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# Effect of starvation on the distribution of positional isomers and enantiomers of triacylglycerol in the diatom *Phaeodactylum tricornutum*

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ABSTRACT

The diatom *Phaeodactylum tricornutum* was cultivated in a standard medium and under sulfur, silicon, nitrogen and phosphorus starvation and its triacylglycerols (TAGs) were analyzed by RP-HPLC/MS-APCI. Nearly 100 molecular species of polyunsaturated TAGs were identified. RP-HPLC was used to isolate positional isomers of TAGs, which were further separated by chiral HPLC. First eluted were those TAGs that have an eicosapentaenoic acid moiety in the *sn*-1 position. The ratios of symmetrical to asymmetrical TAGs in *P. tricornutum* were affected under sulfur-, nitrogen-, phosphorus- and silica-starvation, i.e. in cultivations involving cells in nutrient stress. The ratios of positional TAGs and also the proportions of enantiomers were changed. The ratios of symmetrical to asymmetrical TAGs in the control and under N- and P-starvation were very close. In the control, the ratio of 1,2-dipalmitoyl-3-eicosapentaenoyl-*rac*-glycerol to 1,3-dipalmitoyl-2-eicosapentaenoyl-*rac*-glycerol was 3:1 and the ratio of 1,2-dieicosapentaenoyl-3-palmitoyl-*rac*-glycerol to 1,3-dieicosapentaenoyl-2-palmitoyl-*rac*-glycerol was 9:1. Under N-starvation the ratios were reversed irrespective of the presence or absence of silicate in the medium. A similar pattern was found in P- and S-starvation.



## 1. Introduction

Microalgae have been identified as an attractive source for the production of bioactive lipids and biofuels. Commercially interesting are strains that produce 20% or more of oil relative to their mass. Among microalgae, diatoms were reported to have high lipid content (Borowitzka, 1988). Algal lipids often contain unsaturated fatty acids with multiple double bonds, i.e. polyunsaturated fatty acids (PUFAs). The dominant sources of these PUFAs have so far been fish and fish oils but the acids in fact originate from marine plankton upon which the fish feed (Ackman, 1982). Alternative sources of PUFAs should therefore be looked for first of all among algae (Yongmanitchai and Ward, 1992) in view of the steadily decreasing fishing yields and as a result of climatic changes that could in future favor the cultivation of algae in higher latitudes.

Optimal production of PUFAs or neutral lipids by the diatoms is stimulated when they are stressed by nutrient deficiency of elements such as phosphorus (P), nitrogen (N), and silicon (Si) (Lombardi and Wangersky, 1991; McGinnis et al., 1997). The same is true for the production of triacylglycerols (TAGs) containing these acids (Hu et al., 2008). Although TAGs are biosynthesized mostly under different types of nutritional starvation, systematic comparisons of the yield and molecular species of TAGs produced under these starvation conditions have been missing. The available studies describe only changes in the content of PUFAs, not TAGs (Alonso et al., 2000; Ribalet et al., 2009; Tonon et al., 2002).

According to Tonon et al. (2002) the eicosapentaenoic acid content in *Phaeodactylum tricornutum* ranges from 16.1% for exponential cells to 5.8% of total lipids for stationary ones. Alonso et al. (2000) found 44.7% of eicosapentaenoic acid in the fraction of total TAGs.

The limitation of biosynthesis of PUFAs and TAGs by individual elements has often complex effects; thus, for instance, cells of the Si-starved marine diatom *Thalassiosira pseudonana* accumulated on average 24% more TAGs than those starved for N but the molecular species of the TAGs produced were generally similar regardless of the starvation type employed (Yu et al., 2009).

In the weakly silicified diatom *P. tricornutum* increasing concentration of silicate was found to cause a decrease in the content of eicosapentaenoic acid (Yongmanitchai and Ward, 1991) while in the diatom *Nitzschia laevis* the proportion of eicosapentaenoic acid remained more or less constant (Wen and Chen, 2000) and in the marine diatom *Chaetoceros gracillis* the amount of C20 and C22 ω-3 PUFAs decreased with decreasing silicate availability (Mortensen et al., 1988).

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P-starvation decreased the content of eicosapentaenoic acid in *P. tricornutum* (Yongmanitchai and Ward, 1991), a 10-fold increase in phosphate content increasing the production of eicosapentaenoic acid from 22.6% to 32.4% of total FAs.

Increasing nitrate concentration was accompanied in *P. tricornutum* by increased production of eicosapentaenoic acid and a drop in the production of palmitic acid (Yongmanitchai and Ward, 1991). Under N starvation, *Botryococcus braunii*, *Dunaliella bardawil*, and *D. salina* produced a higher percentage of eicosapentaenoic acid (Benamotz et al., 1985). In contrast, the proportion of polyunsaturated fatty acids in the freshwater algae *Scenedesmus* and *Chlorella* increased at high N concentrations (Piorreck et al., 1984).

Triacylglycerols contain three FAs bound to glycerol by ester bond. Combination of different FAs leads to a large number of molecular species of TAGs. When taking into account both positional isomers and optical enantiomers, the total number is $n^3$, where n is the number of FAs in TAG. Because the biosynthesis of TAGs involves four enzymes, of which three are acyl transferases (Coleman and Douglas, 2004), which specifically esterify each glycerol position, the resulting combinations are not random. The positional distribution but not enantiomer distribution of FAs in TAGs has been the subject of many studies (e.g. Lisa et al., 2011) which were mostly performed on commonly available animal and plant oils, not on algal or cyanobacterial oils. TAGs from both freshwater and sea fish contain PUFAs predominantly in the *sn*-2 position, those from sea mammals in the *sn*-1 or *sn*-3 position. The difference is due to their metabolic pathways or nutritional requirements (Gotoh et al., 2011a). To gain a better understanding of the physical, chemical, and nutritional properties of natural oils and fats, their examination should therefore focus also on the qualitative and quantitative analysis of the TAG molecular species (Karupaiah and Sundram, 2007). The separation of molecular species of TAGs by HPLC has been based predominantly on non-aqueous reversed-phase HPLC on RP C18 columns (Rezanka et al., 2010). However, complete identification has been achieved only by the combination LC-MS, which made it possible to identify many molecular species of TAGs including positional isomers of TAGs in natural fats and oils (Lisa et al., 2007; Rezanka et al., 2011).

Although RP-HPLC can separate positional isomers of TAGs, it cannot be used to separate TAG-enantiomers. These have so far been separated only rarely, and were identified only once in a natural source (palm oil). Using chiral HPLC on a polysaccharide-based chiral stationary phase, Iwasaki et al. (2001) separated without prior derivatization enantiomers of TAGs with very different acyl groups, e.g. 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol (e.g. *sn*-ECC or *sn*-CCE). Nagai et al. (2011) and Gotoh et al. (2011b) used recycle HPLC (6 recyclings) for the separation of 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol and 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (*sn*-POO and/or *sn*-OOP) from palm oil.

The structure and composition of fatty acids in triacylglycerols affects their absorption and distribution in the organism (Mu and Porsgaard, 2005). The best nutritional properties are attributed to structured TAGs (STAGs), i.e. TAGs having in *sn*-2 position polyunsaturated fatty acid (PUFAs). These TAGs contain in *sn*-2 position mostly EPA and DHA and are more readily absorbed than TAGs with the same composition of FAs but in a random distribution. Absorption of STAGs is also faster than that of the parent oils; interestingly, STAGs can protect against hypertriglyceridemia and obesity caused by high fat content in the diet (Takeuchi et al., 2002). STAGs could be potentially used to induce slimming and lower fat accumulation, as well as a reduction of serum cholesterol.

