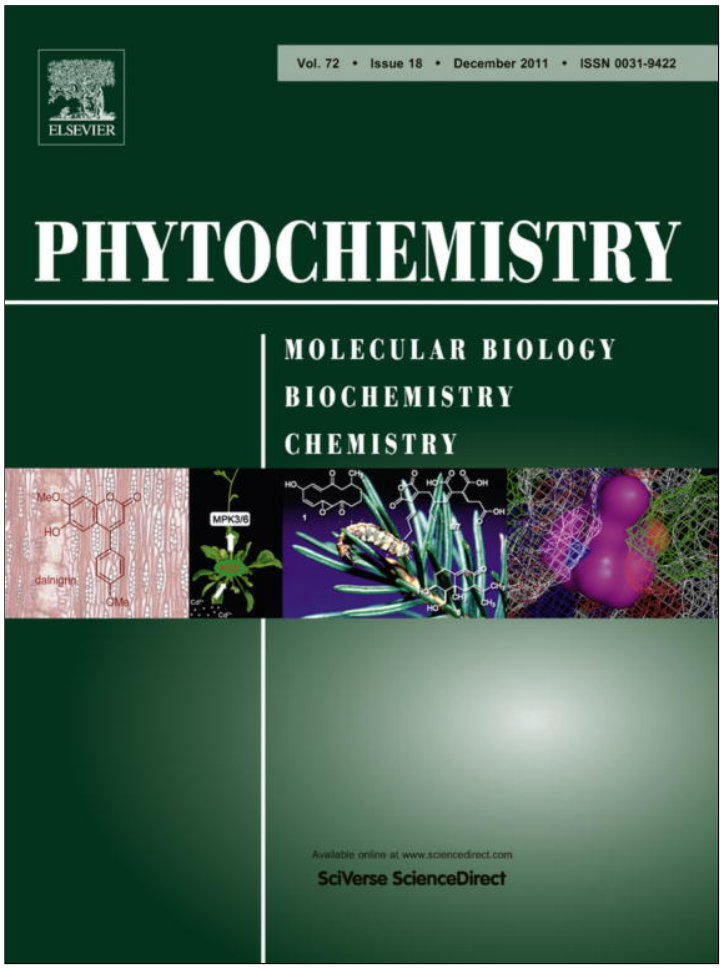

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Effect of nitrogen and phosphorus starvation on the polyunsaturated triacylglycerol composition, including positional isomer distribution, in the alga *Trachydiscus minutus*

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ABSTRACT

The yellow-green alga *Trachydiscus minutus* (Eustigmatophyceae, Heterocontophyta) was cultivated in a standard medium and under nitrogen- and phosphorus-starvation and its triacylglycerols were analyzed by RP-HPLC/MS-APCI. The molecular species of triacylglycerols included a total of 74 triacylglycerols having at least one polyunsaturated fatty acid. Polyunsaturated triacylglycerols were identified for the first time in a yellow-green alga. N-starvation brought about a nearly 50% drop in TAGs containing EPA, and also decreased TAGs containing ARA, while P-starvation had a sizable effect on those TAGs that contain two or three arachidonic acids. In four TAGs containing PUFA, i.e. EEE, EEA, EAA and AAA, N-starvation caused a rapid fivefold increase in ARA content and the ratio of TAGs containing ARA, i.e. AEE to AAA increased tenfold relative to control. Regioisomeric characterization of triacylglycerols containing palmitic, arachidonic (ARA) and eicosapentaenoic acids (EPA) showed that the proportion of positional isomers is affected by N- and P-starvation. N- and P-starvation also changed the ratio of symmetrical to asymmetrical TAGs. Positional isomers exhibited identical ratios of symmetrical and asymmetrical TAGs irrespective of the type of FAs. In control cultivation the major TAGs with a single PUFA were symmetrical ones (PEP or PAP) whose ratio to asymmetrical counterparts (PPE or PPA) was about 3:1, whereas N- and P-starvation yielded opposite ratios, 1:3–1:5. The control cultivation yielded ~90% asymmetrical TAGs with two PUFAs (i.e. PEE and PAA), whereas with N- and P-starvation the ratio of symmetrical to asymmetrical TAGs increased to 2:1 and 3:2, respectively.

1. Introduction

The analysis of the triacylglycerol (TAGs) composition of an oil or fat is a very challenging task because it can contain an enormous number of individual TAG species due to the large number of FA (fatty acid) combinations on the glycerol backbone. For example, oil containing seven different FAs may contain the theoretical number of 126 molecular species excluding optical isomers, or 56 molecular species excluding positional isomers. Simple plant oils contain 5–10 different FAs (Lisa and Holcapek, 2008) while fish oils contain more than 30 different FAs (Mondello et al., 2006) and oil from algae as dietary oil for fish has an intermediate number of FAs, see e.g. Guiheneuf et al. (2009), Hu et al. (2008) and Khozin-Goldberg et al. (2002). The analysis is further complicated by the thermal and oxygen lability of PUFAs (polyunsaturated fatty acids) found in fish and algal oils. Therefore, it is best to use predominantly chromatographic methods, which can separate molecular species of TAGs by RP-HPLC and identify them by soft ionizations MS, as is done with oils containing PUFAs (Byrdwell, 2005). For example, typical plant oils (e.g. sunflower oil) contain around 50 TAGs (Lisa et al., 2009), while thousands of molecular species can be identified in menhaden oil (Francois et al., 2010).

Fish do not synthesise the PUFAs *de novo*, but they derive these compounds from their food composed of marine microorganisms (Cardozo et al., 2007). The fish oil quality depends on fish species and globally available fish resources, which are declining. Moreover, fish tend to accumulate poisonous substances via the food chain, and new sources of PUFAs are therefore required. Thus, microalgae producing all different PUFAs in high amounts might represent an acceptable alternative. In particular there is an increasing interest in EPA (all-Z-eicosa-5,8,11,14,17-pentaenoic acid), which is found in a wide variety of microalgae. Algal oils containing TAGs from different algae under different conditions of cultivation have been widely studied (Cohen, 1994; Wen and Chen, 2003; Yu et al., 2009). However, only a few microalgal species show potential for industrial production, e.g. the red alga *Porphyridium cruentum* and the eustigmatophyte *Nannochloropsis oculata*

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(Cohen, 1999; Sukenik, 1999). In addition, an efficient large scale cultivation system and purification process is required to produce microalgal EPA for commercial purposes (Lebeau and Robert, 2003). Many detailed investigations on growth behavior and PUFA production in several suitable microalgae showed (Guschina and Harwood, 2006) that PUFA production and accumulation in microalgae are complex procedures and difficult to optimize.

