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Temperature dependence of production of structured triacylglycerols in the alga *Trachydiscus minutus*

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ABSTRACT

This study describes the identification of regioisomers and enantiomers of triacylglycerols of C20 polyunsaturated fatty acids (PUFAs) in the alga *Trachydiscus minutus* cultivated at different temperatures using reversed- and chiral-phase liquid chromatography–mass spectrometry. The use of the two different phases contributes to ready identification, both qualitative and semiquantitative, of regioisomers and enantiomers of triacylglycerols containing eicosapentaenoic and arachidonic in the molecule. The ratio of regioisomers and enantiomers of triacylglycerols (TAG) depends on the temperature of cultivation; with lowering temperature the proportion of the achiral TAG increases and the enantiomer ratio diverges from 1:1.

1. Introduction

Unicellular alga *Trachydiscus minutus* (Bourr.) Ettl, strain Lukavský et Přibyl 2005/1 (former *Pseudostaurastrum minutum*) belongs to Eustigmatophyceae as described by Pribyl et al. (2012). It was isolated from cooling pools of the Temelín nuclear power plant (Czech Republic). It contains chlorophyll a, violaxanthin, vaucheriaxanthin ester, and β-carotene. No other chlorophylls were determined. It has a broad temperature adaptability with an optimum growth temperature at 25 °C (Gigova et al., 2012). The life cycle of *T. minutus* includes darkness induced zoosporogenesis (Pribyl et al., 2012).

T. minutus is an interesting organism from the biochemical point of view, because it contains high levels of polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) (Iliev et al., 2010; Rezanka et al., 2010; Lukavský et al., 2010). Currently it is being extensively studied for biotechnological purposes. Cultivation conditions play a key role in biomass production and PUFA composition (Alexandrov et al., 2014; Cepák et al., 2014).

The use of algae as an alternative and renewable source of lipid-rich biomass feedstock for oil production has been intensively explored over the past few decades (Bajpai and Bajpai, 1993; Cao et al., 2012). Many species have the ability to produce substantial amounts (e.g. 20–50% dry cell weight) of TAGs as a storage lipid (Hu et al., 2008). One-fifth of algal strains in the Culture Collection of Algae at Goettingen University (SAG) contain EPA. Most of these strains belong to classes Chlorophyceae, Trebouxiophyceae and Rhodophyceae.

The highest proportions of EPA and arachidonic acid (ARA) (more than 1/3 of total fatty acids) were found in algae of the genera *Cryptomonas*, *Heterococcus*, *Nannochloropsis* and/or *Porphyridium* (Lang et al., 2011).

The effect of temperature on the composition of phospholipid membranes and content of FAs in algae has also been intensively studied (Morgan-Kiss et al., 2006). The lipid composition of the green alga *Botryococcus* was studied at three different cultivation temperatures: suboptimal (18 °C), optimal (25 °C), and supraoptimal (32 °C). The content of polyenoic fatty acids was found to decrease with increasing temperature, e.g. with 18:3*n*−3 to less than half (Kalacheva et al., 2002). Similar results were found also in *Chlorella vulgaris* and *Botryococcus braunii*, where culturing was performed at 18 °C, 25 °C, and 32 °C. The content of linolenic acid also decreased when the cells were cultured at 32 °C compared to culturing at 18 °C while the amount of 18:2*n*−6 was almost doubled (from 14.3% to 28.1% of total FAs) (Sushchik et al., 2003). The effect of temperature on EPA production was studied in the unicellular red algae *Porphyridium purpureum* (Nuutila et al.,

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1997) and *Porphyridium cruentum* (Akimoto et al., 1998). In. *P. cruentum*, ARA content was found to increase with increasing temperature while EPA content decreased (from 13.5% at 20 °C to 3.1% at 32 °C). Another study (Rezanka et al., 1987) of the effect of temperature on the content of PUFAs and specific growth rate of *P. cruentum* showed that the EPA content decreased with increasing temperature whereas the ARA content showed only an insignificant change. Cultivation of *T. minutus* at low (15 °C) and high (40 °C) temperatures was found to increase the lipid content (up to 35.2% dry weight) relative to the cultivation at 25 °C, which is optimal for growth (Gigova et al., 2012).

Structured triacylglycerols (STAGs) can be defined as triacylglycerols (TAGs) that have been modified or restructured from natural oils and fats. The STAGs are synthesized for nutritional use or specific purposes, such as human milk-fat substitutes, low-caloric fats and oils enriched usually by essential fatty acids (FAs) (e.g. linoleic and α-linolenic acid) or long-chain ω-3 polyunsaturated fatty acids (PUFAs) (EPA and ARA). During the last 25 years, a range of animal and human studies have been conducted using STAGs; they constitute the background for many of the clinical studies performed subsequently (Akoh, 2006).

STAGs, i.e. modified or restructured TAGs from natural oils and fats, irrespective of whether they are synthesized or biosynthesized in vivo or in vitro" (Dey and Maiti, 2013; Dey et al., 2014) can be produced by different methods. Chemical interesterification with a catalyst like sodium methoxide results in TAGs with random distribution of the fatty acids within the glycerol backbone. This randomization distributes the fatty acids equally in all three positions of the TAG. Another possibility is a stereospecific organic synthesis which allows the stereospecific esterification of positions *sn*-1, *sn*-2 and *sn*-3 of glycerol (Kristinsson et al., 2014).