The simplest and most direct synthesis of STAGs is transesterification between the original oil and free FAs catalyzed by a 1,3 specific lipase (Xu et al., 1998). This one-step reaction can encounter problems caused by the resistance of PUFAs occupying positions *sn*-1 and *sn*-3 against transesterification. The problem can be partially solved by using a two-step enzyme reaction in which the first step involves the preparation of 2-monoacylglycerols (2-MAGs) by using lipase, and after their separation from free fatty acids the *sn*-1 and *sn*-3 positions are subjected in the second step to enzymatic esterification with the use of 1,3 specific lipases (Irimescu et al., 2001).

An important practical application of STAGs is their use as breast milk substitute. Human milk contains mostly palmitic and oleic acids so that the major TAGs is 1,3-dioleoyl-2-palmitoylglycerol, OPO (Morera Pons et al., 1998). The presence of palmitic acid in *sn*-2 position is very important for absorption of fats in children (Koo et al., 2006). Human milk also contains PUFAs, although their content depends on the consumption of PUFA-rich diet by the mother (Yuhas et al., 2006). The content of PUFAs plays a key role

**Table 1**
Names of FAs from TAGs of *Rhodococcus erythropolis* in control cultivation (CC in% of total FAs) including their abbreviations, carbon numbers (CN), and double bonds (DB).

| Systematic name | Trivial name | Abbreviation | CN:DB | CC (in%)[a] |
|---|---|---|---|---|
| Tetradecanoic | Myristic | M | 14:0 | 5.5 ± 0.5 |
| Hexadecanoic | Palmitic | P | 16:0 | 13.1 ± 1.2 |
| 9-Hexadecenoic | Palmitoleic | Po | 16:1ω9 | 21.8 ± 1.5 |
| 9,12-Hexadecadienoic | Palmitolinoleic | Pl | 16:2ω4 | 4.7 ± 0.4 |
| 6,9,12-Hexadecatrienoic | Palmitolinolenic | Pn | 16:3ω4 | 4.4 ± 0.4 |
| 6,9,12,15-Hexadecatetraenoic | | Pm | 16:4ω1 | 3.9 ± 0.3 |
| Octadecanoic | Stearic | S | 18:0 | 1.4 ± 0.2 |
| 9-Octadecenoic | Oleic | O | 18:1ω9 | 2.7 ± 0.3 |
| 11-Octadecenoic | cis-Vaccenic | V | 18:1ω7 | 0.6 ± 0.2 |
| 9,12-Octadecadienoic | Linoleic | L | 18:2ω6 | 2.1 ± 0.4 |
| 6,9,12-Octadecatrienoic | γLinolenic | γLn | 18:3ω6 | 1.8 ± 0.3 |
| 9,12,15-Octadecatrienoic | αLinolenic | αLn | 18:3ω3 | 0.5 ± 0.2 |
| 6,9,12,15-Octadecatetraenoic | Stearidonic | St | 18:4ω3 | 0.4 ± 0.2 |
| 5,8,11,14-Eicosatetraenoic | Arachidonic | A | 20:4ω6 | 3.8 ± 0.5 |
| 11,14,17-Eicosatrienoic | Dihomolinolenic | | 20:3ω3 | 0.3 ± 0.1 |
| 8,11,14,17-Eicosatetraenoic | Eicosatetraenoic | | 20:4ω3 | 0.2 ± 0.1 |
| 5,8,11,14,17-Eicosapentaenoic | Eicosapentaenoic | E | 20:5ω3 | 29.0 ± 1.2 |
| 7,10,13,16,19-Docosapentaenoic | Docosapentaenoic | Dp | 22:5ω3 | 0.3 ± 0.2 |
| 4,7,10,13,16,19-Docosahexaenoic | Docosahexaenoic | Dh | 22:6ω3 | 3.5 ± 0.5 |

[a] Results are given as mean ± S.D. for three separate determinations.

in the development of the child, especially in the fetal stage, and its provision ensures optimum neurological development during early life (Innis, 2008). It is desirable to increase the content of PUFAs in preterm infants since maternal milk provides less PUFAs than the fetus receives in the uterus (Innis, 2007).

The aim of this study was to elucidate the effect of N-, P-, sulfur (S) and Si-starvation on the TAGs of the marine diatom *P. tricornutum* Bohlin, a widely studied cosmopolitan species found in coastal waters from tropical to cold-temperate regions whose genome has been sequenced (Bowler et al., 2008). It grows well at 15–23 °C, so a strict control of temperature during cultivation is not needed to obtain high yields (Kviderova and Lukavsky, 2003). We employed a two-stage cultivation method to grow the cells: in the first stage, cell growth was performed for maximum cell-mass production, while the second stage focused on maximum lipid production.

## 2. Results and discussion

GC–MS analysis of the methyl esters obtained after hydrolysis of TAGs confirmed the presence of eleven major and some minor fatty acids (Table 1), whose proportion was below 0.2% total FAs in TAGs. Nearly 100 molecular species of TAG were identified, see Table 2; many of them are highly unsaturated with high carbon numbers, as found by using RP-HPLC data from previous analyses (Rezanka et al., 2010, 2011). Although the major peaks were separated on the baseline, the separation of some peaks was insufficient. The mass spectra suggested that several peaks contained two or more overlapping species, see also the analysis of similar TAGs containing PUFAs (Hori et al., 1993, 1994; Lisa et al., 2009; Rezanka et al., 2011). When taking into account only major FAs, see Table 1, the number of theoretically possible molecular species of TAGs is $11^3$, i.e. 1331, of which we identified less than 10%. The highest numbers of TAGs identified so far are of the same order. Thus, e.g., Francois et al. (2010) identified in menhaden fish oil, which contains more than one hundred FAs, 324 TAGs out of the theoretical number of molecular species in this oil, which can exceed half a million. Similarly, Ikeda et al. (2009) identified 103 TAGs in mouse liver. Unlike these studies, the number of TAGs identified in our study includes also enantiomers.

The most common method of separation of molecular species of TAGs (Lisa et al., 2007; Rezanka and Sigler, 2007) has been non-aqueous reversed-phase HPLC on octadecylsilane columns, in which the elution order is dependent on the length of the acyl chain and the number of double bonds in the triacylglycerols (Perona and Ruiz-Gutiérrez, 1999). It identified tens to hundreds of molecular species of TAGs from different plant and/or animal oils and fats (Lisa et al., 2009, 2011).

The authors who analyzed molecular species of TAGs from *P. tricornutum* (Danielewicz et al., 2011; MacDougall et al., 2011; Yu et al., 2009) by ESI or MALDI TOF, did not quantify the identified TAGs and reported only on their molecular species, which are of the order of tens and more or less agree with our data in Table 2, in which the symbol X denotes the TAGs identified by these authors. Quantitative determination of the TAGs in *P. tricornutum* was done by Yongmanitchai and Ward (1993), who separated the triacylglycerol lipid class by RP-HPLC into a minimum of 18 molecular species, each of which was analyzed for composition and positional distribution of fatty acids. Seven TAG molecular species contained ω-3 fatty acids. With a single exception (myristic acid), the *sn*-2 position carried only C16 acids (palmitic and palmitoleic acids) and only two out of the 18 TAGs contained another C16 PUFA.