The presence of high levels of the long-chain ω-3 fatty acids, arachidonic (ARA), EPA and docosahexaenoic (DHA) acids, is identified as one of the major benefits of algae, which are essential to organisms in the upper tiers of food chain (e.g. humans). The uses and applications of these algal oils, i.e. TAGs with PUFAs, are largely dictated by the structure of these TAGs, especially the position of FAs on glycerol backbone. It was described that the positioning of FAs on the backbone of TAG molecules could affect many physical and nutritional properties, oxidative stability, lipid absorption, metabolism and atherogenesis (Cubow, 1996; Cossignani et al., 1999). The beneficial health effects of TAG-constituting PUFAs depend on their amounts in the diet and also on their positions within the TAGs (Krichevsky, 1998; Hunter, 2001; Stanley, 2003). FAs from the *sn*-1 and *sn*-3 positions are released by pancreatic lipase, while FAs at the *sn*-2 position of the glycerol are much better absorbed; e.g. infants absorb better the *sn*-2 position-bound palmitic acid from human milk than the FA from vegetable oils esterified to the *sn*-1,3 positions (Quinlan and Moore, 1993).

The unicellular yellow-green alga *Trachydiscus minutus* (Bourrelly) Ettl is supposed to be a member of Eustigmatophyceae, however, its precise phylogenetic position within the phylum Heterocontophyta (Stramenopila) has to be further investigated by DNA sequence analyses (Adl et al., 2005). The class Eustigmatophyceae contains more than 20 species in 9 genera predominantly freshwater species, although a substantial number is terrestrial. Only a few species are marine. They vary from single-celled flagellates to simple colonial and filamentous forms (Van den Hoek et al., 1996).

The objective of this work is to maximize the production of EPA (about 40% of total lipids, Table 1) in *T. minutus* through manipulation of relevant physiological parameters. In order to enhance the batch PUFA production, the effects of macronutrients, i.e. nitrogen and phosphorus ratios on algal production of fatty acid and mainly for TAGs were investigated (Lukavsky et al., 2010; Rezanka et al., 2010a). Based on our preceding experience with the analysis of uncommon TAGs by RP-HPLC/MS-APCI (Lisa et al., 2007; Rezanka, 1993; Rezanka et al., 2010b; Schreiberova et al., 2010) the acyl lipid composition, i.e. molecular species of TAGs, were also examined by modern analytical methods. We performed the analysis of the algal oil, TAGs containing PUFAs and also six TAGs obtained by organic synthesis. We also characterized regioisomeric TAGs containing palmitic, arachidonic and eicosapentaenoic acids and the proportion of these isomers was found to depend on N- and P-starvation of the alga.

2. Results and discussion

Fig. 1 shows the RP-HPLC with APCI detection of total ion current (from 200 to 1000 Da) of algal TAGs. More than 80 molecular species of TAG were identified; many of them are highly unsaturated with high carbon numbers, as found by using RP-HPLC data from previous analyses (Rezanka et al., 2010b; Schreiberova et al., 2010). Although the major peaks were separated on the baseline, the separation of some peaks was insufficient. From the mass spectra it was suggested that several peaks contained two or more overlapping species, see also the analysis of similar TAGs containing PUFAs (Hori et al., 1993, 1994). GC–MS analysis of the methyl esters obtained after hydrolysis confirmed that some peaks contained overlapping species and yielded the following data for the seven major fatty acids (Table 1).

A guideline for preliminary identification, later confirmed by mass spectra, was the common rule for the elution order of TAGs having the same ECN (equivalent carbon number, ECN = ACN-2xDB (ACN = acyl carbon number; DB = number of double bond(s))), according to which TAGs with more double bond are eluted first (Nikolova-Damyanova, 1997; Mu and Hoy, 2000). When identical molecular weight can be attributed to several structures, e.g. 898 Da, the rule applies that retention is longer for the TAG containing a saturated fatty acid, e.g. LnLE elutes before EEP (Dermaux et al., 1999).

Identification of TAGs containing PUFAs requires LC-MS analysis. For instance, Myher et al. (1996) separated more than 40 molecular species of TAGs from three different sources of PUFAs (marine fish of the genus *Thunnus*, microalga *Crypthecodinium cohnii* and fungus *Mortierella alpina*), but because they used RP-HPLC with evaporative light-scattering detection the peaks could not be identified, or were eluted at different times, though with the same detector (Perona et al., 1998; Perona and Ruiz-Gutierrez, 1999). When the identification of TAGs was done by APCI, the elution of all peaks except the pair ALL and LnLL, described Mu and Hoy (2000), was the same as in our analysis.

The positive ion APCI mass spectra of TAG consisting of PUFA species typically exhibited the $[M+H]^+$ ion and fragment ions of the type $[M+H-RCOOH]^+$ (also called $[DAG]^+$ ions), originating from the loss of one acyl moiety. Further loss of another acyl moiety leads to the monoacylglycerol fragment ions ($[MAG]^+$ is equivalent to $[RCO+74]^+$ ion). The positive ion APCI mass spectrum of EEE shows fragment ions such as $[RCO]^+$ at *m/z* 285, $[RCO+74]^+$ at *m/z* 359 and $[M+H-RCOOH]^+$ at *m/z* 643 were observed with sufficient intensity to analyze the mass spectrum. Increase in the degree of unsaturation of the TAGs was accompanied by an increase in the intensity of protonated molecular ion species $[M+H]^+$ relative to the intensities of the intensities of $[RCO]^+$, $[RCO+74]^+$ and $[M+H-RCOOH]^+$ (Holcapek et al., 1999, 2003; Lisa et al., 2007), see also our mass spectra in Figs. 2 and 3.

Table 1
Fatty acid compositions (%) of *T. minutus* at different mode of cultivation as determined by GC–MS.

FA	Abbreviations	Control	N-starvation	P-starvation	Sig. level[b]
Myristic	M	26.6 ± 1.25[a]	28.1 ± 1.02	24.8 ± 1.12	0.0709545
Palmitic	P	11.6 ± 0.96	9.2 ± 0.43	8.7 ± 0.31	0.0080149
Palmitoleic	Po	11.7 ± 0.91	8.4 ± 0.37	9.1 ± 0.47	0.0044083
Linoleic	L	6.7 ± 0.63	14.2 ± 1.12	8.9 ± 0.39	0.0001871
α-Linolenic	Ln	5.9 ± 0.52	10.1 ± 0.95	3.5 ± 0.19	0.0001357
Arachidonic	A[c]	1.7 ± 0.13	8.1 ± 0.64	5.2 ± 0.24	0.0000122
Eicosapentaenoic	E[d]	35.8 ± 1.68	21.9 ± 0.72	39.8 ± 1.87	0.0000518

[a] Results are given as mean ± S.D. for three separate determinations.
[b] ANOVA (analysis of variance), F test; all FAs except myristic acid exhibited significant differences between the values measured for individual cultivations at 1% significance level.
[c] Abbreviations ARA are given in the text.
[d] abbreviations EPA are given in the text.