STAGs can be also produced by enzymatic interesterification using *sn*-1,3 specific lipases, which results in specific replacement of the fatty acids in the *sn*-1,3 positions of the TAG. The outcome of this process depends primarily on the substrates and the ratio between them, the choice of enzyme, and the process technology (Akoh, 2006). A promising method of preparation and even production of STAGs is the use of different conditions during the cultivation of microorganisms, either algae or yeast (Rezanka et al., 2012, 2013).

Fats and oils having similar fatty acid compositions do not necessarily have the same fatty acid distributions in their TAGs. For instance, EPA and ARA are mainly located in the *sn*-1,3-positions of TAGs from marine mammals whereas they are enriched in the *sn*-2-position of TAGs from fish oil (Nagai et al., 2013). Evidence is accumulating that both the overall fatty acid profile and the intramolecular structure of dietary fats are of importance when considering the nutritional effects of a given fat. Studies on the effects of dietary fatty acids as related to their position on TAGs have been published (Mu and Porsgaard, 2005).

Another important factor that affects the digestion and absorption of oils and fats is their physical state, i.e. the presence of a solid TAG phase. This phenomenon has an effect on the digestion, absorption and metabolism of dietary lipids. It limits the enzymatic hydrolysis of TAG and then their absorption (Michalski et al., 2013).

In contrast to the temperature dependence of FAs composition, similar studies concerning lipids are much fewer (Chen et al., 2008; Olofsson et al., 2012) and, to our mind, the effect of temperature on the ratio of regioisomers and enantiomers of triacylglycerols has not yet been studied at all, unlike studies describing commonly available animal and plant oils (e.g. Lisa et al., 2009; Gotoh et al., 2011; Lisa and Holcapek, 2013).

This study describes the identification of regioisomers and enantiomers of triacylglycerols of C20 PUFAs in the alga *T. minutus* cultivated at different temperatures using reversed- and chiral-phase liquid chromatography–mass spectrometry. The use of the two different phases contributes to ready identification, both qualitative and semiquantitative, of regioisomers and enantiomers of triacylglycerols containing EPA and ARA in the molecule. Our atmospheric pressure chemical ionization mass spectrometry (APCI-MS) method is well suited for direct determination of TAG profiles and for comparing the production of lipids after a change in microorganism cultivation conditions.

2. Results and discussion

2.1. Identification and production of fatty acids

Methyl esters were identified based on their compliance with the retention characteristics of commercially obtained standards by GC–MS and comparison with previously obtained results (Rezanka et al., 2010). The fatty acids were those listed in Table 1. The major acids in control cultivation as well as in the two temperature variations were myristic (M), palmitic (P), palmitoleic (Po), linoleic (L), α-linolenic (Ln), arachidonic acid (A) and eicosapentaenoic (E) acids. It is generally accepted that the content of polyunsaturated fatty acids (PUFAs) decreases with increasing temperature while the amount of saturated or monoenoic FAs increases (Guschina and Harwood, 2006, 2009). Our results describing growth/biomass production (DW g/L), as well as the total fatty acid content (% DW) and the volumetric content (mg/L of culture) for EPA and ARA are presented in Table 1.

We chose several representative articles to compare the influence of culture temperature on the fatty acid content of the algae. When culturing *T. minutus* in the temperature range 15–40 °C, it was found that the production of total lipids is lowest at the optimum temperature, i. e. 25 °C. When culturing *T. minutus* at both limit temperatures (15 and 40 °C) the lipid production was higher, at 15 °C by nearly two thirds (it increased from 21.8% to 35.2%) (Gigova et al., 2012). Conversely, the biomass production was highest at the optimum temperature, i.e. 5 times higher than at 15 °C and more than 4 times higher than at 40 °C. The same trend in biomass production was also described by Cepák et al. (2014), wherein the yield of biomass production was over 10 g/L at 28 °C and only 2 g/L and 1 g/L, respectively, at 20 and 33 °C. Similar conclusions were reached earlier, e.g. by Sukenik (1991) in the genus *Nannochloropsis*, who used the lowest cultivation temperature of 25 °C to produce the highest EPA levels. As already mentioned by Cepák et al. (2014), the highest proportion of EPA was observed at low culture temperature (20 °C), but the production in g/L/day was negligible, mainly due to low biomass production. Similar conclusions were reached in the analysis of the effect of temperature

Table 1
Fatty acid content (%) of triacylglycerols from *T. minutus* cultivated at different temperatures.

