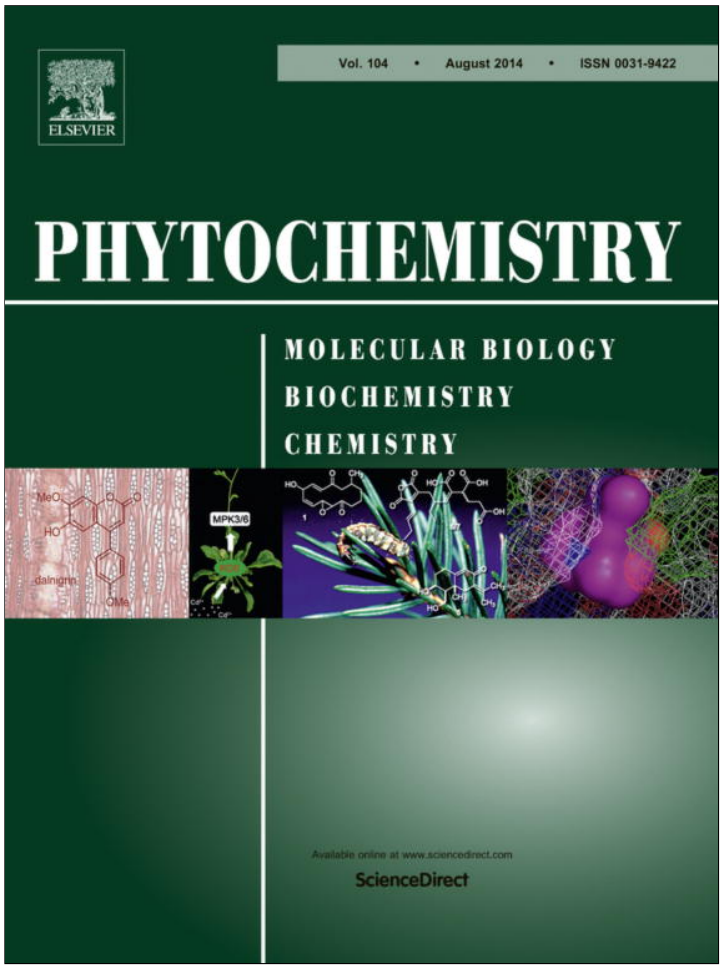

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Production of structured triacylglycerols from microalgae

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ABSTRACT

Structured triacylglycerols (TAGs) were isolated from nine cultivated strains of microalgae belonging to different taxonomic groups, i.e. *Audouinella eugena*, *Balbiania investiens*, *Myrmecia bisecta*, *Nannochloropsis limnetica*, *Palmodictyon varium*, *Phaeodactylum tricornutum*, *Pseudochantransia* sp., *Thorea ramosissima*, and *Trachydiscus minutus*. They were separated and isolated by means of NARP-LC/MS-APCI and chiral LC and the positional isomers and enantiomers of TAGs with two polyunsaturated, i.e. arachidonic (A) and eicosapentaenoic (E) acids and one saturated, i.e. palmitic acid (P) were identified. Algae that produce eicosapentaenoic acid were found to biosynthesize more asymmetrical TAGs, i.e. PPE or PEE, whereas algae which produced arachidonic acid give rise to symmetrical TAGs, i.e. PAP or APA, irrespective of their taxonomical classification. Nitrogen and phosphorus starvation consistently reversed the ratio of asymmetrical and symmetrical TAGs.

1. Introduction

The nutritional value of *n*−3 polyunsaturated fatty acids (PUFAs) found in algae has attracted increasing attention (Khozin-Goldberg et al., 2011). Polyunsaturated fatty acids have beneficial effects on human health; one of the best known of these acids is eicosapentaenoic acid (E, 20:5*n*3) which has been used for years in the prevention of atherosclerosis and thrombosis (Simopoulos, 1991). Arachidonic acid (A, 20:4*n*6) does not belong to essential fatty acids but some mammals lack the ability, or possess it in a very limited degree, to biosynthesize arachidonic acid from linoleic acid. Since the amount of arachidonic acid in the diet is negligible, it is in fact an essential part of their diets (MacDonald et al., 1984). Arachidonic acid plays an important part in cellular signaling as a lipid messenger in the regulation of signaling enzymes or as a precursor of eicosanoids (especially leucotrienes, prostaglandins, prostacyclins and thromboxanes) (Piomelli, 1993).

The position of a fatty acid (FA) in the molecule of triacylglycerols (TAGs) has been described to affect many nutritional properties, oxidative stability, absorption and metabolism in the organism, as well as atherogenesis (Cubow, 1996; Cossignani et al., 1999; Mu and Porsgaard, 2005). FA bound in positions *sn*-1 and *sn*-3 are better hydrolyzed by pancreatic lipase, whereas FA in position *sn*-2 of the glycerol are much better absorbed in the form of monoacylglycerols; thus children absorb better palmitic acid bound in position *sn*-2 and contained in maternal milk than the same FA from plant oils that is bound in positions *sn*-1 or *sn*-3 (Quinlan and Moore, 1993). The best nutritional properties were found with TAGs having PUFAs in position *sn*-2, especially arachidonic and eicosapentaenoic acids; these TAGs are better absorbed than TAGs with the same FA composition in which the FA are accidentally distributed. An interest is currently on the rise in the preparation of structured triacylglycerols (STAGs) containing saturated and short chain FAs in positions *sn*-1 and *sn*-3 and long chain PUFAs in position *sn*-2. It is worth noting that these structured TAGs can protect the organism against hypertriglyceridemia and obesity caused by high dietary fat (Takeuchi et al., 2002). Structured TAGs could be potentially used for inducing weight loss and lower fat accumulation, and for serum cholesterol lowering (Kunesova et al., 2006). The simplest and most direct way to synthesize these STAGs is acidolysis catalyzed by specific *sn*-1, *sn*-3 lipases (Hita et al., 2007). However, the process involves side reactions, e.g. a mutual migration of acyls which reduces the yield of the STAGs. To circumvent this problem, the reaction is performed in two steps. The first step is enzymatic hydrolysis yielding 2-MAGs (monoacylglycerols), which are then esterified in positions *sn*-1 and *sn*-3 by specific lipases. The weak point of the process is the deactivation of lipases caused by ethanol or the ensuing glycerol and an increased stability of esters on primary hydroxyl, i.e. in positions *sn*-1 and *sn*-3 (Soumanou et al., 1998).