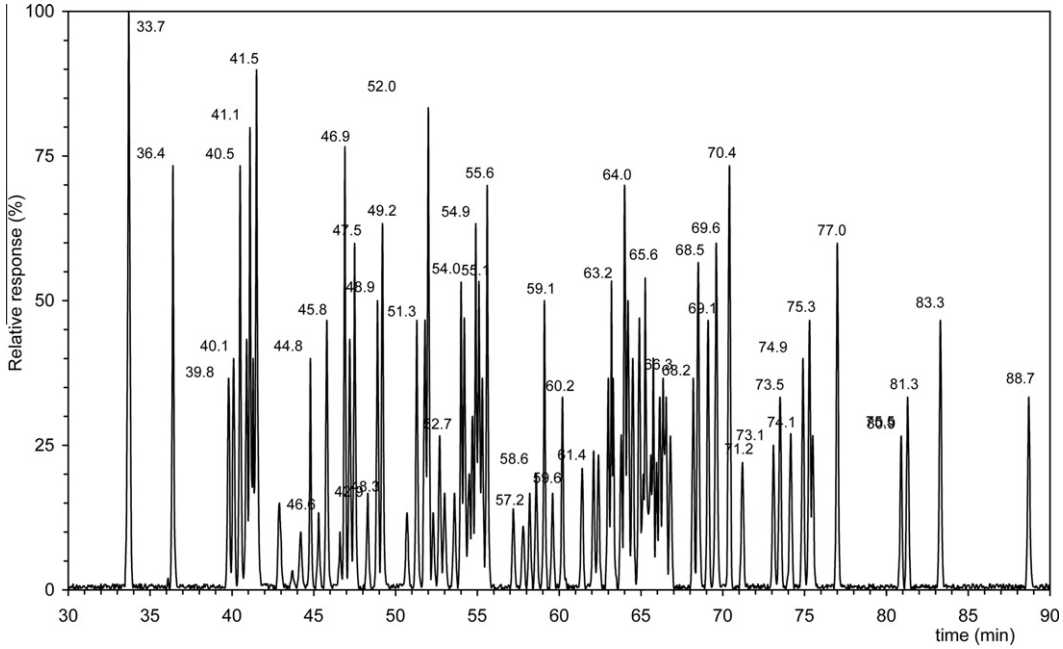

Fig. 1. RP-HPLC/APCI-MS chromatogram of the TAGs from the alga *Trachydiscus minutus*. Numerals above peaks show the retention time of appropriate peak in minutes.

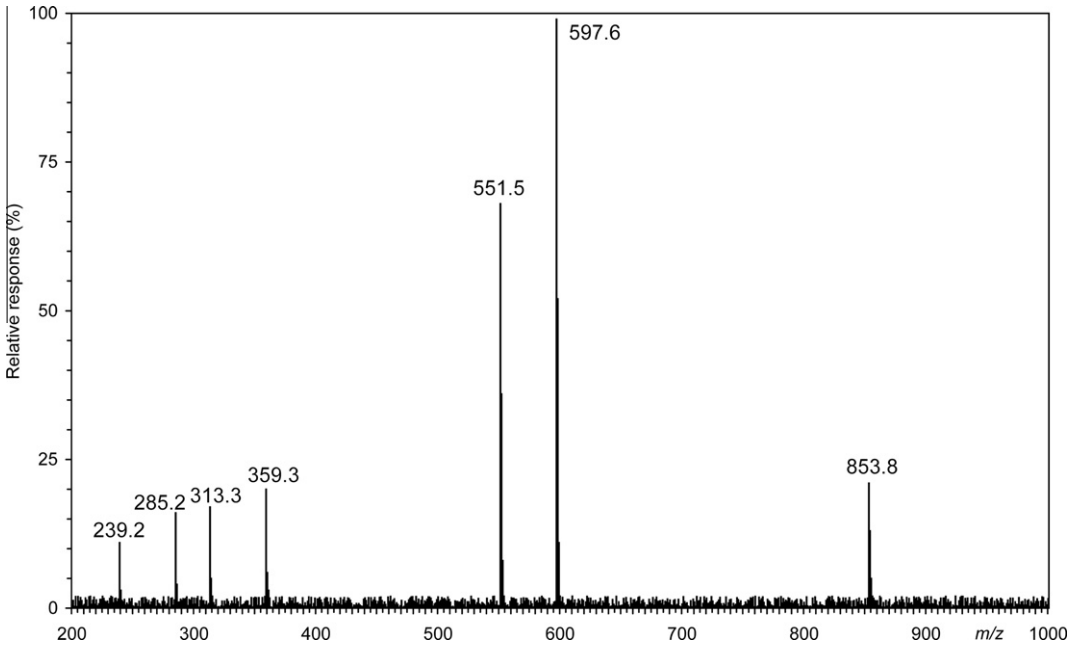

Fig. 2. Mass spectrum (APCI) of natural PPE.

The APCI mass spectrum of peak at RT 51.3 min represents ALnL, as indicated by the following ions: $[M+H–RCOOH]^+$ at *m/z* 621 (ALn), *m/z* 597 (LnL) and *m/z* 623 (AL); $[M+H]^+$ at *m/z* 901; RCO^+ at *m/z* 261 (Ln), *m/z* 263 (L) and *m/z* 287 (A). Although it is possible to determine the molecular species of TAGs from other peaks by the above method, e.g. peaks in the range of 51.8–56.0 min, the identification is very difficult. This is best illustrated on the peak with the RT interval of 51.8–52.0 min, in which the front of this peak contains ELL with $[M+H]^+$ at *m/z* 901 and the structure was determined based on the following ions: $[M+H–RCOOH]^+$ at *m/z* 621 (EL) and *m/z* 599 (LL). In contrast, the TAG of the latter part of this peak contained AEP with $[M+H]^+$ at *m/z* 901, which was determined from the following ions: $[M+H–RCOOH]^+$ at *m/z* 599 (AP), *m/z* 597 (EP) and *m/z* 645 (EA).