FA		10 °C	25 °C	32 °C
La	Lauric	0.1	0.2	0.3
M	Myristic	12.3	24.8	29.3
P	Palmitic	8.5	10.4	13.1
Po	Palmitoleic	6.3	10.9	9.8
S	Stearic	0.2	0.4	0.8
O	Oleic	0.3	0.5	0.9
L	Linoleic	2.9	5.3	6.9
Ln	α-Linolenic	6.3	5.8	4.9
A	Arachidonic	7.2	4.1	1.6
E	Eicosapentaenoic	55.7	37.0	31.5
B	Behenic	0.2	0.6	0.9
	Dry weight (g/L)	1.8	9.6	4.7
	Total FAs (% dw)	36.1	33.2	25.8
	EPA (mg/L)	361.9	1179.3	382.0
	ARA (mg/L)	46.8	130.7	19.4

on other, more or less related algae. For example the linolenic acid content in green algae of the genus *Chlorella* and *Botryococcus* decreases to half when culture temperature rises from 18 to 32 °C (Sushchik et al., 2003). Similarly, the hexadecatrienoic acid content in *Chlorella* decreased from 11.4% to 3.2%, i.e. almost 4 times, on temperature change from 13 to 38 °C, whereas the content of myristic acid was essentially unchanged, 2.8 versus 3.1%, upon a temperature change from 4 to 30 °C (Teoh et al., 2013). Analogously, the hexadecatetraenoic acid content in the genus *Chlamydomonas* decreased from 16.5 to 3.2% for a temperature change from 13 to 33 °C and the stearic acid content increased from 8.6% to 24.9%. The spectrum of fatty acids was studied in the unicellular red alga *P. cruentum* grown at 21, 24 and 28 °C. The proportion of unsaturated fatty acids (L, A and E) increased with decreasing cultivation temperature while the production of saturated acids (P) declined (Rezanka et al., 1987).

Table 1 shows the fatty acid content of the alga *T. minutus* cultivated in our experiments. The differences in percent proportion between our results and those of an important study published by Cepák et al. (2014) are mainly caused by two distinct basic experimental conditions – different culture temperatures (10, 25 and 32 °C versus 20, 28 and 33 °C), and the fact that Cepák et al. (2014) analyzed total fatty acids, while we analyzed only FAs contained in TAGs, see below. Yet Table 1 clearly shows that the same phenomenon took place in both cases, i.e. loss of PUFAs and an increase in the production of saturated FAs in cultivations at higher temperatures. This statement is entirely consistent with the results of PCA analysis of both our data and the findings of Cepák et al. (2014), see Figs. 1S and 2S.

2.2. Identification and production of triacylglycerols

Unlike the analysis and identification of fatty acids, which is the subject of thousands of studies (13,500 according to the Web of Science), the analysis and identification of TAGs is much less intensively studied (630 studies according to the Web of Science). Out of this number, papers describing analysis of diastereoisomers and enantiomers of TAGs can be counted in units. This analysis is not a trivial task, see e.g. the studies cited in this article. The method most commonly used for analyzing the molecular species of the TAGs is liquid chromatography–tandem mass spectrometry (LC–MS/MS) using soft ionization techniques. Either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) is most commonly used, non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) being the most commonly used LC technique. Alternatively, one can also use direct injection of a mixture of TAGs into mass spectrometer, but this method cannot identify the regioisomers and certainly not enantiomers. Based on our previous experience with identifying regioisomers and enantiomers we used here two methods, which proved to be successful, viz. APCI and chiral chromatography with MS/MS APCI. NARP-LC/tandem MS allowed us to identify dozens of molecular species, see Table 2 and Fig. 1.

Positive ion APCI mass spectra of TAGs typically exhibited $[M+H]^+$ ion and fragment ions of type $[M+H-RCOOH]^+$ ($[DAG]^+$), resulting from the loss of an acyl chain from TAG. Monoacyl glycerol ($[MAG]^+$) is equivalent to $[RCO+74]^+$ ion. The positive ion APCI mass spectrum (EEA, for abbreviations of FAs see Table 1), i.e. the peak at RT 26.4 min shows $[M+H]^+$ ion at *m/z* 947.7 and the fragment ions $[M+H-RCOOH]^+$ at *m/z* 645.5 $[EA]^+$ and at *m/z* 643.5 $[EE]^+$. Further ions at *m/z* 361.3 $[A+74]^+$ and at *m/z* 359.3 $[E+74]^+$ or $[RCO]^+$ at *m/z* 287.2 [arachidonoyl] and at *m/z* 285.2 [eicosapentaenoyl] were also present. These ions were observed with sufficient intensity to analyze the mass spectrum.

Further TAGs were identified in a similar manner; the identification was often complicated by the coelution of several TAGs,

Table 2
Relative quantities (%), retention time (RT, min), equivalent carbon number (ECN) and acyl carbon number (ACN) of molecular species of TAGs identified in alga *T. minutus*.

