosynthesis of TAGs.

Conclusion

In this study, we showed that the temperature of cultivation affects the ratio of regioisomers and enantiomers of TAGs. The reason for this effect is as yet unknown. The data in Fig. 9 and mainly in Fig. 10 or 4S show that the PCA can be used as a highly sensitive tool for identifying the effect of cultivation conditions on the production of a specific compound by a microbial strain and may help biotechnologists in finding the most suitable production conditions.

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