As mentioned above, TAGs from fish tend to bind PUFAs at the *sn*-2 position, whereas TAGs from sea mammals bind PUFAs at the *sn*-1 or *sn*-3 position (Ikeda et al., 1998). This shows that each

**Table 2**
Relative quantities (%) of total acyl groups identified in *P. tricornutum* control cultivation, their retention times, acyl carbon number, number of double bonds, and equivalent chain number.

| RT | ECN | ACN:DB | TAG | % | I[a] |
|---|---|---|---|---|---|
| 15.7 | 26 | 52:13 | Pm-E-Pm[b] | 0.7 | |
| 18.0 | 28 | 52:12 | Pn-E-Pm | 0.8 | |
| 18.6 | 28 | 56:14 | Pm-E-E | 1.9 | |
| 19.3 | 28 | 58:15 | Pm-E-Dh | 0.7 | |
| 21.6 | 30 | 56:13 | Pn-E-E | 1.9 | + |
| 21.9 | 30 | 60:15 | E-E-E | 7.6 | |
| 22.4 | 30 | 62:15 | A-E-Dh | 0.7 | |
| 23.2 | 30 | 62:16 | E-E-Dh | 1.9 | |
| 24.1 | 30 | 64:17 | Dh-E-Dh | 0.7 | |
| 24.1 | 32 | 52:10 | Po-E-Pm | 1.5 | |
| 24.5 | 32 | 60:9 | M-E-Pm | 0.8 | |
| 25.0 | 32 | 54:11 | Po-Dh-Pm | 0.4 | |
| 25.8 | 32 | 58:13 | Ln-E-E | 1.8 | |
| 26.4 | 32 | 60:14 | A-E-E | 3.9 | |
| 26.8 | 34 | 48:7 | Pn-Pn-Po | 0.4 | + |
| 27.3 | 34 | 52:9 | Po-E-Pn | 1.6 | + |
| 28.5 | 34 | 54:10 | P-Dh-Pm | 0.2 | + |
| 29.5 | 34 | 56:11 | Po-E-E | 3.5 | + |
| 30.2 | 34 | 54:10 | M-E-E | 1.9 | + |
| 30.7 | 34 | 58:12 | Po-E-Dh | 1.5 | + |
| 31.4 | 34 | 56:11 | M-E-Dh | 0.7 | |
| 33.1 | 34 | 58:12 | A-E-Ln | 0.6 | |
| 34.2 | 34 | 58:12 | E-E-L | 1.8 | + |
| 34.8 | 34 | 56:11 | Po-E-E | 3.3 | |
| 35.6 | 34 | 56:11 | Ln-E-Ln | 0.6 | |
| 36.5 | 36 | 60:12 | O-E-Dh | 0.7 | |
| 37.4 | 36 | 58:11 | P-E-Dh | 1.0 | + |
| 38.1 | 36 | 58:11 | O-E-E | 1.9 | + |
| 39.0 | 36 | 56:10 | P-E-E | 2.7 | + |
| 40.2 | 36 | 58:11 | A-E-L | 0.6 | |
| 41.8 | 36 | 56:10 | A-E-Po | 1.5 | |
| 42.5 | 36 | 56:10 | Ln-E-L | 0.6 | |
| 42.9 | 36 | 56:10 | E-E-P | 2.6 | |
| 43.5 | 36 | 54:9 | Ln-E-Po | 1.3 | + |
| 44.3 | 36 | 54:9 | A-E-M | 0.8 | |
| 44.9 | 36 | 52:8 | Ln-E-M | 0.7 | |
| 45.1 | 36 | 48:6 | Po-Pm-Po | 1.1 | |
| 46.7 | 38 | 56:9 | A-A-Po | 0.4 | + |
| 47.8 | 38 | 56:9 | L-E-L | 0.6 | |
| 48.0 | 38 | 56:9 | A-E-P | 1.0 | + |
| 48.3 | 38 | 54:8 | Ln-A-Po | 0.3 | |
| 49.0 | 38 | 54:7 | Po-E-L | 1.5 | + |
| 50.0 | 38 | 54:8 | Ln-E-P | 1.0 | + |
| 50.2 | 38 | 52:7 | Ln-Po-Ln | 0.3 | + |
| 50.5 | 38 | 52:7 | Po-E-Po | 2.7 | + |
| 50.9 | 38 | 52:7 | M-E-L | 0.7 | |
| 51.3 | 38 | 50:6 | M-E-Po | 1.6 | + |
| 51.6 | 38 | 48:5 | M-E-M | 0.8 | + |
| 53.8 | 40 | 56:8 | A-A-P | 0.2 | |
| 54.6 | 40 | 54:7 | Po-A-L | 0.3 | |
| 55.1 | 40 | 54:7 | Ln-A-P | 0.2 | + |
| 55.6 | 40 | 54:7 | P-E-L | 1.0 | + |
| 55.9 | 40 | 54:7 | Po-E-O | 1.5 | + |
| 56.2 | 40 | 52:6 | Po-A-Po | 1.1 | |
| 57.4 | 40 | 52:6 | Ln-Po-L | 0.3 | |
| 58.1 | 40 | 52:6 | Ln-P-Ln | 0.1 | + |
| 59.0 | 40 | 52:6 | Po-E-P | 2.0 | + |
| 59.2 | 40 | 50:5 | Ln-Po-Po | 1.0 | + |
| 59.3 | 40 | 50:5 | M-A-Po | 0.4 | |
| 60.0 | 40 | 50:5 | M-E-P | 1.1 | + |
| 60.2 | 40 | 48:4 | Ln-M-Po | 0.4 | + |
| 60.7 | 42 | 52:5 | L-Po-L | 0.3 | + |
| 60.9 | 42 | 50:4 | Po-Po-L | 1.0 | + |
| 61.0 | 42 | 52:5 | Ln-P-L | 0.1 | + |
| 61.2 | 42 | 54:6 | P-A-L | 0.2 | |
| 61.3 | 42 | 52:5 | L-Po-L | 0.3 | |
| 61.5 | 42 | 52:5 | Po-A-P | 0.8 | + |
| 61.6 | 42 | 52:5 | P-E-P | 1.6 | + |
| 61.9 | 42 | 50:4 | Ln-Po-P | 0.7 | + |
| 62.3 | 42 | 50:4 | M-A-P | 0.2 | |
| 62.4 | 42 | 48:3 | Po-Po-Po | 2.8 | + |
| 62.8 | 42 | 48:3 | Po-M-L | 0.4 | + |

(*continued on next page*)

**Table 2** (*continued*)

| RT | ECN | ACN:DB | TAG | % | I[a] |
|---|---|---|---|---|---|
| 64.2 | 42 | 48:3 | Ln-M-P | 0.2 | |
| 64.5 | 42 | 46:2 | Po-M-Po | 1.2 | + |
| 65.6 | 42 | 44:1 | M-M-Po | 0.6 | + |
| 65.9 | 44 | 50:3 | O-Po-Po | 1.1 | + |
| 66.7 | 44 | 50:3 | L-P-Po | 0.7 | + |
| 67.3 | 44 | 48:2 | P-Po-Po | 1.6 | + |
| 67.9 | 44 | 48:2 | L-M-P | 0.2 | |
| 68.1 | 44 | 50:3 | Ln-P-P | 0.4 | + |
| 68.5 | 44 | 44:0 | P-M-M | 0.3 | |
| 68.8 | 44 | 52:4 | O-Po-L | 0.3 | |
| 69.1 | 44 | 52:4 | P-A-P | 0.4 | + |
| 69.5 | 44 | 50:3 | Po-P-L | 0.7 | |
| 71.3 | 44 | 48:2 | P-M-L | 0.2 | |
| 71.5 | 44 | 46:1 | Po-M-P | 0.8 | |
| 73.0 | 44 | 44:0 | M-M-P | 0.3 | |
| 73.3 | 46 | 52:3 | O-Po-O | 0.4 | + |
| 74.1 | 46 | 52:3 | O-P-L | 0.2 | + |
| 74.3 | 46 | 50:2 | O-M-O | 0.1 | |
| 74.5 | 46 | 50:2 | O-P-Po | 0.8 | |
| 75.3 | 46 | 50:2 | P-P-L | 0.4 | |
| 75.6 | 46 | 48:1 | P-Po-P | 1.1 | + |
| 75.8 | 46 | 48:1 | P-M-O | 0.2 | |
| 76.2 | 46 | 46:0 | P-M-P | 0.6 | + |
| 78.8 | 48 | 52:2 | O-P-O | 0.2 | + |
| 80.1 | 48 | 50:1 | O-P-P | 0.4 | + |
| 81.3 | 48 | 48:0 | P-P-P | 0.8 | |