RT	TAG	ECN	ACN	10 °C	25 °C	32 °C
21.9	EEE	30	60:15	3.9	1.8	1.1
25.8	LnEE	32	58:13	2.2	1.8	1.1
26.4	AEE	32	60:14	2.2	2.1	1.1
29.5	PoEE	34	56:11	2.3	2.1	1.6
30.2	MEE	34	54:10	2.7	2.6	2.5
32.9	AELn	34	58:12	0.6	0.4	0.1
33.6	AAE	34	60:13	0.6	0.4	0.3
34.2	EEL	34	58:12	2.3	2.1	1.7
34.8	PoEE	34	56:11	2.3	2.1	1.6
35.6	LnELn	34	56:11	1.3	1.2	1.3
35.8	AAA	36	60:12	1.2	1.1	1.2
38.1	AALn	36	58:11	1.6	1.5	1.7
39.0	PEE	36	56:10	2.4	2.1	1.6
40.2	AEL	36	58:11	1.4	1.2	1.3
41.8	AEPo	36	56:10	1.4	1.3	1.2
42.4	ALnLn	36	56:10	0.7	0.4	0.3
42.5	LnEL	36	56:10	1.4	1.3	0.3
42.9	EEP	36	56:10	2.3	2.1	1.6
43.0	LnLnLn	36	54:9	0.7	0.5	0.3
43.5	LnEPo	36	54:9	1.4	1.4	1.2
44.3	AEM	36	54:9	1.8	1.7	1.7
44.9	LnEM	36	52:8	1.8	1.7	1.8
45.4	AAL	38	58:10	0.7	0.5	0.3
46.7	AAPo	38	56:9	0.6	0.5	0.4
47.4	ALnL	38	56:9	0.8	0.5	0.4
47.8	LEL	38	56:9	1.5	1.3	1.5
48.0	AEP	38	56:9	1.4	1.3	1.2
48.3	LnAPo	38	54:8	0.6	0.5	0.5
49.0	PoEL	38	54:7	1.5	1.4	1.3
49.1	LnLnL	38	54:8	0.9	0.5	0.4
49.8	AAM	38	54:8	1.1	1.0	0.9
50.0	LnEP	38	54:8	1.4	1.3	1.2
50.2	LnPoLn	38	52:7	0.7	0.6	0.4
50.5	PoEPo	38	52:7	1.5	1.5	1.2
50.7	ALnM	38	52:7	1.2	0.9	1.1
50.9	MEL	38	52:7	1.9	1.7	2.0
51.0	LnLnM	38	50:6	1.2	0.9	1.1
51.3	MEPo	38	50:6	1.9	1.9	1.7
51.6	MEM	38	48:5	2.0	2.3	2.9
53.4	ALL	40	56:8	0.5	0.9	0.4
53.8	AAP	40	56:8	0.6	0.5	0.4
54.3	LnLL	40	54:7	0.5	1.0	0.5
54.6	PoAL	40	54:7	0.5	0.8	0.7
55.1	LnAP	40	54:7	0.5	0.7	0.5
55.6	PEL	40	54:7	1.4	1.5	1.4
56.2	PoAPo	40	52:6	0.6	0.6	0.8
57.4	LnPoL	40	52:6	0.6	0.8	0.7
58.1	LnPLn	40	52:6	0.6	0.7	0.4
58.5	AML	40	52:6	1.1	1.3	1.1
59.0	PoEP	40	52:6	1.5	1.5	1.2
59.2	LnPoPo	40	50:5	0.6	0.7	0.5
59.3	MAPo	40	50:5	1.1	1.1	1.2
59.6	LnML	40	50:5	1.0	1.4	1.2
60.0	MEP	40	50:5	1.9	1.9	1.7
60.2	LnMPo	40	48:4	1.1	1.2	1.2
60.5	AMM	40	48:4	1.6	1.7	1.6
60.6	LnMM	40	46:3	1.3	1.5	2.0
60.7	LLPo	42	52:5	0.6	0.9	0.5
60.8	LLL	42	54:6	0.5	1.1	0.8
60.9	PoPoL	42	50:4	0.8	0.7	0.8
61.0	LnPL	42	52:5	0.6	0.8	0.7
61.2	PAL	42	54:6	0.5	0.8	0.7
61.3	LPoL	42	52:5	0.2	0.3	0.5
61.5	PoAP	42	52:5	0.5	0.6	0.8
61.6	PEP	42	52:5	1.5	1.5	1.2
61.9	LnPoP	42	50:4	0.7	0.7	0.7
62.2	MLL	42	50:4	1.5	1.2	1.3
62.3	MAP	42	50:4	1.2	1.1	1.2
62.4	PoPoPo	42	48:3	0.5	0.6	0.9
62.8	PoML	42	48:3	1.3	1.2	1.4
64.2	PoMPo	42	46:2	1.3	1.2	1.3
64.5	LnMP	42	48:3	1.2	1.1	1.2
65.6	MMPo	42	44:1	0.9	1.5	2.4

(*continued on next page*)

Table 2 (*continued*)

RT	TAG	ECN	ACN	10 °C	25 °C	32 °C
66.1	MMM	42	42:0	0.5	1.4	2.7
66.7	MML	42	46:2	1.8	1.6	1.7
66.9	LPPo	44	50:3	0.2	0.4	0.5
67.3	PPoPo	44	48:2	0.5	0.6	1.1
67.9	LMP	44	48:2	1.1	1.3	1.7
68.1	LnPP	44	50:3	0.5	0.7	0.9
68.5	PMM	44	44:0	0.0	0.1	0.2
69.1	PAP	44	52:4	0.6	0.6	0.7
69.5	PoPL	44	50:3	0.6	0.8	1.0
71.3	PML	44	48:2	0.1	0.3	0.8
71.5	PoMP	44	46:1	1.0	1.3	1.8
71.6	PLL	44	52:4	0.5	0.9	1.5
73.0	MMP	44	44:0	1.3	1.7	2.4
75.3	PPL	46	50:2	0.6	0.7	1.2
75.6	PPoP	46	48:1	0.3	0.6	1.2
76.2	PMP	46	46:0	0.1	0.3	1.1
81.3	PPP	48	48:0	0.1	0.1	0.5