STAGs are most often prepared from fish oils (cod liver, tuna, anchovy/sardine or bonito oils). Unfortunately, these oils are a

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hardly suitable material because fish are positioned nearly at the end of the food chain (see, e.g., Gotoh et al., 2011). Two processes of STAG preparation have been described: in a two-step process, 2-MAGs are produced from TAGs with the aid of a lipase and their reesterification by a different lipase gives rise to STAGs (Irimescu et al., 2001; Halldorsson et al., 2003; Munio et al., 2009; Suarez et al., 2010). Some steps ensuring purification of the intermediates have been added to provide a four-step process, which produces STAGs of a higher purity (Robles et al., 2011). All currently used methods of preparation of STAGs are time- and effort-intensive and the experiments with using STAGs as dietary supplements or even as the only source of dietary lipids have therefore been conducted only to a limited extent since the amount of available STAGs is low.

Natural TAGs are known to exist in a large number of different molecular species. For instance, the number of possible molecular species for TAG with a mere five different fatty acids is 75 without optical isomers, or 125 if the enantiomers are included, see Table 1. Since the biosynthesis of TAGs involves three acyl transferases (Coleman and Douglas, 2004), each of which esterifies only one of the three hydroxyls of glycerol, the resulting combinations are not random. The distribution of positional isomers, but not enantiomers, was the subject of many studies (e.g. Lisa and Holcapek, 2008; Lisa et al., 2011), which were however performed on commonly available animal and plant oils but not on algal oils.

An increase in the number of the fatty acids present in the TAGs results in an increasing number of molecular species, which may reach astronomical values. Thus for 20 fatty acids, which are approximately present in most microbial and algal oils (Khozin-Goldberg et al., 2002; Hu et al., 2008; Guiheneuf et al., 2009; Lang et al., 2011), the formula in Table 1 gives the total number of 8000. So far, nobody has succeeded in identifying more than hundreds of molecular species in one sample; see, e.g., Araujo et al. (2014) who identified 762 molecular species in fish oil.

Table 1
The number of possible TAGs.

Description	Number of possible TAGs
Without isomers	$x = (y^3 + 3y^2 + 2y)/6$
Without enantiomers	$x = (y^3 + y^2)/2$
All isomers	$x = y^3$

x is the number of TAGs, y is the number of FAs in TAGs.

Non-aqueous reversed-phase liquid chromatography (NARP-LC) makes use of a combined separation according to both the degree of unsaturation and the length of the acyl chain of the fatty acids. Though an excellent separation of TAGs by NARP-LC has been known for many years, many practical and theoretical aspects of the process are still not clear (Hellmuth et al., 2011). Analysis of regioisomers, i.e. TAG isomers in which the acyl chains are in different positions (e.g. AAB and/or ABA), or the double bonds in one or more acyl chains are in different positions (α- and γ-linolenic acids), is one of the most complicated tasks in TAG analysis.

APCI-MS (atmospheric-pressure chemical ionization mass spectrometry) or ESI (electrospray ionization), including the use of tandem mass spectrometry (MS^n) are well suited for the identification of the components and their quantification provided the calibration is carefully done (Byrdwell, 2005; Leskinen et al., 2010).

These techniques can be used in combination with liquid chromatography or with a direct injection of TAGs into the instrument, and they provide information on molecular species including a regiospecific but not stereospecific separation of fatty acids in *sn*-TAGs, which have so far been separated only rarely.

By using polysaccharide-based chiral LC and a chiral stationary phase, Iwasaki et al. (2001) separated without preceding derivatization enantiomers of TAGs with widely different acyl groups, e.g. 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol (e.g. *sn*-ECC or *sn*-CCE) (Nagai et al., 2011). Gotoh et al. (2011) used chiral LC for separating 1,2-dioleoyl-3-palmitoyl-*sn*-glycerol and 1-palmitoyl-2,3-dioleoyl-*sn*-glycerol (*sn*-POO and or *sn*-OOP) from palm oil.

Here we separated and isolated, by means of non-aqueous reversed-phase liquid chromatography/mass spectrometry atmospheric pressure chemical ionization (NARP-LC/MS-APCI) and

Table 2
Composition of fatty acids from TAGs of the nine algae in control cultivation (in% of total FAs) including their retention characteristics (ECL – equivalent chain lengths). *Aud eug* – *Audouinella eugena*, *Bal inv* – *Balbiania investiens*, *Pse* sp. – *Pseudochantransia* sp., *Tho ram* –*Thorea ramosissima*, *Myr bis* – *Myrmecia bisecta*, *Pal var* – *Palmodictyon varium*, *Nan lim* – *Nannochloropsis limnetica*, *Pha tri* – *Phaeodactylum tricornutum*, *Tra min* – *Trachydiscus minutus*.