To facilitate the identification of molecular species, which can be poorly separated by chromatography despite the use of two RP-HPLC columns in series with a total of 52,000 theoretical plates/500 mm, we used the method employing selected ion monitoring (SIM). The interval from 51.5 to 52.5 min is shown as an example. SIM was measured for the following values: *m/z* 571 $[LnPo]^+$, 597 $[EP; APo]^+$, 599 $[AP; LL]^+$, 621 $[EL; ALn]^+$, and 645 $[EA]^+$ (see Fig. 4), including total ion current (TIC). The figure clearly shows that the SIM method has a practical use and, in combination with MS/MS (see below), enables a correct identification

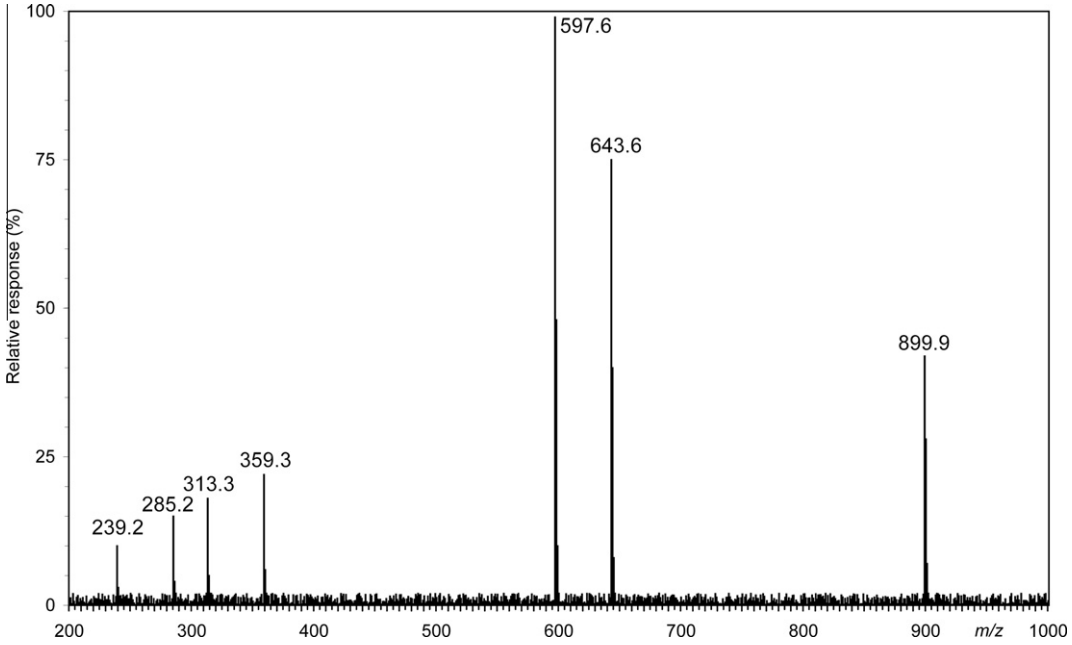

Fig. 3. Mass spectrum (APCI) of natural EEP.

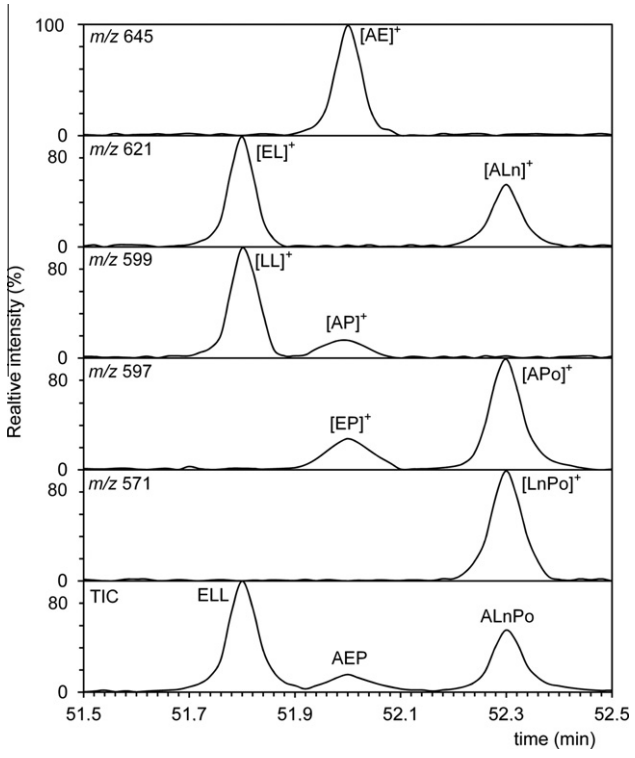

Fig. 4. Selected ion monitoring in the interval from 51.5 to 52.5 min. SIM was measured for the following values: m/z 571 $[LnPo]^+$, 597 [EP; APo]$^+$, 599 [AP; LL]$^+$, 621 [EL; ALn]$^+$, and 645 $[EA]^+$.

of all molecular species including those whose separation by RP-HPLC was not successful. MS/MS can be used to identify even such TAGs that have the same molecular weight but obtain different FAs, see ELL and AEP.

The other peaks shown in Fig. 1 were similarly identified by MS/MS. For instance, the incompletely separated peaks with RT from 65.1 to 65.7 min were identified based on SIM and MS/MS as a total of 7 molecular species having ECN = 42 and the following TAG structures: LLL, APL, PoLL, LnPL, APoP, EPP and PoPoL.

Separation of the four positional isomers that we have synthesized (*sn*-PPE, *sn*-PEP, *sn*-EEP and *sn*-EPE) was performed under the same conditions as the analysis of algal oil. In agreement with literature data we succeeded in separating all four positional isomers in less than 70 min. In all studies published so far on the subject (Momchilova et al., 2006; Gotoh et al., 2011) the symmetrical positional isomer had always a shorter retention time than its asymmetric counterpart, which was confirmed also in this study. This fact corresponds also to the observation of some authors (Momchilova et al., 2004), who separated *sn*-PEP and *sn*-PPE 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. They 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.

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 (Byrdwell, 2005; Mottram, 2005); however, the intensity of this ion can be affected by many factors, the most important being the number and positions of the bond(s) (Lisa et al., 2007, 2009). The mass spectra show the differences between two regioisomeric dublets, i.e. EEP–EPE and PPE–PEP, respectively. The mass spectra exhibited $[M+H]^+$ ions and DAG fragment ions $[M+H-RCOOH]^+$, which were formed depending on the positional distribution of FAs in TAG as reported previously for 18:3($n-3$)/18:2/18:2 and 18:3($n-6$)/18:2/18:2 (Leskinen et al., 2008). All four TAGs, i.e. EEP–EPE and PPE–PEP, showed the following DAGs ratios: the ratio of intensities of DAGs for EEP, i.e. $[EE]^+/[EP]^+$, was 75:100, the $[EE]^+/[EP]^+$ ratio for EPE was 32:100, $[PP]^+/[EP]^+$ ratio for PPE was 68:100 and the $[PP]^+/[EP]^+$ ratio for PEP was 23:100. Holcapek et al. (2010) 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 (Byrdwell, 2005; Mottram, 2005), viz. that the ratio of intensities of $[AA]^+/[AB]^+$ in AAB

type TAGs (asymmetrical TAGs) is ⅔:1, whereas in ABA (symmetrical TAGs) the ratio of intensities of DAG ions $[AA]^+/[AB]^+$ is ⅓:1.

Not even the recent study (Francois et al., 2010), which identified hundreds of TAGs from fish oil, succeeded in identifying all of them despite the use of two-dimensional LC, i.e. supercritical fluid chromatography with silver-ion and octadecyl silica with APCI identification. The authors assume that as much as thousands of molecular species can be identified in menhaden fish oil, which contains more than 100 FAs.