[a] Identified by Danielewicz et al. (2011), Yongmanitchai and Ward (1993), Yu et al. (2009), but without any quantification.
[b] The molecular species denote the *sn*-1(3), -2, -3(1) positions.

natural fat or oil is characterized by the distribution of FA on the glycerol backbone (Hunter, 2001). Nutrient starvation affects not only the ratio of positional TAG isomers, but also the proportion of individual enantiomers. To separate individual enantiomers by chiral HPLC it was necessary, in keeping with the technique used for the analysis of palm oil (Nagai et al., 2011), to separate not only other molecular species of TAGs (about a hundred compounds, see Table 2), but also symmetrical positional isomers of TAGs which do not have a center of chirality. Semipreparative RP-HPLC (see Figs. 1 and 2) was used to isolate from the mixture of *P. tricornutum* TAGs two racemic mixtures, i.e. PPE and PEE (more exactly a mixture of *sn*-PPE/*sn*-EPP and *sn*-EEP/*sn*-PEE). The separated peaks were detected by APCI-MS using the full scan mode.

Like in previous chiral separations of TAGs (Iwasaki et al., 2001; Nagai et al., 2011; Gotoh et al., 2011b), we also used a polysaccharide-based chiral stationary phase, i.e. 3,5-dimethylphenyl carbamate modified β-cyclodextrin. Also, like in these studies methanol was much better in separating individual enantiomers than other mobile phases such as acetonitrile and *n*-hexane–isopropanol.

Mass spectra of two out of the three possible TAGs, i.e. *sn*-PPE and *sn*-PEP are given in Figs. 3A and 3B. Both *sn*-EPP enantiomers have identical mass spectra (Nagai et al., 2011). The APCI mass spectra also confirm the positional isomeric purities of *rac*-PPE and *sn*-PPE, because *rac*-PPE and *sn*-PPE formed DAG ions at the same ratio, *m/z* 551 and *m/z* 597, corresponding to $[PP]^+$ and $[PE]^+$, respectively. As indicated by the above mass spectra, the addition of *sn*-PPE (internal standard) to *rac*-PPE increased the abundance of the later eluted peak (RT = 161.2 min); this proved that *sn*-PPE is eluted after *sn*-EPP. The same elution order, i.e. the elution of two enantiomers containing two saturated and one unsaturated FAs, the earlier eluting ECC and the later eluting CCE on a column with the stationary phase with the cellulose tris-(3,5-dimethylphenylcarbamate) chiral selector, or the first eluted *sn*-OPP and the later eluted *sn*-PPO, was previously reported by Iwasaki et al. (2001) and Nagai et al. (2011).

Semipreparative RP-HPLC of *rac*-EEP and its separation from other TAGs, especially from *sn*-EPE, is shown in Fig. 2. Chiral separation of synthetic *rac*-EEP, synthetic *sn*-EEP and a natural mixture of *rac*-EEP from the N-starvation cultivation of *P. tricornutum* is shown in Fig. 4. In agreement with literature data (Iwasaki et al., 2001) the peaks were again eluted in the order *sn*-PEE and *sn*-EEP (Fig. 4A). This was again confirmed by the mass spectrum of synthetic *sn*-EPE (Fig. 5) and by adding synthetic *sn*-EEP as internal standard (Fig. 6). The mass spectra of both enantiomers (*sn*-PEE and *sn*-EEP) were identical (spectra not shown). The above data show that *rac*-PPE and *rac*-EEP can be separated on a chiral

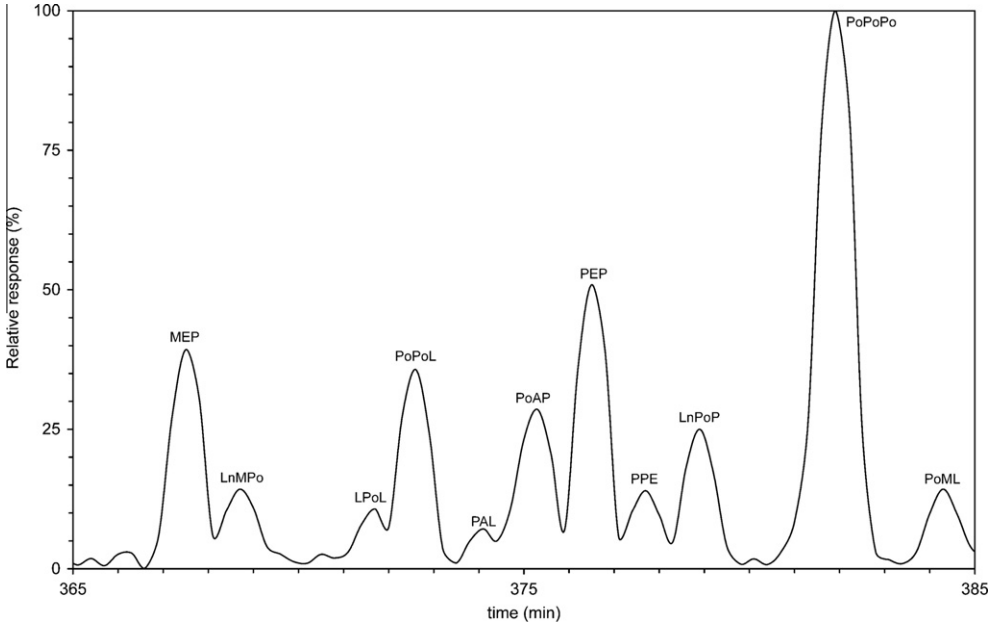


**Fig. 1.** Semipreparative RP-HPLC of *rac*-PPE and its separation from other TAGs, especially from *sn*-PEP - N-starvation.

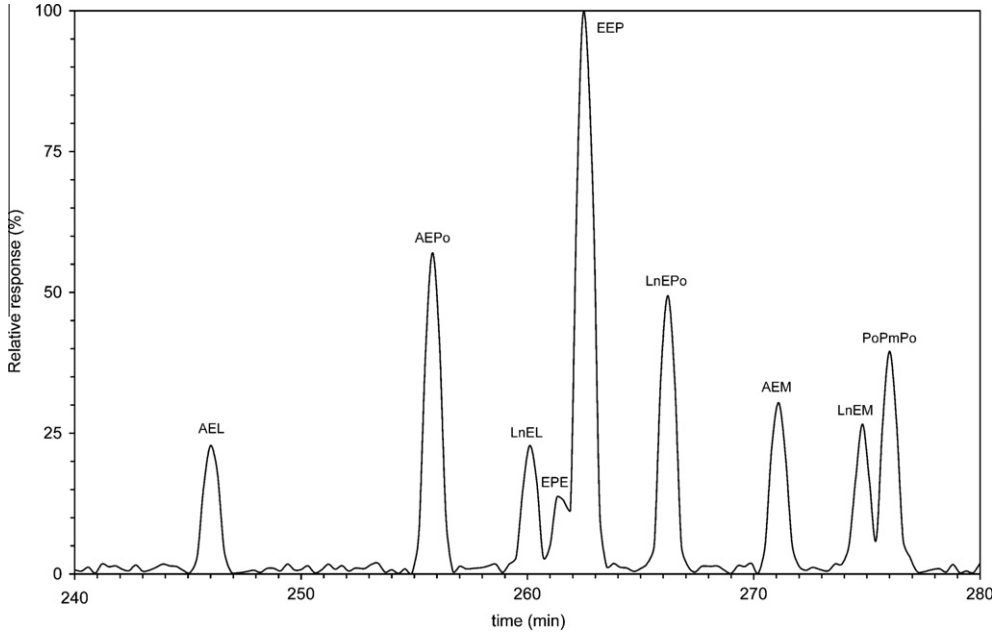


**Fig. 2.** Semipreparative RP-HPLC of *rac*-EEP and its separation from other TAGs, especially from *sn*-EPE - control cultivation.

column; *sn*-EPP and *sn*-EEP, which have an eicosapentaenoic acid moiety in the *sn*-1 position, are eluted before *sn*-PPE and *sn*-PEE, respectively.