FAME	ECL	Rhodophyta				Chlorophyta		Chromophyta		
		Aud eug	*Bal inv*	*Pse spe*	*Tho ram*	*Myr bis*	*Pal var*	*Nan lim*	*Pha tri*	*Tra min*
14:0	14.000	1.8	2.4	1.0	1.7	1.4	1.3	5.2	6.5	23.9
14:1ω5	14.397	0.2	0.1	0.2	0.5	0.0	0.3	0.4	0.0	0.0
15:0	15.000	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0
16:0	16.000	20.6	19.6	14.9	21.0	9.0	16.5	12.6	11.9	11.8
16:1ω9	16.191	3.7	3.1	1.8	2.2	3.1	0.9	0.0	26.7	8.5
16:1ω7	16.252	0.0	0.0	0.0	0.0	0.0	0.0	26.0	0.0	0.0
16:2ω4	16.844	0.3	0.0	0.0	0.0	0.0	0.0	0.9	5.5	0.0
17:0	17.000	0.1	0.0	0.1	0.0	0.0	0.1	0.2	0.0	0.2
16:3ω4	17.185	0.2	0.1	0.0	0.0	0.0	0.0	5.2	5.2	0.1
16:4ω1	17.749	0.2	0.1	0.0	0.0	0.0	0.0	0.8	1.1	0.0
18:0	18.000	0.4	0.7	4.9	2.4	1.2	0.8	0.3	0.5	0.3
18:1ω9	18.156	0.0	14.2	8.3	6.4	9.5	4.4	6.4	6.4	3.7
18:1ω7	18.228	12.8	10.8	2.3	0.5	7.9	0.0	3.1	3.1	1.1
18:2ω6	18.630	0.0	0.0	2.8	5.9	8.8	7.3	3.3	1.4	5.4
18:3ω6	18.941	0.0	0.0	1.7	0.8	1.1	1.7	0.7	0.9	0.5
18:3ω3	19.278	0.0	0.0	4.8	3.3	2.7	3.1	0.6	0.6	4.3
18:4ω3	19.588	6.7	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0
20:3ω6	20.861	0.0	0.0	4.1	0.0	0.0	0.0	0.0	0.0	0.7
20:4ω6	21.090	49.9	45.4	45.7	49.8	52.1	59.7	4.8	3.3	3.1
20:3ω3	21.263	0.7	0.2	1.4	0.2	0.0	1.0	0.4	0.4	0.6
20:4ω3	21.527	0.1	0.3	2.5	2.2	0.0	0.6	0.0	0.2	1.8
20:5ω3	21.751	1.7	2.8	3.5	3.1	3.2	2.3	27.5	22.4	33.9
22:4ω6	23.510	0.5	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0
22:5ω3	23.766	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.0
22:6ω3	24.043	0.0	0.0	0.0	0.0	0.0	0.0	0.8	1.8	0.1

chiral LC the positional isomers and enantiomers of TAGs with two polyunsaturated, i.e. arachidonic and eicosapentaenoic acids and one saturated, i.e. palmitic acid. Algae that produce eicosapentaenoic acid were found to biosynthesize more asymmetrical TAGs, i.e. PPE or PEE, whereas algae which produced arachidonic acid gave rise to symmetrical TAGs, i.e. PAP or APA, irrespective of their taxonomical classification. The effect of nitrogen and phosphorus starvation on the ratios of positional isomers and enantiomers of TAGs was also tested.

2. Results and discussion

2.1. Algal strains

Nine algal species producing arachidonic or eicosapentaenoic acid as one of the major FAs were analyzed by GC–MS. The contents of three major FAs, i.e. arachidonic, eicosapentaenoic, and palmitic acids, and also of minor FAs in TAGs are given in Table 2. All three algae overproducing eicosapentaenoic acid belong to Chromophyta, albeit to different classes – Eustigmatophyceae (*Nannochloropsis* and *Trachydiscus*) and Bacillariophyceae (*Phaeodactylum*). On the other hand, *Audouinella*, *Balbiania*, *Pseudochantransia* and *Thorea* (division Rhodophyta), *Myrmecia* and *Palmodictyon* (division Chlorophyta) are producers of arachidonic acid. As noted by Lang et al. (2011), out of the more than 2000 algal strains from the SAG culture collection (Culture Collection of Algae at Goettingen University) nearly 17% were found to contain arachidonic acid and more than 20% contained eicosapentaenoic acid.

2.2. Analysis of fatty acids and triacylglycerols

Table 1S (Supplements) and Fig. 1S give the amounts of almost 100 TAGs from the red alga *Thorea ramosissima*. Major peaks and a number of minor ones were separated without any problems. This

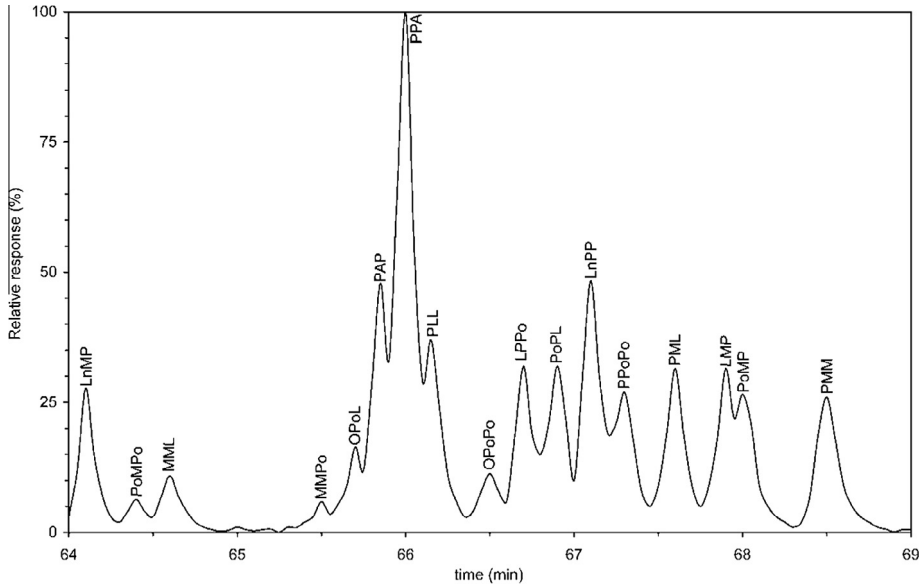

Fig. 1. Semipreparative RP-HPLC of APP and its separation from other TAGs (*Thorea ramosissima*).

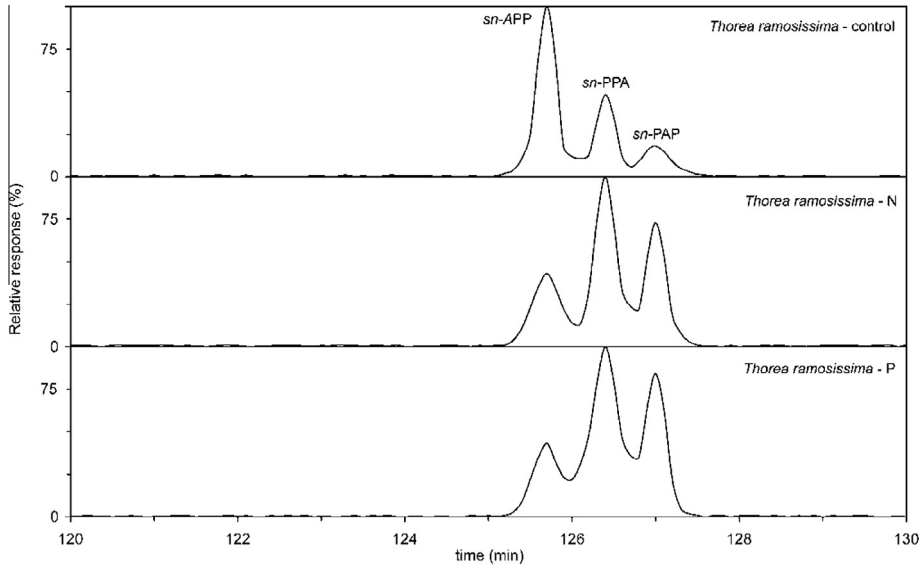

Fig. 2. Chiral separation of a natural mixture of *sn*-APP, *sn*-PPA, and *sn*-PAP from from the control and N- and P-starvation cultivation of *Thorea ramosissima*.