Although there still remain some difficulties in the quantitative analysis of various TG molecular species, in that the intensities of their fragment ions are unclear (the yields of fragment ions were found to vary with the degree of unsaturation), it was concluded that RP-HPLC/APCI-MS is a useful method for the composition analysis of complex natural oils containing PUFAs.

One of the most common methods for increasing the production of TAGs in algae is nutrient limitation in the medium, especially in nitrogen and phosphorus. As repeatedly described, nitrogen and phosphorus starvation induces an accumulation and shifts in the quality of lipids, chiefly TAGs, in many microalgae irrespective of their taxonomical classification.

In the green microalga *Parietochloris incisa* (Trebouxiophyceae), nitrogen starvation brings about a strong increase in the arachidonic acid production (Solovchenko et al., 2008). However, the increase in ARA is not accompanied by changes in the content of EPA (Khozin-Goldberg et al., 2002).

In keeping with previous data, the proportion of EPA in the red microalga *Porphyridium cruentum* decreases during N starvation while the proportion of ARA increases (Cohen, 1990).

Controversial data relative to a haptophyte alga were obtained with the marine phytoflagellate *Pavlova lutheri* (Haptophyceae) (Carvalho et al., 2006). Neither P nor N starvation had any significant effect on the production of EPA, yet at high phosphorus concentration (0.05 g/L), the production of EPA in the culture was higher (of the order of mg/L) with increasing nitrogen concentration.

A different pattern was found in the class Eustigmatophyceae. In the microalga *Monodus subterraneus* (previously Xanthophyceae), the content of both ARA and EPA declines during both N and P starvation (Cohen, 1994; Khozin-Goldberg and Cohen, 2006). The starvation of the eustigmatophyte *Nannochloropsis* sp. for both N and P also reduces the level of ARA as well as EPA (Hu and Gao, 2006).

The production of total FAs, EPA or ARA in algal lipids or TAGs as dependent on nutrient starvation has been widely studied, albeit with variable results (see above). By contrast, the composition of TAGs, i.e. identification of molecular species of TAGs in algae, has hardly been looked into. As opposed to tens of plant oils or some fish oils, in which such identification of TAGs has been performed (Lisa and Holcapek, 2008; Francois et al., 2010), only two papers have so far dealt with the identification of algal molecular species of TAGs (Yongmanitchai and Ward, 1993; Yu et al., 2009). In the former study the diatom *Phaeodactylum tricornutum* was found to contain 18 TAGs; their lipase-catalyzed cleavage after HPLC separation showed that, except for C16 PUFAs, PUFAs were preferentially bound in positions 1 or 3 on glycerol backbone. The latter study of the same species identified more than 30 molecular species of TAGs by using ESI-MS/MS. No data were given on their regiospecificity and, moreover, the cultivation of the diatom was performed under nitrate-limited conditions but no data on the analysis of TAGs from control cultivation were presented.

Table 2 gives 82 molecular species of TAGs from *T. minutus*. The major constituents (74 molecular species) are polyunsaturated TAGs; the remaining TAGs containing saturated and monounsaturated FAs. The lack of PUFA TAG standards is a limiting factor for quantitation. The table illustrates also the effect of N- and P-starvation. In keeping with the values for FAs in Table 1, N-starvation brought about a nearly 50% drop in TAGs containing EPA and increased TAGs with 2 or 3 ARA and palmitic acid, while P-starvation had a sizable effect only on those TAGs that contain two or three arachidonic acids. These differences cannot be statistically evaluated since no commercial standards of PUFA TAGs are available and the TAGs thus cannot be significantly quantified. Unlike other publications on plant and fish oils, we identified TAGs containing only one PUFA, such as EEE, AAA or LnLnLn. This is understandable because, for example, the content of EPA is more than 30% of total FAs. What are the different molecular species of TAGs containing PUFAs is illustrated by the molecular species of TAGs containing only EPA and ARA. If we compare the four molecular species, i.e. EEE, EEA, EAA and AAA, N-starvation caused a rapid increase in ARA content (from 1.7% to 8.1%, see Table 1). The ratio of TAGs containing ARA, i.e. AEE to AAA, also increased (from 0.7 to 7) relative to control, see Table 2.

This contrasts with TAGs containing ARA and palmitic acid, whose proportion more than doubled during N-starvation, P-starvation having again a small effect on the proportion of these TAGs.

We succeeded in separating and identifying positional isomers of TAGs containing palmitic acid, ARA and EPA. When using four synthesized standards (see Section 4) we showed that the ratio of symmetrical to asymmetrical TAGs varies depending on cultivation conditions, i.e. on N- and P-starvation (Table 3). These three cultivations differed markedly in the percentages of positional isomers of TAGs. Interestingly, the proportions of EPA and ARA, and thereby of appropriate TAGs, varied in an opposite manner, whereas positional isomers exhibited identical ratios of symmetrical and asymmetrical TAGs irrespective of the type of FAs (Table 3). In control cultivation the major TAGs with a single PUFA were asymmetrical (PPE or PPA) whose ratio to symmetrical ones (PEP or PAP) was about 3:1, whereas N- and P-starvation yielded opposite ratios, 1:3–1:5. We explained this effect as being due to the fact that during N and P starvations also the PtdCho pool becomes insufficient (see the Kennedy pathway) (Snyder et al., 2009; Coleman and Lee, 2004) so that one of the biosynthetic pathways that results in polyunsaturated TAGs is thus probably disabled or at least limited.

It should be noted that the current knowledge of the pathways (e.g. the Kennedy pathway) and enzymes involved in TAG synthesis in algae is very limited (Hu et al., 2008). The acylation of *sn*-glycerol-3-phosphate (PtdOH) is catalyzed by acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase. The second acylation is catalyzed by acyl-CoA:*lyso*-phosphatidic acid acyltransferase. The third acyl-CoA-dependent acylation is catalyzed by acyl-CoA:diacylglycerol acyltransferase or phospholipid:diacylglycerol acyltransferase, which uses phospholipids as acyl donors. Phospholipid:diacylglycerol acyltransferase which catalyzes the acyl-CoA-independent synthesis of TAG using PtdCho as an acyl donor and *sn*-1,2-DAG as an acyl acceptor, has also been described. This reaction may be useful in transferring unusual fatty acids from PtdCho into TAG (Zhang et al., 2009).

The biosynthesis is complicated by the substrate specificity of individual transferases in terms of the unsaturation of the substrate FAs (Zhu et al., 2009). The same holds for another two transferases – the acyl-CoA specificity of microsomal LPAAT activity (Sorensen et al., 2005). Controversial results were obtained with the specificity for 18:1, which exceeded that for 22:1 (Bernerth and Frentzen, 1990), while the opposite was found by Cao and Huang (1987).

All these facts document that, as mentioned above, the biosynthesis of molecular species or positional isomers is an exceedingly complex process and only fragments of the relevant biosynthetic pathways are currently known.