Fig. 4A and B shows the partial chromatograms of natural mixtures of appropriate TAGs from control cultivation and N-starved cultivation of *P. tricornutum*. Table 3 shows that under S-, N-, P- and Si-starvation, i.e. cultivations involving cells in nutrient stress, both the ratios of positional TAGs, i.e. *sn*-PPE – *sn*-PEP and *sn*-EEP – *sn*-EPE, and also the proportions of individual enantiomers are changed.

As previously found in the alga *Trachydiscus* (Rezanka et al., 2011), the ratios of symmetrical and asymmetrical TAGs, i.e. *rac*-EPE to *rac*-PEE and *rac*-PEP to *rac*-PPE in *P. tricornutum* are also affected. Both these microorganisms belong to the same phylum (Ochrophyta) though to different classes (see Table 3 containing also the previously published proportions of TAGs for comparison (Rezanka et al., 2011)) and the ratios of symmetrical to asymmetrical TAG in the control and under N- and P-starvation are very close. Thus in the control the ratio of *rac*-PPE to *rac*-PEP is 3:1 in both the diatom and in *Trachydiscus*, and the same holds for the ratio of *rac*-PEE to *rac*-EPE, which is 9:1 in the diatom and 10:1 in *Trachydiscus*.

Under N-starvation the ratio is opposite to that in the control irrespective of the presence or absence of silicate in the medium. A similar pattern is found under P-starvation, in which the ratio further increases in both *P. tricornutum* and *Trachydiscus*. No changes in the ratio were found under Si-starvation since Si is used by diatoms for the formation of frustules and does not enter

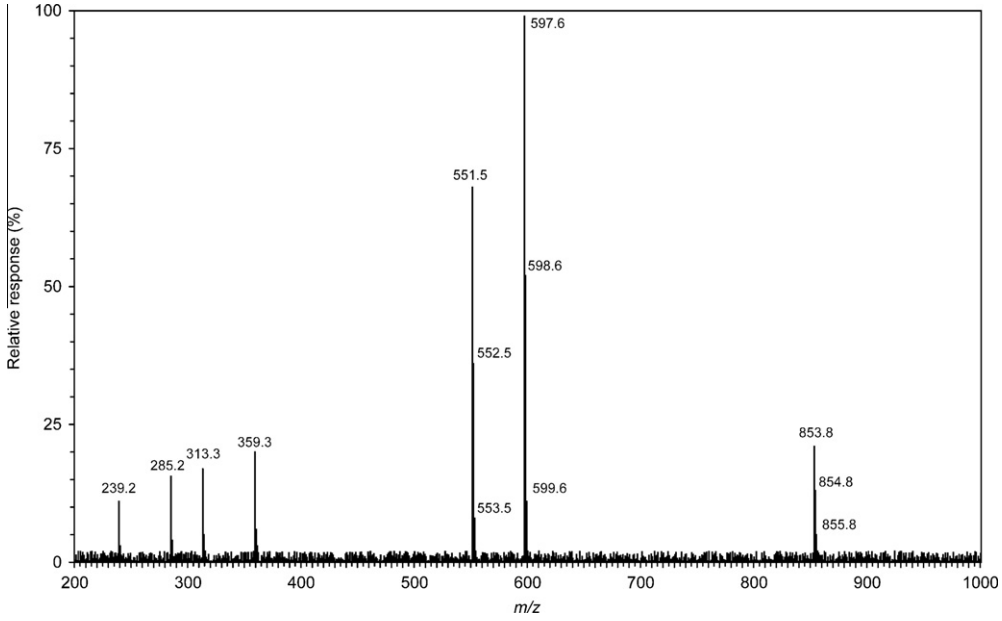


**Fig. 3A.** Mass spectrum (APCI) of synthetic *sn*-PPE.

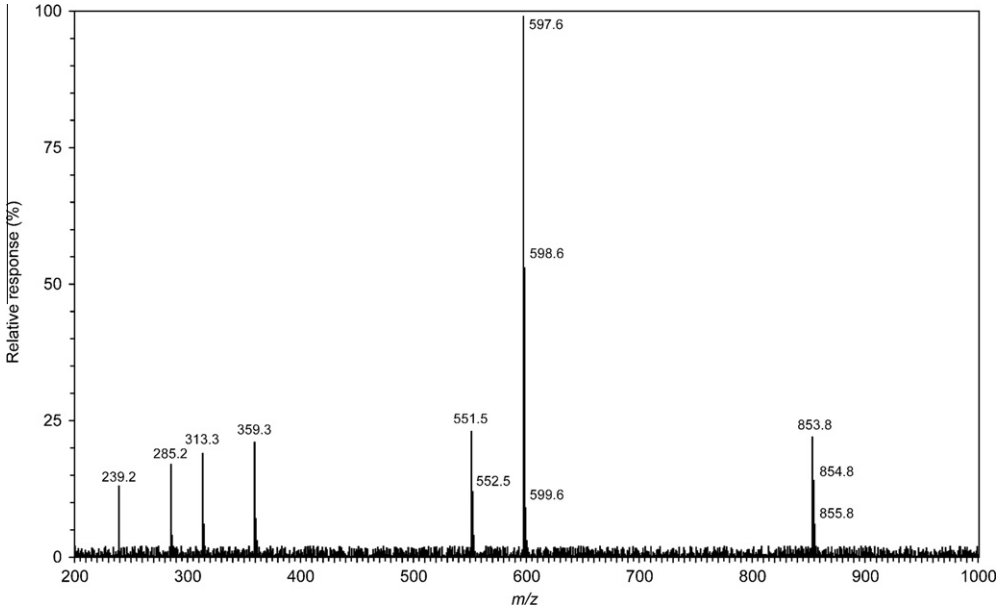


**Fig. 3B.** Mass spectrum (APCI) of synthetic *sn*-PEP.

directly into metabolism. Moreover, *Phaeodactylum* can also grow in a frustule-free form (Round et al., 2007). S-starvation has a similar effect as N- and P-starvation, causing an increase of the ratio in favor of symmetrical TAGs.

The effect of sulfur absence on the ratios of individual asymmetrical enantiomers is near that of nitrogen absence. The ratios of enantiomers of TAGs after chiral HPLC separation attest to a different effect of N- and P-starvation on enantiomer ratios within the asymmetrical TAGs; this points to a different action of N and P on their biosynthesis. P-starvation causes an inversion of enantiomer ratios relative to the control, whereas under N-starvation the ratios are nearer the control, e.g. there is more EEP than PEE (Table 3).

TAG-enantiomers have been separated and identified by chiral chromatography only as mono- and diacyl-glycerols after their partial hydrolysis and derivatization (Kuksis and Itabashi, 2005). The method was modified by Sempore and Bezard (1991) to identify two enantiomers, i.e. *sn*-OLL, *sn*-LLO, and a third symmetrical *sn*-LOL in, e.g., peanut and cottonseed oils; their ratio was 16:72:12. The stereospecific analysis of TAGs from sunflower oil (Boukhchina et al., 2003) showed that all analyzed TAGs, i.e. PLL, OOL, OLL and POL contain all theoretically possible stereoisomers including enantiomers. Thus OLL was identified to be composed of three TAGs, i.e. *sn*-OLL, *sn*-LLO and *sn*-LOL in a ratio of 24:63:13. Redden et al. (1996) also found that the dilinoleoyl-mono-γ-linolenin TAGs from evening primrose oil have γ-1inolenic acid distributed asymmetrically, with 19.5%, 33.8% and 47.2% γ-1inolenic acid found at the *sn*-1, *sn*-2 and *sn*-3 positions, respectively; this implies the formation of all three TAGs, i.e. *sn*-LLG, *sn*-GLL and *sn*-LGL, where L is linoleic acid and G is γ-linolenic acid. No explanation of this phenomenon was offered.