included also the molecular species explored in this study, i.e. TAGs containing eicosapentaenoic, arachidonic and palmitic acids. To our best knowledge, none of the algal species producing arachidonic acid (which can reach 50% total FAs) has so far been analyzed in terms of TAGs, and the comparison of the data on the production of TAGs by algae producing large amounts of eicosapentaenoic acid with our data on algae producing abundantly arachidonic acid is therefore virtually impossible.

Depending on the source of oil, PUFAs are known to be bound either in position *sn*-1 and *sn*-2 (Fig. 2Sa, for structural formula) or in positions *sn*-1 or *sn*-3 (Fig. 2Sb), in other words on primary carbon atoms or on the secondary carbon atom. This shows that natural oils and fats are characterized, in addition to the content of fatty acids, also by different stereochemistry of substituents. Several methods can be used to obtain oils and fats with a required stereochemistry of the substituent fatty acids. These include, e.g., trans-esterification by specific lipases (Quinlan and Moore, 1993) or a direct alteration of the biosynthesis of structured TAGs by changing the physiological conditions of the cultivation, as previously described with two algal species (Rezanka et al., 2011, 2012) producing eicosapentaenoic acid.

As described repeatedly in the literature (Mottram, 2005), determination of regioisomers does not present any particular problems. Briefly, based on the intensities of ions $[M+H-R_iCOOH]^+$, i.e. ions of the diacylglycerol type, one can determine if the TAG is a 1,2-isomer (2,3-isomer) or 1,3-isomer derived from the original TAGs (see, e.g., Mottram, 2005; Byrdwell, 2005).

Although the final phase of identification is done by mass spectrometry, it is in many cases convenient to use, prior to the final identification, also separation by silver-LC (Lisa et al., 2009) or NARP-LC (Lisa and Holcapek, 2008), especially in the case of complex mixtures of natural fats and oils.

A completely different situation is encountered when separating and identifying enantiomers – optical isomers of TAGs. If the TAGs are asymmetrical, i.e. when positions *sn*-1 and *sn*-3 of prochiral glycerol are esterified by different fatty acids, then these TAGs

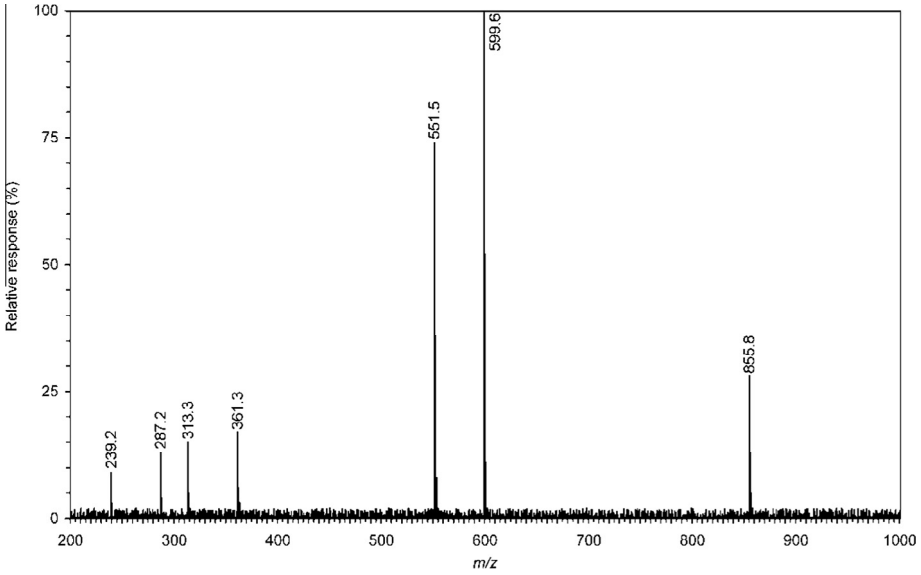

Fig. 3. Mass spectrum (APCI) of natural *sn*-PPA.

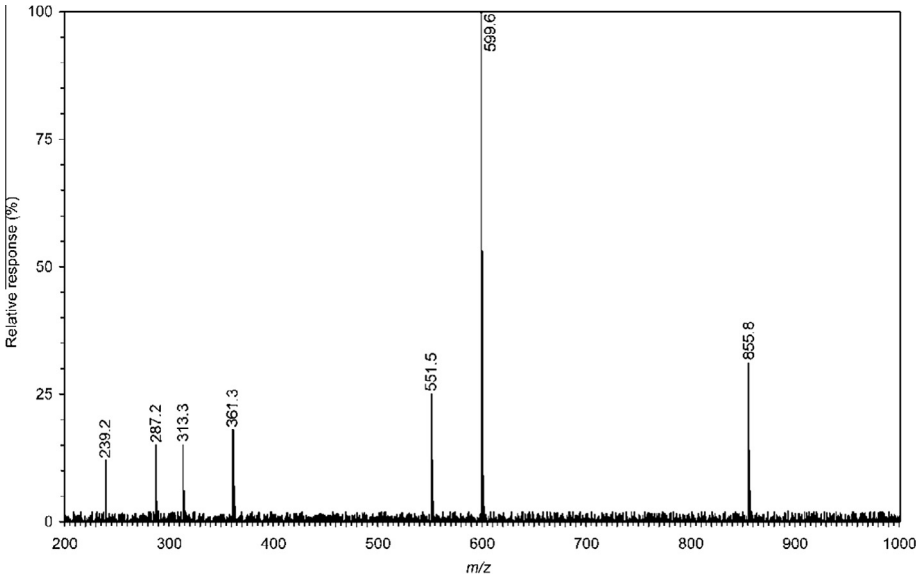

Fig. 4. Mass spectrum (APCI) of natural *sn*-PAP.