see Table 2 and Fig. 1. For instance, three TAGs, AMM, LnMM, and LLPo, were coeluted to form the peak at retention time 60.6 ± 0.1 min. They, as well as other coeluted peaks, were identified by tandem MS, which clearly identified peaks of the $[DAG]^+$ type, i.e. $[AM]^+$, at *m/z* 571.5, $[LnM]^+$ at *m/z* 545.5, and $[LL]^+$ at *m/z* 599.5. TAGs often occur as regioisomers, i.e. positional isomers on the glycerol backbone (Mottram, 2005). The overlapping of TAG peaks means that overlapping of total ion current (TIC) takes place, see also Fig. 1. However, if one uses neutral loss scan tandem mass spectrometry (NLS), see Fig. 2, then one can distinguish and thus also quantify, e.g. TAGs having nearly identical retention times, such as, e.g., RT 42.9 min (EEP) and RT 43.0 min (LnLnLn). To quantify ions of the $[DAG]^+$ type – ($[EP]^+$ and ($[LnLn]^+$) one can thus use in fact NLS-E and NLS-Ln, which have different *m/z*, see Table 2.

To identify the enantiomers and diastereoisomers TAGs one must use standards, since it is known that regioisomers and both enantiomers have the same *m/z* values of ions but not the same abundance of the ions. An achiral TAG, e.g. *sn*-EAE, differs as a diastereomer in its mass spectrum from each of the two enantiomers (*sn*-EEA and *sn*-AEE). Mass spectra of enantiomers and regioisomers are demonstrated on the example of di-eicosapentaenoyl arachidonoins-and di-arachidonoyl-eicosapentaenoins (Figs. 3S–10S, see Supplements). One can use for the purpose the well-known rule (Byrdwell, 2005; Mottram, 2005), which says that, based on the intensities of ions $[M+H-RiCOOH]^+$, it is possible to determine whether a TAG is a 1,2-isomer (2,3-isomer) or a 1,3-isomer. Conversely, enantiomers have almost identical mass spectra, see Figs. 4S and 5S, so it cannot be determined from the mass spectra which enantiomer is present and has to be differentiated on the basis of comparison of retention times of the standards.

To prepare standards of the individual enantiomers of TAGs, we used an already described method, the principle of which consists in the hydrolysis of commercially available diacylphosphatidylcholine by lipase C and esterification of the free OH group of the corresponding diacylglycerol with a fatty acid (Rezanka et al., 2013). As is evident from Fig. 2, optical purity, i.e. the yield of the desired enantiomer, always exceeded 95%.

The elution order of the individual enantiomers and regioisomers can partly be predicted, because, as described previously (Nagai et al., 2011; Rezanka et al., in press), the enantiomer having a more unsaturated FA at the *sn*-1 position of the glycerol backbone is eluted earlier than the other enantiomer. Achiral TAG is eluted as the last of the three. So, for example, the elution order of di-arachidonoyl-*sn*-eicosapentaenoin is *sn*-EAA, *sn*-AAE and *sn*-AEA. This sequence however holds only under strictly defined conditions, i.e. a certain type of stationary and mobile phases, temperature, etc., see also Nagai et al. (2011), where the mere substitution of CH_3 group for Cl group in the stationary phase led to an inverse order of elution of enantiomers. These findings indicate that, to ensure correct identification, at least one of the two enantiomers must be obtained either from commercial sources or by organic synthesis. This statement does not apply to regioisomers, as they can be identified based on the abundance of ions of type ($[AA]^+$ to $[AB]^+$), see above. Identification of most regioisomers having the same fatty acyls in the *sn*-2 and *sn*-1 (*sn*-3) positions therefore usually does not pose a problem.

Selected ion monitoring (SIM) was used to determine the appropriate enantiomers and regioisomers, see Fig. 3 acquired for a mixture of two TAGs, i.e. di-arachidonoyl-eicosapentaenoins and di-eicosapentaenoyl-arachidonoins obtained from algae cultured at various temperatures (10, 25 and 32 °C).