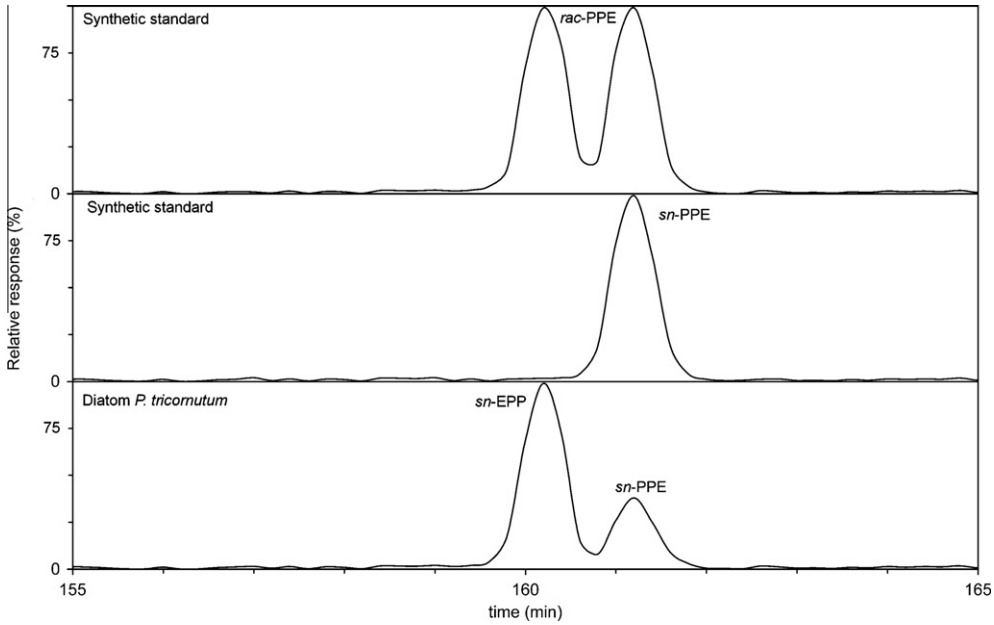


**Fig. 4A.** Chiral separation of synthetic *rac*-PPE, synthetic *sn*-PPE and a natural mixture of *rac*-PPE from from the control cultivation of *P. tricornutum*.

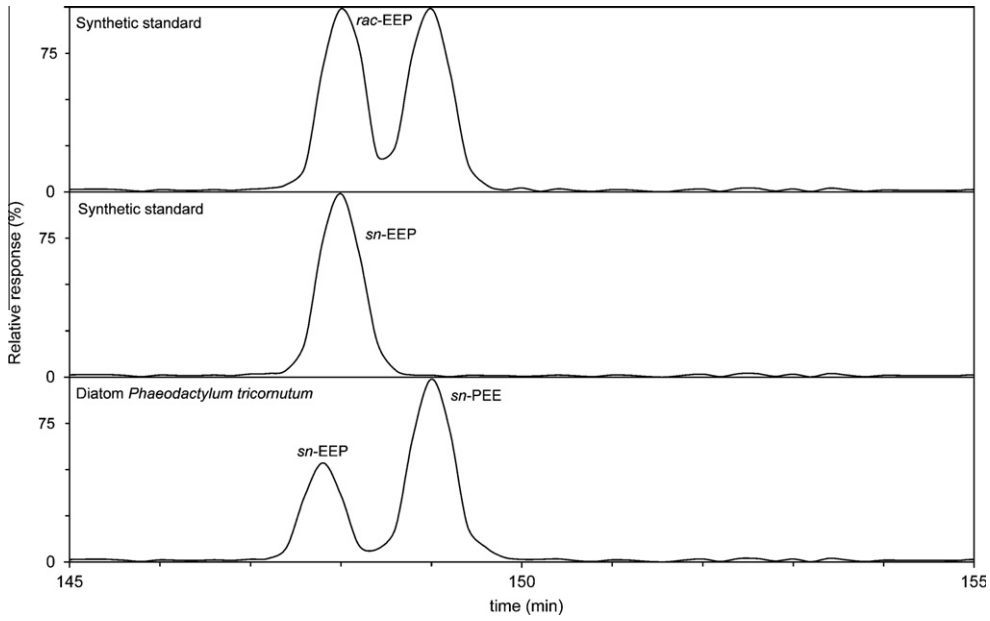


**Fig. 4B.** Chiral separation of synthetic *rac*-EEP, synthetic *sn*-EEP and a natural mixture of *rac*-EEP from the N-starvation cultivation of *P. tricornutum*.

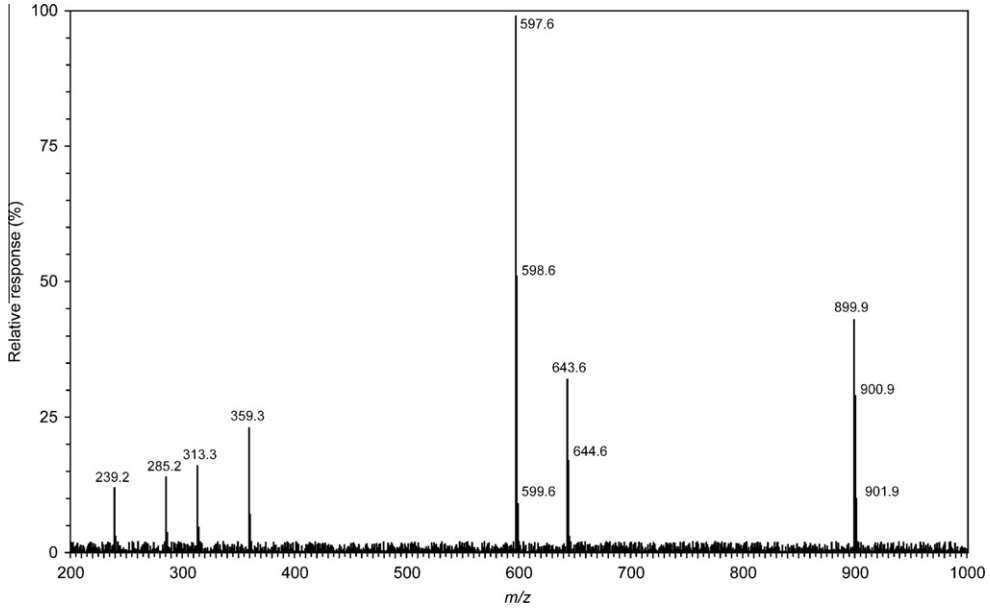


**Fig. 5.** Mass spectrum (APCI) of synthetic *sn*-EPE.

The full explanation of why both enantiomers of TAGs are synthesized by the diatom is still missing.

Another, highly improbable, possibility is the reduction of dihydroxyacetone phosphate by, e.g., glycerol-1-phosphate dehydrogenase (EC 1.1.1.261) to glycerol-1-phosphate, an enantiomer of glycerol-3-phosphate. This biosynthesis has been described in all Archaebacteria (Koga and Morii, 2007) and also in the bacterium *Bacillus subtilis* (Guldan et al., 2008).