form enantiomers (R,S-isomers). Their separation is very difficult, as signified by the low number of studies dealing with this problem. Their identification is also complicated for the following reasons. Optical rotation, commonly used for determining the purity of enantiomers, cannot be used for TAGs since enantiomers give $[\alpha]_D$ only negligibly different from 0; thus, e.g., 1-oleoyl-2, 3-palmitoyl-*sn*-glycerol had $[\alpha]_D^{20} = 0.00$ (c 7.05, $CHCl_3$) (Stamatov and Stawinski, 2007). Gronowitz and Herslof (1979) measured the optical rotatory dispersion and circular dichroism curves of unsaturated TAGs and found that, for instance, at 300 nm 1,2-dilinoleyl-3-olein has a zero molecular rotation. Hence, at wavelengths shorter that the wavelength of sodium no optical rotation occurs and this method is thus not suitable for identification of individual enantiomers. Also, no pure enantiomers of TAGs are commercially available.

We therefore performed a successful synthesis of at least one enantiomer of TAGs containing eicosapentaenoic acid (Rezanka et al., 2011) and now also an enantiomer containing arachidonic acid (Figs. 2Sa and 2Sb). Briefly, phospholipase C was used to obtain, from commercially available phosphatidylcholine, one of the diacylglycerol enantiomers, which was then esterified to yield a corresponding TAG (for structural formula, see Fig. 2S). This enabled us to obtain regioisomers of TAGs having one or two PUFAs (eicosapentaenoic or arachidonic acids) and palmitic acid.

The mass spectra of both regioisomers (but not enantiomers), i.e. *sn*-PPA and *sn*-PAP, after semipreparative (Fig. 1) and chiral separation (Fig. 2) are shown in Figs. 3 and 4. All chromatograms and mass spectra were obtained from TAGs of the alga *T. ramosissima.* Both enantiomers, i.e. *sn*-APP and *sn*-PPA, have nearly identical mass spectra, as described by Lisa and Holcapek (2013) for 1-arachidin-2,3-olein and 1,2-olein-3-arachidin. APCI mass spectra confirm the purity of positional isomers, since both *rac*-PPA and *sn*-PPA form ions $[PP]^+$ and $[PA]^+$ at *m*/*z* 551 and *m*/*z* 599 with the same intensity. Addition of *sn*-PPA (internal standard) enhances the

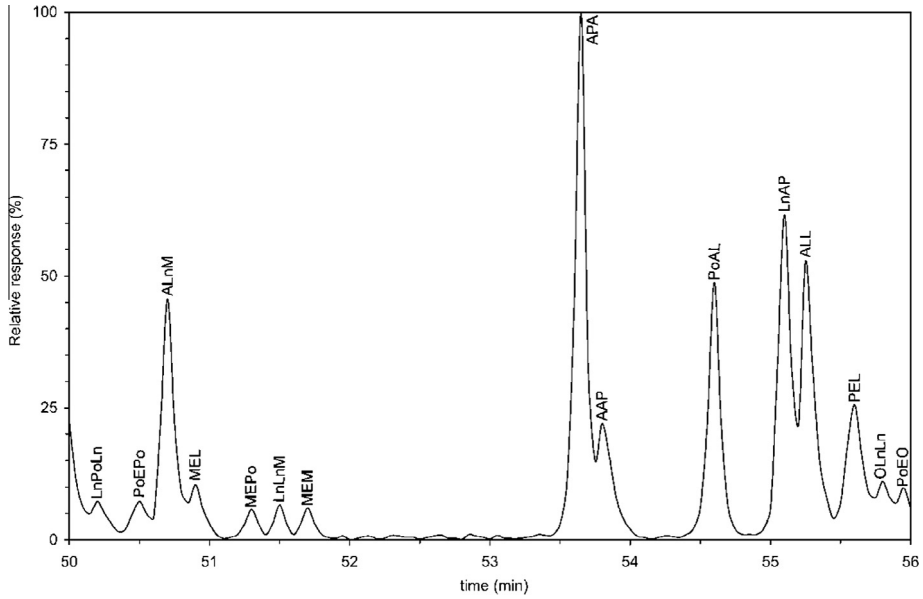

Fig. 5. Semipreparative RP-HPLC of AAP and its separation from other TAGs from *Thorea ramosissima.*

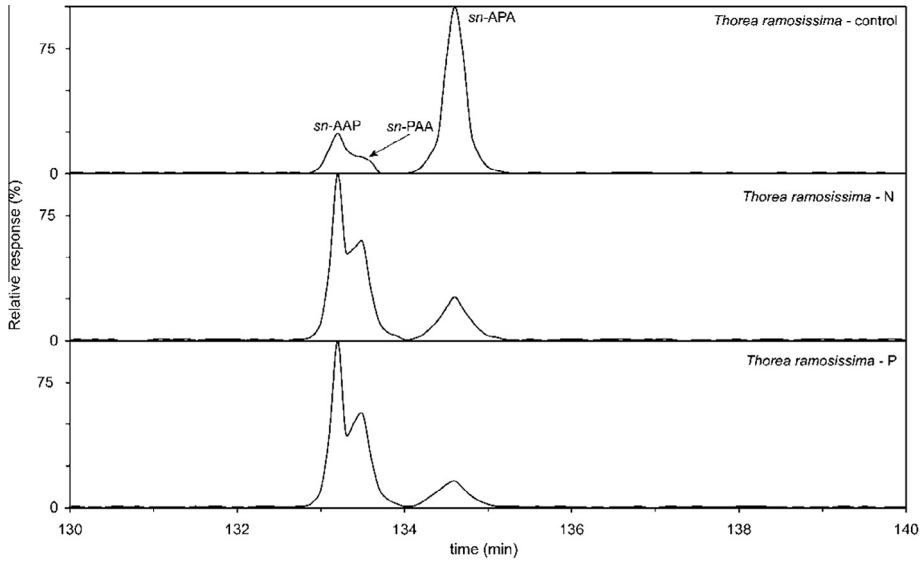

Fig. 6. Chiral separation of a natural mixture of *sn*-PAA, *sn*-AAP, and *sn*-APA from from the control and N- and P-starvation cultivation of *Thorea ramosissima.*

intensity of the later eluted peak with RT = 65.85 min; this confirms that the order of elution is APP–PPA, as described by Iwasaki et al. (2001) and Nagai et al. (2011) for similar enantiomers having two saturated and one unsaturated FA.