Table 2
Relative quantities (%) of TAGs identified in *T. minutus* at different mode of cultivation, their retention times, acyl carbon number, number of double bonds, and equivalent chain number.

Peak No.	RT	TG[a]	ACN	DB	ECN	Control%[b,c]	-N%	-P%
1	33.7	E-E-E	60	15	30	4.0 ± 0.18	2.9 ± 0.14	4.3 ± 0.21
2	36.4	A-E-E	60	14	32	2.0 ± 0.10	1.4 ± 0.07	2.4 ± 0.12
3	39.8	Ln-E-E	58	13	32	2.4 ± 0.11	1.5 ± 0.08	2.3 ± 0.11
4	40.1	A-A-E	60	13	34	1.1 ± 0.06	1.3 ± 0.07	1.4 ± 0.07
5	40.5	A-Ln-E	58	12	34	1.2 ± 0.06	1.1 ± 0.06	1.3 ± 0.07
6	40.9	E-E-L	58	12	34	2.3 ± 0.11	1.6 ± 0.08	2.5 ± 0.13
7	41.1	Po-E-E	56	11	34	2.3 ± 0.12	1.5 ± 0.08	2.5 ± 0.12
8	41.3	Ln-Ln-E	56	11	34	1.4 ± 0.08	1.2 ± 0.06	1.3 ± 0.07
9	41.5	M-E-E	54	10	34	2.8 ± 0.13	2.3 ± 0.11	3.0 ± 0.14
10	42.9	A-A-A	60	12	36	0.1 ± 0.02	0.7 ± 0.04	0.4 ± 0.02
11	43.7	A-A-Ln	58	11	36	0.3 ± 0.02	0.7 ± 0.04	0.4 ± 0.02
12	44.2	A-E-L	58	11	36	1.3 ± 0.07	1.2 ± 0.06	1.5 ± 0.08
13	45.3	A-Ln-Ln	56	10	36	0.4 ± 0.02	0.8 ± 0.04	0.3 ± 0.02
14	45.8	A-Po-E	56	10	36	1.4 ± 0.07	1.1 ± 0.05	1.5 ± 0.07
15	46.6	Ln-E-L	56	10	36	1.4 ± 0.08	1.3 ± 0.06	1.5 ± 0.07
16	46.9	E-E-P	56	10	36	2.3 ± 0.12	1.5 ± 0.08	2.5 ± 0.12
17	47.2	Ln-Ln-Ln	54	9	36	0.5 ± 0.03	0.8 ± 0.04	0.3 ± 0.02
18	47.5	Ln-Po-E	54	9	36	1.5 ± 0.08	1.1 ± 0.06	1.5 ± 0.08
19	48.3	A-M-E	54	9	36	1.8 ± 0.09	1.6 ± 0.09	2.0 ± 0.11
20	48.9	Ln-M-E	52	8	36	1.9 ± 0.10	1.7 ± 0.08	2.0 ± 0.10
21	49.2	A-A-L	58	10	38	0.3 ± 0.02	0.8 ± 0.04	0.5 ± 0.03
22	50.7	A-A-Po	56	9	38	0.4 ± 0.02	0.7 ± 0.04	0.5 ± 0.03
23	51.3	A-Ln-L	56	9	38	0.4 ± 0.03	0.9 ± 0.05	0.5 ± 0.03
24	51.8	E-L-L	56	9	38	1.4 ± 0.08	1.4 ± 0.07	1.6 ± 0.08
25	52.0	A-E-P	56	9	38	1.4 ± 0.07	1.1 ± 0.06	1.5 ± 0.08
26	52.3	A-Ln-Po	54	8	38	0.5 ± 0.03	0.7 ± 0.04	0.5 ± 0.03
27	52.7	Ln-Ln-L	54	8	38	0.5 ± 0.03	1.0 ± 0.05	0.4 ± 0.02
28	53.0	Po-E-L	54	8	38	1.5 ± 0.08	1.2 ± 0.06	1.6 ± 0.08
29	53.6	A-A-M	54	8	38	0.8 ± 0.04	1.2 ± 0.06	1.1 ± 0.05
30	54.0	Ln-E-P	54	8	38	1.5 ± 0.08	1.1 ± 0.05	1.4 ± 0.07
31	54.2	Ln-Ln-Po	52	7	38	0.7 ± 0.04	0.8 ± 0.04	0.4 ± 0.02
32	54.5	Po-Po-E	52	7	38	1.6 ± 0.08	1.1 ± 0.06	1.6 ± 0.08
33	54.7	A-Ln-M	52	7	38	1.0 ± 0.06	1.3 ± 0.07	1.0 ± 0.05
34	54.9	M-E-L	52	7	38	1.9 ± 0.09	1.8 ± 0.09	2.1 ± 0.10
35	55.1	Ln-Ln-M	50	6	38	1.1 ± 0.06	1.3 ± 0.07	1.0 ± 0.05
36	55.3	M-Po-E	50	6	38	2.1 ± 0.10	1.6 ± 0.08	2.1 ± 0.11
37	55.6	M-M-E	48	5	38	2.5 ± 0.12	2.2 ± 0.11	2.7 ± 0.13
38	57.2	A-L-L	56	8	40	0.4 ± 0.02	1.0 ± 0.06	0.6 ± 0.04
39	57.8	A-A-P	56	8	40	0.4 ± 0.03	0.7 ± 0.04	0.5 ± 0.03
40	58.2	Ln-L-L	54	7	40	0.5 ± 0.03	1.1 ± 0.06	0.6 ± 0.04
41	58.6	A-Po-L	54	7	40	0.6 ± 0.04	0.9 ± 0.05	0.6 ± 0.03
42	59.1	A-Ln-P	54	7	40	0.5 ± 0.03	0.8 ± 0.04	0.5 ± 0.03
43	59.6	E-P-L	54	7	40	1.5 ± 0.08	1.3 ± 0.07	1.6 ± 0.08
44	60.2	A-Po-Po	52	6	40	0.7 ± 0.05	0.7 ± 0.04	0.7 ± 0.04
45	61.4	Ln-Po-L	52	6	40	0.7 ± 0.04	0.9 ± 0.05	0.6 ± 0.03
46	62.1	Ln-Ln-P	52	6	40	0.7 ± 0.04	0.8 ± 0.04	0.4 ± 0.03
47	62.4	A-M-L	52	6	40	1.0 ± 0.05	1.4 ± 0.08	1.2 ± 0.06
48	63.0	Po-E-P	52	6	40	1.6 ± 0.09	1.1 ± 0.06	1.6 ± 0.08
49	63.2	Ln-Po-Po	50	5	40	0.8 ± 0.04	0.7 ± 0.04	0.5 ± 0.03
50	63.3	A-M-Po	50	5	40	1.1 ± 0.06	1.2 ± 0.06	1.2 ± 0.07
51	63.8	Ln-M-L	50	5	40	1.1 ± 0.05	1.5 ± 0.08	1.1 ± 0.06
52	64.0	M-E-P	50	5	40	2.1 ± 0.11	1.6 ± 0.09	2.2 ± 0.13
53	64.2	Ln-M-Po	48	4	40	1.2 ± 0.06	1.3 ± 0.07	1.1 ± 0.06
54	64.5	A-M-M	48	4	40	1.5 ± 0.08	1.8 ± 0.09	1.7 ± 0.09
55	64.9	Ln-M-M	46	3	40	1.6 ± 0.09	1.8 ± 0.10	1.4 ± 0.08
56	65.1	L-L-L	54	6	42	0.6 ± 0.04	1.2 ± 0.07	0.7 ± 0.04
57	65.2	A-P-L	54	6	42	0.6 ± 0.03	0.9 ± 0.05	0.6 ± 0.02
58	65.3	Po-L-L	52	5	42	0.7 ± 0.04	1.0 ± 0.05	0.5 ± 0.02
59	65.4	Ln-P-L	52	5	42	0.7 ± 0.04	0.9 ± 0.05	0.6 ± 0.04
60	65.5	A-Po-P	52	5	42	0.7 ± 0.03	0.7 ± 0.04	0.6 ± 0.03
61	65.6	E-P-P	52	5	42	1.6 ± 0.09	1.1 ± 0.06	1.6 ± 0.09
62	65.8	Po-Po-L	50	4	42	0.8 ± 0.04	0.9 ± 0.05	0.7 ± 0.04
63	65.9	Ln-Po-P	50	4	42	0.8 ± 0.04	0.8 ± 0.04	0.6 ± 0.03
64	66.1	M-L-L	50	4	42	1.2 ± 0.06	1.6 ± 0.09	1.3 ± 0.06
65	66.3	A-M-P	50	4	42	1.1 ± 0.05	1.3 ± 0.06	1.2 ± 0.06
67	66.8	M-Po-L	48	3	42	1.3 ± 0.06	1.4 ± 0.07	1.3 ± 0.07
68	68.2	Ln-M-P	48	3	42	1.2 ± 0.07	1.3 ± 0.07	1.1 ± 0.06
69	68.5	M-Po-Po	46	2	42	1.4 ± 0.07	1.2 ± 0.06	1.3 ± 0.06
70	69.1	M-M-L	46	2	42	1.7 ± 0.08	2.0 ± 0.08	1.6 ± 0.09
71	69.6	M-M-Po	44	1	42	1.8 ± 0.08	1.8 ± 0.09	1.8 ± 0.09
72	70.4	M-M-M	42	0	42	2.1 ± 0.09	2.3 ± 0.11	1.7 ± 0.08
73	71.2	P-L-L	52	4	44	0.6 ± 0.04	1.0 ± 0.05	0.7 ± 0.04
74	73.1	A-P-P	52	4	44	0.7 ± 0.04	0.7 ± 0.04	0.6 ± 0.04