The change in the center of chirality on carbon *sn*-2 and thus the formation of both enantiomers could take place by intramolecular transesterification – see, e.g., the *in vitro* experiment of Iwasaki et al. (2001), in which interesterification of tricapryloylglycerol with ethyleicosapentaenoate under catalysis by *Rhizomucor miehei* lipase gave rise to an asymmetrical pair of TAGs, i.e. 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol and 1,2-dicapryroyl-3-eicosapentaenoyl-*sn*-glycerol. Further, a third pathway involving diacylglycerol transferase has been recognized in maturing plant seeds. The existence of still another, as yet undescribed biosynthetic pathway cannot be fully excluded.

The ratios of individual enantiomers of TAGs were affected by N-, P-, and S-starvation, see Table 3. The most probable possibility for the appearance of both enantiomers in different ratios appears to be intramolecular transesterification, although the current data do not allow us to exclude other possibilities.

Based on the three-point attachment model (Pirkle and Pochapsky, 1989), which explains the contact between the stationary phase and the chiral substrate as being due to hydrophobic interactions of long-chain fatty acid(s) and Π–Π interaction between the stationary phase (benzene ring) and Π-electrons of double bonds in TAGs, we propose that separation will be successful

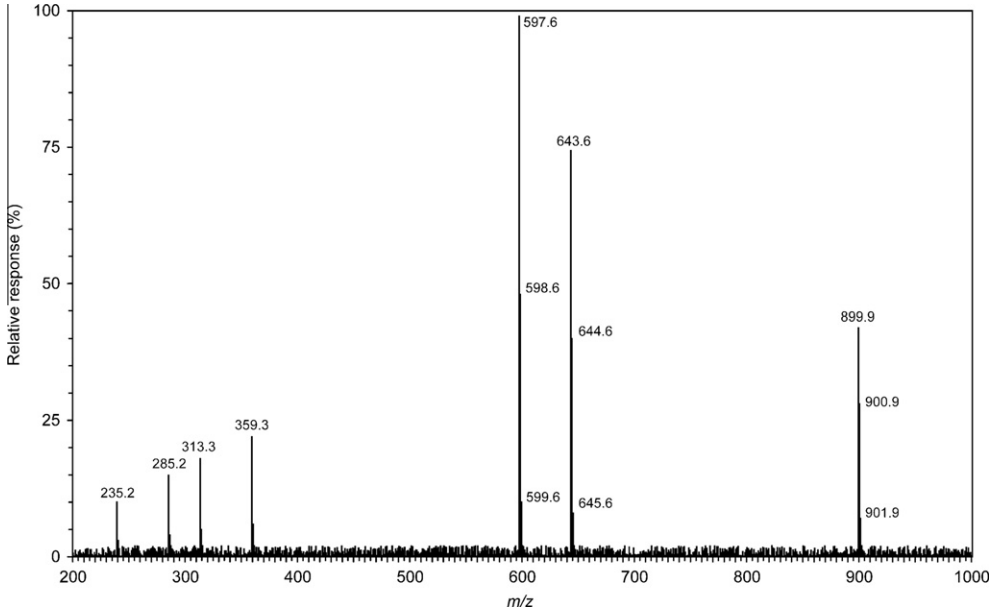


**Fig. 6.** Mass spectrum (APCI) of synthetic *sn*-EEP.

**Table 3**
The ratios of three TAGs, i.e. *sn*-EEP, *sn*-PEE, *sn*-EPE, *sn*-PPE, *sn*-EPP, and *sn*-PEP under different types of nutrient starvation.

| Starvation type | *sn*-EEP | *sn*-PEE | **Σ asym**[#] | *sn*-EPE | **Σ sym** | *sn*-EPP | *sn*-PPE | **Σ asym** | *sn*-PEP | **Σ sym** |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 57 | 29 | **86** | 14 | **14** | 41 | 34 | **75** | 25 | **25** |
| -P-Si | 6 | 16 | **22** | 78 | **78** | 5 | 10 | **15** | 85 | **85** |
| -N-Si | 23 | 9 | **32** | 68 | **68** | 23 | 10 | **33** | 67 | **67** |
| -S-Si[*] | 19 | 7 | **26** | 74 | **74** | 18 | 7 | **25** | 75 | **75** |
| -P+Si | 6 | 10 | **16** | 84 | **84** | 7 | 13 | **20** | 80 | **80** |
| -N+Si | 21 | 11 | **32** | 68 | **68** | 22 | 8 | **30** | 70 | **70** |
| -S+Si[*] | 14 | 7 | **21** | 89 | **89** | 16 | 6 | **22** | 78 | **78** |
| Control *Trachydiscus* | | | **91** | | **9** | | | **75** | | **25** |
| -P *Trachydiscus* | | | **33** | | **67** | | | **16** | | **84** |
| -N *Trachydiscus* | | | **40** | | **60** | | | **25** | | **75** |

[*] N/P 7.82 (16.04 mg N/L and 2.05 mg P/L), Last three rows – similar values for *Trachydiscus* (Rezanka et al., 2011) for comparison.
[#] Bold values are the sums of enantiomers (*sn*-EEP + *sn*-PEE and/or *sn*-EPP + *sn*-PPE) given here for comparison with the previously measured values (Rezanka et al., 2011), where only positional isomers and not enantiomers were measured.

mostly with TAGs that substantially differ in acyl groups, as described by Iwasaki et al. (2001). Our separation of TAGs containing both saturated straight long-chain(s) and polyunsaturated fatty acids on a chiral column was accomplished without the use of recycle runs employed by Gotoh et al. (2011b) for molecular species of TAGs containing oleic and palmitic acids. Further progress in chiral separations of asymmetrical TAGs will consist in determining the TAGs that can be separated in a single run, i.e. without recycle. An important prerequisite is the acquisition (synthesis) of commercially inaccessible standards; this represents a serious problem since even chiral DAGs or MAGs are virtually unavailable.

The ultimate step in successful chiral separation of enantiomers of asymmetric TAGs is to our mind the elucidation of both the biosynthesis and utilization of these TAGs, which can have considerable impact on dietetics.

## 3. Conclusion

By using RP-HPLC in combination with APCI-MS we identified individual positional isomers in the diatom *P. tricornutum*, both in control cultivation and under N-, P-, S- and Si-starvation. We also resolved on a chiral stationary phase racemic mixtures of asymmetric TAGs, namely two pairs of TAGs, i.e. *sn*-PPE, *sn*-EPP and *sn*-EEP, *sn*-PEE, into the individual enantiomers in a single run. With the exception of Si, nutrient starvation reversed the ratios of symmetrical and asymmetrical TAGs, whereas the ratios of individual enantiomers were affected by various types of starvation in a different way.

By affecting the cultivation conditions, one can thus directly produce structured TAGs (STAGs) having PUFA in position *sn*-2, since in the control the proportion of TAG having PUFA in *sn*-2 position PUFA (eicosapentaenoic acid) is a mere 25% whereas under phosphorus and silica starvation it increases to 85% of total TAG containing two palmitic acids and one eicosapentaenoic acid. Likewise, for palmito-dieicosapentaenoine (EEP and PEE against EPE), cultivation under S-, Si-starvation produced the highest amounts of the nutritionally beneficial TAG having palmitic acid in position *sn*-2 (Table 3).

## 4. Experimental

### 4.1. Standards and instrumentation

Acetonitrile, 2-propanol, THF, hexane, dichloromethane, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 1,2-dieicosapentaenoyl- *sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl- *sn*-glycerol, and phospholipase C from *Clostridium perfringens* (*C. welchii*) Type I, lyophilized

powder, 10–50 units/mg protein, were purchased from Sigma–Aldrich (Prague, CR). 1,2-Dieicosapentaenoyl-3-palmitoyl-*rac*-glycerol (EEP), 1,3-dieicosapentaenoyl-2-palmitoyl-*rac*-glycerol (EPE), 1,2-dipalmitoyl-3-eicosapentaenoyl-*rac*-glycerol (PPE), 1,3-dipalmitoyl-2-eicosapentaenoyl-*rac*-glycerol (PEP) were obtained previously (Rezanka et al., 2011)

A Perkin–Elmer (Perkin–Elmer, Norwalk, CT, USA) model 1310 IR spectrophotometer was used for scanning IR spectroscopy as neat films. High resolution MS spectra were recorded using a VG 7070E-HF spectrometer (70 eV). NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz ($^1$H) and 125.7 MHz ($^{13}$C).