The same approach was used with another pair of regioisomers and enantiomers. Semipreparative separation of a natural mixture from the cultivation of *Thorea* under N- and P-starvation is shown in Fig. 5. As described by Iwasaki et al. (2001), individual enantiomers were again eluted in the order *sn*-AAP and *sn*-PAA. This was confirmed by chiral separation of a natural mixture of *sn*-PAA, *sn*-AAP and *sn*-APA from the control and N- and P-starvation cultivation of *T. ramosissima* (Fig. 6) and mass spectra of both symmetrical and asymmetrical TAGs, see Figs. 7 and 8. Our results thus show that *rac*-PPA and *rac*-PAA can be separated on a chiral column, TAGs having arachidonic acid in position *sn*-1 (*sn*-APP and *sn*-AAP) being eluted before TAGs having palmitic acid in position *sn*-1 (*sn*-PPA and *sn*-PAA). The importance of using at least one of the two enantiomers for identification has been documented in the study by Nagai et al. (2013) in which a mere change in substituents in the stationary phase, such as replacing methyl for chlorine, (3,5-dimethylphenyl carbamate versus 3-chlor-5-methylphenyl carbamate) reverses the order of elution of *sn*-PEE and *sn*-EEP and the first eluting TAG is then that having saturated acid in position *sn*-1.

Identification of enantiomeric TAGs, but not regioisomeric TAGs, or the fatty acyls in positions *sn*-1, *sn*-2 and *sn*-3, has so far been performed in a few studies conducted with plant oils such as sunflower (Boukhchina et al., 2003), peanut (Sempore and Bezard, 1991), evening primrose and borage oils (Redden et al., 1995). The content of one of the TAGs which is most commonly encountered in plants, dioleoyl-linoleoyl-glycerol, is given in Table 3.

2.3. Abundance of individual triacylglycerols isomers in algae

As seen from Table 3, all three isomers, i.e. *sn*-LLO, *sn*-OLL and *sn*-LOL, are always present, albeit in different proportions. This

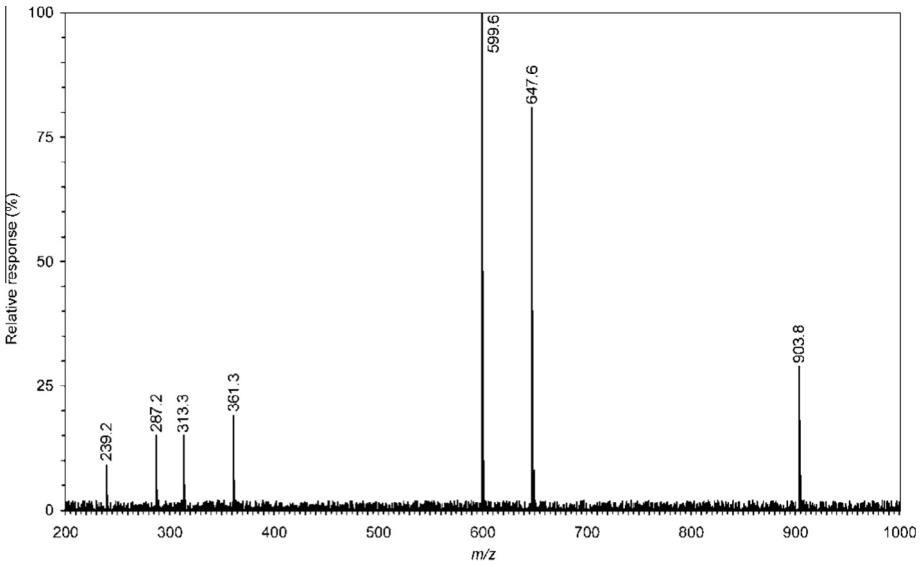

Fig. 7. Mass spectrum (APCI) of natural *sn*-AAP.

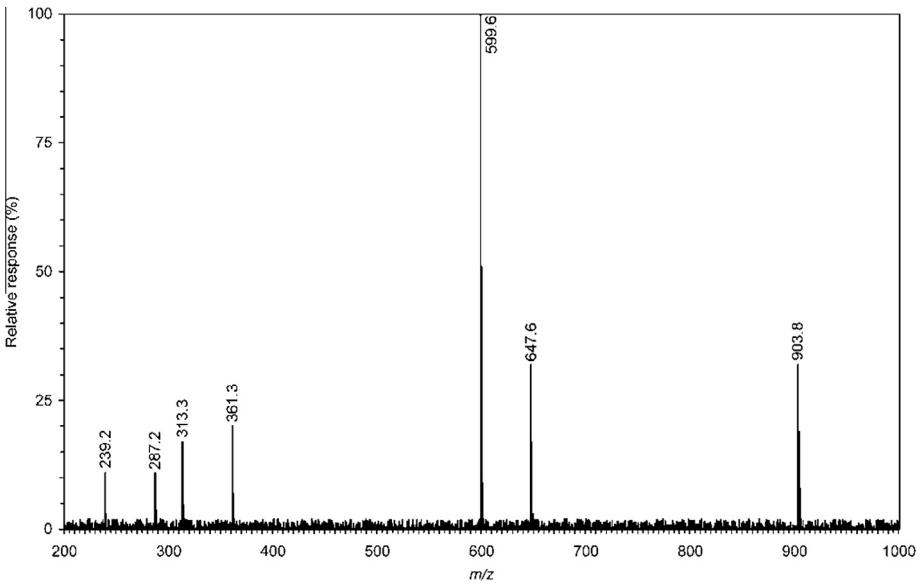

Fig. 8. Mass spectrum (APCI) of natural *sn*-APA.