(*continued on next page*)

Table 2 (*continued*)

Peak No.	RT	TG[a]	ACN	DB	ECN	Control%[b,c]	-N%	-P%
75	73.5	Po-P-L	50	3	44	0.9 ± 0.05	0.9 ± 0.05	0.7 ± 0.04
76	74.1	Ln-P-P	50	3	44	0.8 ± 0.03	0.8 ± 0.04	0.6 ± 0.03
77	74.9	Po-Po-P	48	2	44	1.0 ± 0.04	0.7 ± 0.04	0.5 ± 0.02
78	75.3	M-P-L	48	2	44	1.2 ± 0.04	1.4 ± 0.08	1.3 ± 0.07
79	75.5	M-Po-P	46	1	44	1.5 ± 0.06	1.3 ± 0.07	1.1 ± 0.06
80	77.0	M-M-P	44	0	44	1.8 ± 0.07	1.8 ± 0.08	1.4 ± 0.07
81	80.9	P-P-L	50	2	46	0.8 ± 0.05	0.9 ± 0.04	0.7 ± 0.04
82	81.3	Po-P-P	48	1	46	1.0 ± 0.06	0.7 ± 0.04	0.7 ± 0.03
83	83.2	M-P-P	46	0	46	1.4 ± 0.07	1.3 ± 0.06	1.0 ± 0.05

[a] The TAGs do not indicate any regiospecificity.
[b] Results are given as mean ± S.D. for three separate determinations.
[c] Quantitation was based on the $[M+H]^+$ ion.

Table 3
Ratios of TAG positional isomers consisted of EPA, ARA and palmitic acid in the alga *T. minutus*.

TAG ratio	Control	-N	-P
PEP/PPE	1:3	3:1	5:1
PAP/PPA	1:3	3:1	4:1
EPE/PEE	1:10	2:1	3:2
APA/PAA	1:10	2:1	3:2

Table 4
^{13}C NMR signals of regioisomeŕs TAGs.

TAG	α C=O	β C=O	α′ C=O
PPE	173.3	172.5	172.9
PPE	173.3	172.5	173.3
EPE	173.4	173.0	173.7
EEP	173.4	173.3	173.4

In the case of TAG with two PUFAs the control cultivation yielded ~90% asymmetrical TAGs (i.e. PEE and PAA), whereas with N- and P-starvation the ratios of symmetrical to asymmetrical TAGs increased to 2:1 or 3:2, respectively. At present, this finding defies explanation.

However, a similar disproportion has already been found in nature. Only one paper describes the stereospecific analysis of a natural (not model) mixture of TAGs containing EPA (Gotoh et al., 2011). Unfortunately, the polyunsaturated TAGs were isolated from marine fishes and mammals. The PEP to PPE ratio in fish, which are nearer to algae in the food chain, was 1:4 do 2:1 while in marine mammals feeding on fish it was not symmetrical since PEP was not identified at all. A completely different situation was described in TAGs with two PUFAs (i.e. EPE and EEP); here the ratio in the mammals was 4:1 while in fish it was 1:10–1:4.

3. Conclusion

Using modern analytical techniques, such as RP-HPLC/MS-APCI along with tandem MS or SIM, enabled us to identify 82 molecular species of TAGs in the alga *T. minutus*. The vast majority of them (74) include at least one PUFA. Using GC–MS of methyl esters, we found that the content of EPA and ARA is dependent on N- and P-starvation. A similar effect was observed for TAGs analyzed by LC–MS. It was found that the ratio of the four molecular species, i.e. EEE, EEA, EAA and AAA, during N-starvation rapidly increases.

However, the most important finding seems to be that N- and P-starvation has a major impact on regiostereospecifity of TAGs, i.e. the biosynthesis of positional isomers, whether symmetrical (EPE, PEP, APA, PAP), or asymmetrical. This fact can be used to prepare targeted stereoisomers of TAGs containing polyunsaturated fatty acids.