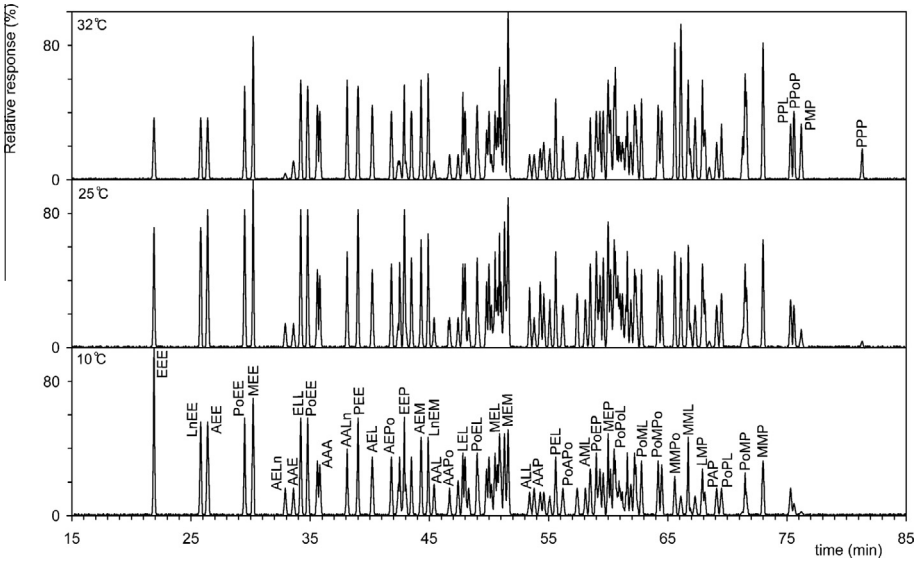

Fig. 1. RP-LC/APCI-MS chromatogram total ion current (TIC) of the TAGs from the alga *T. minutus*, cultivated at 10, 25 and 32 °C.

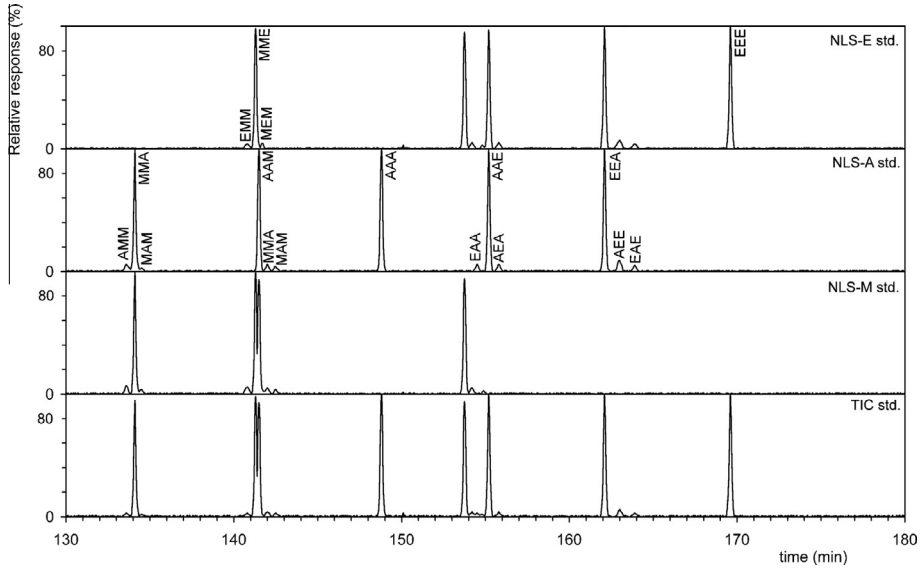

Fig. 2. Chiral LC of synthetic standards, see Table 3. The prefix "*sn*" was omitted in order to abbreviate the acronyms in the figure. TAGs containing myristic, arachidonic and eicosapentaenoic acids as revealed by the neutral loss scan for $[M+H]^+$ – 228, $[M+H]^+$ – 304, and $[M+H]^+$ – 302 Da, respectively, during APCI of synthetic TAGs.

Fig. 3 illustrates an obvious dependence of the change in the ratios of individual enantiomers and regioisomers on cultivation temperature. In the case of di-arachidonoyl-eicosapentaenoin it is clearly seen that the percent proportion of achiral TAG (*sn*-AEA) decreases with increasing temperature of cultivation, while Table 2 and Fig. 1, i.e. the results obtained from NARP-LC/APCI-tandem MS obviously show that the total content of di-arachidonoyl-eicosapentaenoin decreases with increasing cultivation temperature. Next examined was the TAG di-eicosapentaenoyl-arachidonoin. Here both Table 3 and Fig. 3 shows that the proportion of achiral TAG (*sn*-EAE) has an increasing tendency with temperature increasing from 10 °C to 25 °C and then it decreases at 32 °C, the sum of asymmetric (*sn*-EEA and *sn*-AEE) to symmetrical *sn*-EAE having a rising characteristic, i.e. 0.56:0.74:1.20. Here again there is a sharp reduction in the proportion of the di-eicosapentaenoyl-arachidonoin, which is shown in Fig. 1 and in Table 2. It is also confirmed by the data of Table 1, which clearly point to the loss of both C20 PUFAs with increasing temperature of cultivation. SIM and tandem MS were similarly used to identify and further semi-quantify 4 trios of enantiomers and regioisomers of TAGs (di-eicosapentaenoyl myristoin-, di-arachidonoyl-myristoin, di-myristoyl-eicosapentaenoin, and di-myristoyl-arachidonoin). The results are shown in Table 3.