Table 3
The ratios of 3 TAGs in different types of plant oils.

TAG in%	Sunflower[a]	Peanut[b]	Evening-primrose[c]	Borage[c]
sn-LLO	63	73	64	32
sn-OLL	24	15	15	27
sn-LOL	13	12	21	41

[a] Boukhchina et al. (2003).
[b] Sempore and Bezard (1991).
[c] Redden et al. (1995).

was confirmed also by Lisa and Holcapek (2013), who separated the TAGs of hazelnut oil directly by chiral chromatography, and identified all three dioleoyl-linoleoyl-glycerols. The biosynthetic aspects of this phenomenon have not yet been clarified. When stated in simple terms, TAGs are synthesized by 3 different acyltransferases of the Kennedy pathway. The specificity of these acyltransferases in the biosynthesis of TAGs determines the content and position of FAs in the resulting TAG. The synthesis of the TAG from glycerol-3-phosphate and FA is represented by a series of mutually linked reactions; glycerol-3-phosphate is first acylated by glycerol-3-phosphate acyltransferase to lyso-phosphatidic acid, which is acylated by lysophosphatidate acyltransferase, which uses acyl-CoAs as their substrates to form phosphatidic acid.

This acid is then hydrolyzed by phosphohydrolase to yield diacylglycerol, which, under the action of the third acyltransferase, is then transferred to TAG. However, it should be noted that the structure of the biosynthesized TAG depends on many other factors, especially on the availability of the appropriate acyl-CoA (i.e. on the biosynthesis of appropriate FA), on other modifications such as potential trans-esterifications of the resulting TAG, on the catabolism (specific degradation of some TAGs), etc. There are three principal reactions leading to the formation of all three isomers: (a) the previously published (Guldan et al., 2008) reduction of dihydroxyacetone phosphate to glycerol-1-phosphate, (b) intramolecular transesterification, or (c) the action of diacylglycerol transferase. The knowledge of individual biosynthetic pathways, appropriate enzymes and/or genes would then make it possible to manipulate the production of algal oil in order to achieve the best biological and nutritional properties.

All the data from preceding studies (Rezanka et al., 2011, 2012) and from the present measurements performed on the six arachidonic acid producing algal species and three eicosapentaenoic acid producing strain are summarized in Table 4.

All six algae producing arachidonic acid in amounts as high as 50% of total FAs produce also TAGs containing this acid and palmitic acid in very similar proportions (see Table 2). The ratio of symmetrical TAGs (APA or PAP) in control cultivations is always higher that the ratio of asymmetrical counterparts, i.e. two pairs of corresponding enantiomers (*sn*-AAP and *sn*-PAA, or *sn*-APP and *sn*-PPA). In the case of nitrogen and phosphorus limitation the ratios of symmetrical to asymmetrical TAGs are inverted but the starvation has no effect on the ratios of enantiomers. A completely different situation is observed in the alga *Trachydiscus*, the producer of both PUFAs – arachidonic and eicosapentaenoic acids. In terms of arachidonic acid, palmito-diarachidonoin shows the same pattern as in all the above six overproducers while the other TAG, dipalmito-arachidonoin, shows an inverted ratio of symmetric to asymmetric TAGs. Comparison of overproducers of eicosapentaenoic (Lang et al., 2011) and arachidonic acid shows their very low similarity. In fact, each of the three algae has unique and individual features in terms of the production of palmitic and eicosapentaenoic acids.

Table 4
The ratios of TAGs (in%) containing palmitic, arachidonic and eicosapentaenoic acids in control cultivation (C) under nitrogen (-N) and phosphorus (-P) starvation. For chiral separation of *sn*-EEP, *sn*-PEE, *sn*-EPE, *sn*-EPP, *sn*-PPE, and *sn*-PEP see Figs. 3S and 4S.

	sn-AAP	*sn*-PAA	*sn*-APA	**Σ asym**[#]	**Σ sym**	*sn*-APP	*sn*-PPA	*sn*-PAP	**Σ asym**	**Σ sym**
Audouinella eugena C	9	14	77	**23**	**77**	19	23	58	**42**	**58**
Audouinella eugena -N	24	28	48	**52**	**48**	39	43	18	**82**	**18**
Audouinella eugena -P	27	32	41	**59**	**41**	35	45	20	**80**	**20**
Balbiania investiens C	11	11	78	**22**	**78**	14	15	69	**31**	**69**
Balbiania investiens -N	28	37	35	**65**	**35**	42	52	6	**94**	**6**
Balbiania investiens -P	25	41	34	**66**	**34**	43	49	4	**96**	**4**
Myrmecia bisecta C	5	7	88	**12**	**88**	3	3	93	**7**	**93**
Myrmecia bisecta -N	24	32	44	**56**	**44**	28	31	41	**59**	**41**
Myrmecia bisecta -P	24	27	49	**51**	**49**	32	37	31	**69**	**31**
Palmodictyon varium C	19	23	58	**42**	**58**	21	26	53	**47**	**53**
Palmodictyon varium -N	36	38	26	**74**	**26**	34	42	24	**76**	**24**
Palmodictyon varium -P	37	41	22	**78**	**22**	36	38	26	**74**	**26**
Pseudochantransia sp. C	6	11	83	**17**	**83**	9	10	81	**19**	**81**
Pseudochantransia sp. -N	34	39	27	**73**	**27**	29	31	40	**60**	**40**
Pseudochantransia sp. -P	41	47	12	**88**	**12**	28	34	38	**62**	**38**
Thorea ramosissima C	7	24	69	**31**	**69**	11	29	60	**40**	**60**
Thorea ramosissima -N	32	54	14	**86**	**14**	34	46	20	**80**	**20**
Thorea ramosissima -P	33	58	9	**91**	**9**	37	44	19	**81**	**19**
Trachydiscus minutus C				**9**	**91**				**75**	**25**
Trachydiscus minutus -N				**66**	**34**				**25**	**75**
Trachydiscus minutus -P				**60**	**40**				**20**	**80**
	sn-EEP	sn-PEE	sn-EPE	**Σ asym**	**Σ sym**	sn-EPP	sn-PPE	sn-PEP	**Σ asym**	**Σ sym**
Nannochloropsis limnetica C	38	36	26	**74**	**26**	31	50	19	**81**	**19**
Nannochloropsis limnetica -N	22	23	55	**45**	**55**	12	9	79	**21**	**79**
Nannochloropsis limnetica -P	17	21	62	**38**	**62**	19	15	66	**34**	**66**
Phaeodactylum tricornutum C	57	29	14	**86**	**14**	41	34	25	**75**	**25**
Phaeodactylum tricornutum -N	23	9	68	**32**	**68**	23	10	67	**33**	**67**
Phaeodactylum tricornutum -P	6	16	78	**22**	**78**	5	10	85	**15**	**85**
Trachydiscus minutus C	41	50	9	**91**	**9**	37	38	25	**75**	**25**
Trachydiscus minutus -N	15	25	47	**40**	**60**	11	14	75	**25**	**75**
Trachydiscus minutus -P	13	20	67	**33**	**67**	6	10	84	**16**	**84**

