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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

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