For TAGs having two myristic acids in the molecule, i.e. di-myristoyl-eicosapentaenoin and di-myristoyl-arachidonoin the content of achiral TAG, i.e. *sn*-MEM and *sn*-MAM remains substantially constant. In both cases the content of the two pairs of enantiomers is rapidly increasing with increasing cultivation temperature. It can therefore be concluded that cultivation temperature has a significant impact not only on the amount of PUFAs (a phenomenon known for decades) but also on the content of TAG (which has also been reported by Olofsson et al., 2012) and especially on the ratio of enantiomers and regioisomers of the relevant molecular species. Unfortunately, the research in this area has so far been fully focused on those TAGs that are useful in the food industry (Craven and Lencki, 2013) and not on the oil bodies in living cells of photosynthesizing plants, not even on oleaginous crops such as sunflower, canola, soya, etc. So far, all research has been directed toward understanding the adaptation of membranes (membrane fluidity) at low temperatures, completely neglecting the above-mentioned oil bodies (Morgan-Kiss et al., 2006).

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. From the above findings it is clear that the achiral TAGs are preferably synthesized at low temperatures, implying a different activity of the enzymes acyl-CoA: *sn*-glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA: lyso-phosphatidic acid acyltransferase (LPAAT), and acyl-CoA: diacylglycerol acyltransferase (DGAT) at various temperatures.

This phenomenon is demonstrated using a principal component analysis, where the first axis scores of the samples are clearly correlated with the temperature of cultivation (Fig. 4). PCA analysis of all 18 measured abundant TAGs is shown in Fig. 11S, even if the image is not as illustrative as Fig. 4.

Large differences between the proportion of enantiomers and regioisomers were detected by Nagai et al. (2011) for marine fish (skipjack tuna, sardine) and mammals (steller sea lion, harp seal). Basically they are final predators in the food chain that begins with phytoplankton which is the source of C20 polyunsaturated fatty acids – arachidonic and eicosapentaenoic acid. A similar phenomenon was recorded also in algae grown under both stressful conditions and under different nutritional conditions (Rezanka et al., 2012), and in strains of the genus *Stichococcus* obtained from locations with different temperature conditions (Svalbard, a lake in a temperate region, thermal springs) (Rezanka et al., in press).

Based on the knowledge about the influence of temperature on the crystalline structure of TAGs (Craven and Lencki, 2013) it is clear that TAGs exhibit polymorphism and usually crystallize in three polymorphic forms, exhibiting with increasing temperature increasing stability of the sub-cells, i.e. $\alpha \rightarrow \beta' \rightarrow \beta$, where β is the most stable form.

However, natural TAGs are always a mixture of different molecular species that differ in chain length and number of double bond(s), and therefore they are a mixture of polymorphic forms that can coexist simultaneously. The crystalline structure of TAGs is important for fluidity of oil bodies in the cells of living organisms, since each crystalline form presents unique properties with respect to plasticity and solubility. The crystallization and polymorphic behavior for achiral, chiral racemic, and chiral-enantiopure TAGs is remarkably different (Craven and Lencki, 2013). All

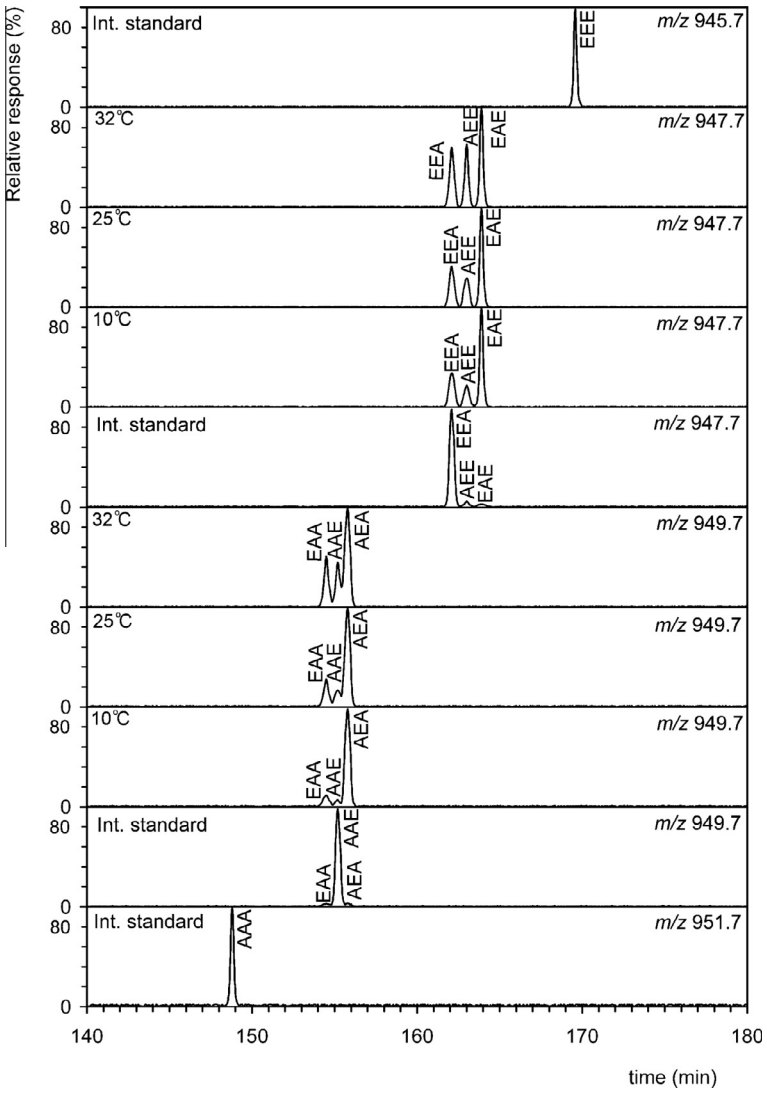

Fig. 3. Chiral-LC of TAGs (SIM – selected ion monitoring) from *T. minutus*, cultivated at various temperatures (10, 25 and 32 °C).