[#] Bold values are the sums of enantiomers (e.g. *sn*-AAP plus *sn*-PAA or *sn*-APP plus *sn*-PPA, etc.).

This large disparity can be ascribed most of all to the considerable taxonomic distance between producers of eicosapentaenoic acid (3 algae) a arachidonic acid (6 algae), though, as described above, all these differences cannot be explained in this way. Thus for instance *Trachydiscus* and the six arachidonic acid producing algae have an identical course of palmito-diarachidonoin production. We therefore assume that, in addition to phylogenetical similarity or dissimilarity, an important action is exerted by the preferences of different acyltransferases for appropriate acyl-CoAs. However, it should be noted that comparison with other algae is still missing and more data are necessary to confirm the patterns observed in this study.

3. Conclusion

As already described for evening-primrose and borage oils (Redden et al., 1995), the different proportion of molecular species, i.e. different content of enantiomers and regioisomers, is likely to lead to substantial changes in the pharmacological properties and effects of algal oils. Oils with an identical content of FAs but a different structure of TAGs can have different biological properties that can play an important part in absorption, biological properties and distribution of FAs in tissue lipids of animals feeding on the algae. Substantial differences in positional isomers, i.e. enantiomers and regioisomers of individual molecular species of TAGs, can thus lead to different biological activities of algal oils prepared in cultivations performed under different physiological conditions. Our study thus can point out one of the possible ways to the production of structured TAGs that can be used for human and animal nutrition.

4. Experimental

4.1. Standards and instrumentation

Acetonitrile, 2-propanol, THF (tetrahydrofuran), hexane, dichloromethane, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 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).

1,2-Diarachidonoyl-*sn*-glycero-3-phosphatidylcholine was purchased from Avanti Polar Lipids, Inc., AL, USA.

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 red algae *Audouinella eugenea* (strain SAG 52.94 (Skuja) Jao), *Balbiania investiens* (strain SAG 50.93 (Lenormand) Sirodot), *Pseudochantransia* sp. (strain SAG 19.96 F. Brand), *T. ramosissima* (strain SAG 46.94 Bory de St. Vincent), the green algae *Myrmecia bisecta* (strain SAG 2043 Reisigl) and *Palmodictyon varium* (strain SAG 3.92 (Nägeli) Lemmerman), the eustigmatophycean algae *Nannochloropsis limnetica* (strain SAG 18.99 Krienitz, Hepperle, Stich et Weiler) and *Trachydiscus minutus* (strain CCALA 931 (P. Bourrelly) H.Ettl) and the diatom *Phaeodactylum tricornutum* (CCMP632 Coughlan) were obtained from the SAG Culture Collection of Algae (Göttingen, Germany), the Culture Collection of Autotrophic Organisms at the Institute of Botany, ASCR (Třeboň, Czech Republic) and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Maine, USA). Experimental cultures were inoculated with algae that had been grown in WC medium with vitamin B solution (Guillard and Lorenzen, 1972) (red algae), Zehnder medium (Staub, 1961) (green and eustigmatophycean algae) or Zehnder medium with the addition of NaCl (20 g L^{-1}) (*P. tricornutum*) at room temperature (20 °C) and under daylight conditions. Under these conditions the strains were precultivated for about 3 months. To assess the influence of nitrogen and phosphorus starvation, full media (see above) and their two modifications were used. In nutrient depleted media, nitrogen and phosphorus concentration were reduced to 0 mg L^{-1}. Batch cultures with high inoculum density were established and they were maintained in 100 mL Erlenmeyer glass flasks in temperature and light conditions mentioned above. The cultures were stirred three times a day and the supply of CO_2 was provided by diffusion from the air. After 10 days of cultivation, the biomass was harvested and lyophilized, and the samples were stored in aluminium foil until solvent extraction.

4.3. Enzymatic synthesis of 1,2-diarachidonoyl-sn-glycerol

1,2-Diarachidonoyl-*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), 2×10^{-3} M in 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-diarachidonoyl-*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 arachidonic 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*-PPA was 46.9 mg, i.e. 55%.

The general procedure for the synthesis of *sn*-AAP was as follows: under an inert atmosphere, a solution of 1,2-diarachidonoyl-*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*-AAP was 5.2 mg, i.e. 58%.

The ^{1}H NMR spectra of both TAGs, i.e. PPA and AAP 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. FAMEs analysis

The total TAGs (~5 mg) were saponified overnight in 10% KOH–MeOH at room temperature. A fatty acid fraction obtained from the saponification was partitioned between alkali solution (pH 9) and diethyl ether to remove basic and neutral components. The aqueous phase containing fatty acids was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using BF_3/MeOH.

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) and previously published papers (Rezanka, 1993; Rezanka and Podojil, 1984).

4.6. TAGs analysis by RP-HLPC/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 ID, 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, 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, vol/vol); linear from 5 min to 110 min ACN/iPrOH 30:70 (vol/vol); 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, Ontario, 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 200–1100. 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.

CID ions 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 Q2. Scanning range of Q3 was m/z 200–1100 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.

4.7. TAGs analysis by Chiral LC/MS-APCI

The LC system used for separation in the chiral mode was the same as that used in the reversed-phase mode. TAGs (~1 μg/μL in methanol) were chromatographed on two Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl 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% of during 180 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, 25 °C, and 10 μL, respectively. Other separation conditions used were as in the RP-LC, see above.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.04.013.

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