### 4.2. Cultivation

The diatom *P. tricornutum* (strain CCMP632 Coughlan) was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA). Experimental cultures were inoculated with cells that had been grown in full Zehnder liquid medium (Staub, 1961) with the addition of NaCl (20 g $L^{-1}$) at room temperature and irradiance of ~100 μmol $m^{-2}$ $s^{-1}$ (final dry weight 0.43 mg $L^{-1}$). The initial dry weight of batch cultures was 0.26 mg $L^{-1}$. We employed a two-stage cultivation method to grow the cells: in the first stage, cell growth was for maximum cell-mass production, while the second stage focused on maximum lipid production.

To assess the influence of nitrogen and phosphorus starvation, full Zehnder liquid medium with NaCl (see above) and three other modifications of this medium were used. In nutrient depleted media, nitrogen and phosphorus concentrations were reduced to 0 mg $L^{-1}$. Furthermore, the effect of silicon and sulfur starvation was tested by (a) adding of aqueous solution of $Na_2SiO_3$ (1 ml $L^{-1}$) to all the four media mentioned above and by (b) substituting $MgSO_4$ by $MgCl_2$ in full Zehnder liquid medium without and with the addition of silicon. Altogether, 10 batch cultures were established. They were maintained in 2 L glass flasks at 15 °C under continuous light of 160 μmol $m^{-2}$ $s^{-1}$ provided by Osram cool-white tubes. The cultures were mixed three times a day and the supply of $CO_2$ was provided by diffusion. The cells were harvested in the stationary phase after 19 days of cultivation, dry mass was determined by filtration with pre-weighed Synpor filters (pore diameter 0.6 μm) and drying to constant mass at 105 °C. Samples were lyophilized and stored in aluminum foil until solvent extraction.

### 4.3. Enzymatic synthesis of 1,2-dieicosapentaenoyl-sn-glycerol

1,2-Dieicosapentaenoyl-*sn*-glycero-3-phosphocholine was treated with phospholipase C (Type I from *C. perfringens* (*C. welchii*)) as described by Christie (1989). In brief, phospholipase C from *C. welchii* (3 mg) in 0.5 M tris(hydroxymethyl)methylamine (tris) buffer (pH 7.5; 2 mL), containing $2 \times 10^{-3}$ M calcium chloride, was added to phosphatidylcholine (15 mg) in diethyl ether (2 mL). The mixture was shaken at room temperature for 3 h and extracted three times with diethyl ether (4 mL portions). The ether layer was dried over anhydrous sodium sulfate before being evaporated in a stream of nitrogen at ambient temperature. Pure 1,2-dieicosapentaenoyl-*sn*-glycerol was obtained by preparative TLC on a layer of silica gel G impregnated with boric acid (10%, w/w), with hexane-diethyl ether (50:50, v/v) as solvent system. The appropriate band was located under UV light and was eluted from the adsorbent with diethyl ether (Christie, 1989).

### 4.4. Preparation of mixed FA composition TAGs

The esterification of glycerol derivatives with acids was described previously (Ziegler and Berger, 1979; Halldorsson et al., 2003). Briefly, a solution of 1,2-dipalmitoyl-*sn*-glycerol (57 mg, 0.1 mmol) and eicosapentaenoic acid (33 mg, 0.11 mmol) as a free acid in dichloromethane (1.0 mL) was added to DMAP (6.1 mg, 50 μmol) and EDCI (18.6 mg, 0.12 mmol) in dichloromethane (1.0 mL) under an inert atmosphere (argon). The resulting solution was stirred at room temperature for 15 h. The reaction was stopped by passing the reaction mixture in ether/dichloromethane (10:90) through a short column packed with silica gel. Solvent removal in vacuo afforded the pure product as yellowish oil. The yield of the target *sn*-PPE was 46.9 mg, i.e. 55%. NMR spectra of *rac*-PPE, which is identical with *sn*-PPE were published previously (Rezanka et al., 2010):

The general procedure for the synthesis of *sn*-PEE was as follows: under an inert atmosphere, a solution of 1,2-dieicosapentaenoyl-*sn*-glycerol (6.6 mg, 0.01 mmol) and palmitic acid (2.8 mg, 0.011 mmol) as a free acid in dichloromethane (1.0 mL) was added to DMAP (0.1 mg, 8 μmol) and EDCI (3.7 mg, 0.024 mmol) in dichloromethane (1.0 mL). For further procedure, see above. The yield of the target *sn*-PEE was 5.2 mg, i.e. 58%.

The $^1$H NMR spectra of all four TAGs, i.e. PPE, PEP, EPE and EEP are practically identical; minor differences in chemical shifts of carboxyl were obtained only in $^{13}$C NMR spectra in agreement with the literature (Halldorsson et al., 2003; Fauconnot et al., 2006).

### 4.5. Isolation of TAGs

The extraction procedure was based on the method of Bligh and Dyer (1959), 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 of both cyanobacterial strains 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 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; 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), and acidic lipids with 30 mL of chloroform–methanol–concentrated aqueous ammonia (70:30:2) containing 0.4% (w/v) ammonium acetate. The eluate of neutral lipids was reduced in volume, mixed with 1 mL of hexane and the mixture was stirred for 15 min. The solution was filtered, hexane was 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.

### 4.6. FAMEs analysis

The total TAGs (~5 mg) were saponified overnight in 10% KOH–MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and $Et_2O$ 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 (Dembitsky et al., 1992a,b).

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of 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 to 180 °C and at 2 °C/min to 280 °C, which

was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague).

### 4.7. RP-HLPC/MS-APCI

HPLC 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 ID, 5 μm particle size, in series. This setup provided us with a high-efficiency column – approximately 26,000 plates/250 mm. A quadrupole mass spectrometer system Navigator (Finnigan MAT, San Jose, CA, USA) was used for analysis. The instrument was fitted with an atmospheric pressure chemical ionization source (vaporizer temperature 390 °C, capillary heater temperature 260 °C, corona current 7 μA, sheath gas – high-purity nitrogen, pressure 0.45 MPa, and auxiliary gas (also nitrogen) flow rate 15 mL/min). Positively charged ions with *m/z* 200–1000 were scanned with a scan time of 0.5 s. The whole HPLC flow (0.35 mL/min) was introduced into the APCI source without any splitting. TAGs were separated using a gradient solvent program with acetonitrile (MeCN) and 2-propanol (iPrOH) as follows: initial MeCN/iPrOH (99:1, vol/vol); linear from 5 min to 120 min MeCN/iPrOH 30:70, vol/vol); held until 30 min; the composition was returned to the initial conditions over 10 min. A peak threshold of 0.07% intensity was applied to the mass spectra. Data acquisition and analyses were performed using PC with MassLab 2.0 for Windows XP applications/operating software.

### 4.8. Chiral HPLC

The HPLC system used for separation in the chiral mode was the same as that used in the reverses-phase mode. TAGs (~1 μg/μL in methanol) were chromatographed on an Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin) chiral HPLC column 5 μm, 25 cm × 4.6 mm from Sigma. Mobile phase ratio 99:1 (methanol: 10 mM ammonium acetate, pH 6.5) was used. The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 μL, respectively. Other separation conditions used were as in the RP-HPLC, see above.

## Acknowledgements

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