4. Experimental

4.1. Standards and instrumentation

Acetonitrile, 2-propanol, Mg turnings, THF, hexane, dichloromethane, glycerol, 4-dimethylaminopyridine (DMAP) and EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), DL-α-palmitin (1-palmitin), 1,3-dipalmitin (1,3-dipalmitoyl-glycerol), 1,2-dipalmitin (1,2-dipalmitoyl-rac-glycerol) and cis-5,8,11,14,17-eicosapentaenoic acid were purchased from Sigma–Aldrich (Prague, CR). High resolution MS spectra were recorded using a VG 7070E-HF spectrometer (70 eV). HR-FAB-MS (positive ion mode) were obtained with a PEG-400 matrix. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C).

4.2. Preparation of mixed FA composition TAGs

The esterification of glycerol derivatives with acids was described previously (Ziegler and Berger, 1979; Haraldsson et al., 2000; Halldorsson et al., 2003). Briefly, the general procedure for the synthesis of TAGs containing one EPA and two palmitic acids was as follows: under an inert atmosphere (argon), a solution of 1,3-palmitoylglycerol(1,2-palmitoylglycerol) (57 mg, 0.1 mmol) and EPA (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). 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 a yellowish oil. The yield of the target TAGs was (46.9 mg, i.e. 55% for PPE and 50.3 mg, i.e. 59% for PEP, respectively). NMR spectra of PPE:

^1H NMR (500.1 MHz, $CDCl_3$, δ, ppm) 5.44–5.33 (m, 10H, =CH), 5.30–5.22 (m, 1H, $CH_2CH(O-)CH_2$), 4.29 (dd, J = 11.8, 4.3 Hz, 2H, $-OCH_2CHCH_2O-$), 4.14 (dd, J = 11.9, 5.9 Hz, 2H, $-OCH_2CHCH_2O-$), 2.88–2.75 (m, 8H, $=CCH_2C=$), 2.36 (t, J = 7.6 Hz, 2H, CH_2COO in EPA), 2.30 (t, J = 7.6 Hz, 4H, CH_2COO in P), 2.15–2.01 (m, 4H, $=CHCH_2CH_2$ and $CH_3CH_2CH=$), 1.75–1.63 (m, 2H, CH_2CH_2COO in EPA), 1.66–1.53 (m, 4H, CH_2CH_2COO in P), 1.40–1.17 (m, 48H, CH_2), 0.97 (t, J = 7.5 Hz, 3H, CH_3 in EPA) and 0.88 (t, J = 6.8 Hz, 6H, CH_3 in P). ^{13}C NMR (125.7 MHz, $CDCl_3$, δ, ppm) 173.3 (1C, OC=O in *sn*-1 P), 172.9 (1C, OC=O in *sn*-3 EPA), 172.5 (1C, OC=O in *sn*-2 P), 131.9–126.9 (10C, =CH), 69.3 (1C, $CH_2CH(O-)CH_2$), 62.6 (1C, EPA–CH_2CH), 62.5 (1C, $CHCH_2$-P), 34.4 (1C, CH_2COO in *sn*-2 P), 34.2 (1C, CH_2COO in *sn*-1/3 P), 33.8 (1C, CH_2COO in *sn*-1/3 EPA), 32.3 (2C, $CH_2CH_2CH_3$ in P), 30.1–29.5 (20C, CH_2), 27.6–

26.0 (5C, CH_2CH=), 25.3 (2C, CH_2CH_2COO in *sn*-1 and *sn*-2 P), 25.1 (1C, CH_2CH_2COO in *sn*-3 EPA), 23.1 (2C, CH_2CH_3 in P), 23.0 (1C, CH_2CH_3 in EPA), 14.5 (2C, CH_3 in P), 14.4 (1C, CH_3 in EPA). IR ν_{max} 3020 (s, C–H), 2920 (vs C–H), 2850 (vs C–H), 1735 (vs C=O) cm^{-1}. HREIMS (*m/z*): 852.7210 $[M]^+$, calc. for $[C_{55}H_{96}O_6]^+$ 852.7207.

The general procedure for the synthesis of TAGs containing two EPA and one palmitic acid was as follows: under an inert atmosphere, a solution of 1-palmitoylglycerol (2-palmitoylglycerol) (33 mg, 0.1 mmol) and EPA (66.4 mg, 0.22 mmol) as a free acid in dichloromethane (1.0 mL) was added to DMAP (9.8 mg, 80 μmol) and EDCI (37.2 mg, 0.24 mmol) in dichloromethane (1.0 mL). For further procedure see above. The yield of the target TAGs was 52.1 mg, i.e. 58% for PEE and 54.8 mg, i.e. 61% for EPE, respectively.

The ^1H 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 (Haraldsson et al., 2000; Halldorsson et al., 2003; Fauconnot et al., 2006), see Table 4.

4.3. Cultivation

The alga *T. minutus* (strain Lukavsky et Pribyl 2005/1) was obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany ASCR, Treboň, Czech Republic. The experimental cultures were inoculated with cells that had been grown in full Zehnder liquid medium (Staub, 1961) at room temperature and irradiance of 100 μmol m^{-2} s^{-1} (final dry weight 0.5 mg L^{-1}). The initial dry weight of cultures was 0.027 mg L^{-1}. To assess the influence of N and P starvation, full Zehnder liquid medium, medium without N and medium without P were used. Batch cultures were maintained in 2 L glass flasks at 27 °C under continuous light of 160 μmol m^{-2} s^{-1} (cool-white tubes, Osram). The cultures were mixed three times a day and the supply of CO_2 was provided only by diffusion. After 17 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. Cells were centrifuged and lyophilized, and the samples were stored in aluminum foil until solvent extraction.

4.4. 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 2-propanol does not serve as a substrate for phospholipases (Kates, 1986). The alcohol-water mixture was cooled and one part chloroform was added and the lipids from the lyophilized cells of different cultivations of *T. minutus* 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.

First, total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35 cc (Waters; with 10 g of silica normal-phase), and TAGs were subsequently eluted from the cartridge with 40 mL of chloroform–methanol (9:1). The eluate was evaporated and the oil samples were dissolved in an acetonitrile-2-propanol–hexane mixture (1:1:1, v/v/v), which was injected for HPLC analysis.

4.5. FAMEs analysis

The 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 CH_2N_2. GC–MS of fatty acid methyl ester (FAME) mixture was done on a Finnigan 1020 B in EI mode. 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. All spectra were scanned within the range of *m/z* 50–500. The structures of FAMEs were confirmed by comparison of retention times and fragmentation patterns with those of the standard FAMEs (Supelco, Prague).

4.6. 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 (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 5 to 120 min ACN/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.

Statistically significant differences between treatments and the control were computed by ANOVA (analysis of variance) and F test by using the IBM SPSS Statistics version 19 software (IBM, USA).

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