Table 3
Relative quantities (%) of regioisomers and enantiomers of TAGs from *T. minutus*.

TAG	10 °C	25 °C	32 °C
AMM	0.23	0.26	0.33
MMA	0.13	0.30	0.39
MAM	1.19	1.09	0.91
EMM	0.34	0.50	0.67
MME	0.22	0.40	0.73
AAM	0.19	0.21	0.18
MEM	1.45	1.40	1.52
MAA	0.10	0.17	0.23
AMA	0.80	0.64	0.45
AAA	1.19	1.10	1.19
EEM	0.35	0.41	0.57
MEE	0.17	0.42	0.71
EAA	0.06	0.07	0.08
EME	2.22	1.74	1.21
AAE	0.04	0.04	0.07
AEA	0.54	0.25	0.17
EEA	0.48	0.52	0.29
AEE	0.31	0.38	0.30
EAE	1.40	1.21	0.49
EEE	3.92	1.84	1.08

TAGs (achiral and chiral, racemic mixture or enantiomers) can, in principle, adopt α and β′ forms, whereas only achiral and racemic mixtures of chiral TAGs can form the β polymorph. Enantiopure TAG cannot form the β polymorph because both enantiomers must be present in the unit cell.

The life functions of the cell have to be retained throughout these transitions. One of the possibilities how to achieve this is to keep oil bodies in a fluid – non-crystalline state, which could be achieved by transitions between crystalline forms. Regretfully, the data on the melting point of crystalline forms of TAG regioisomers as well as enantiomers are available for only a few of them. Despite this, some data have been gathered (Serebrennikov et al., 1961), e.g. for SLO and SOL, in which the melting points for individual α and β forms are −2 to −4 °C and −15 to −17 °C and −11.5 to −13 °C and −18 to −19 °C, respectively. Although this holds for two regioisomers, i.e. compounds having the same FAs and the same molecular weight, it is surprising that a mere change in the positions of two FA (O and L) on glycerol backbone shifts the melting point by as much as 10 °C.

The composition of TAGs and/or the proportion of individual regioisomers and enantiomers are thus seen to affect whether the oil bodies are, at a certain temperature, fluid or crystalline. We can therefore hypothesize that an alga could regulate their adjustment to the temperature changes by changing the content of FA and phospholipids in the membranes but also by changing TAG fluidity, i.e. transition between α → β′ → β forms and back in oil bodies.

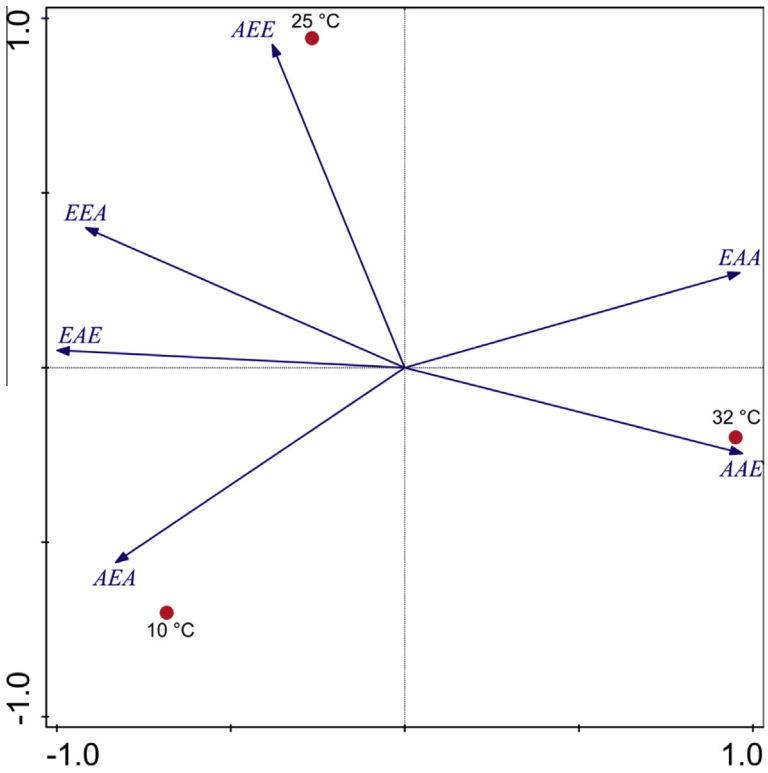

Fig. 4. Principal component analysis (PCA) of TAG composition in the alga *T. minutus* cultivated at 10 °C, 25 °C and 32 °C. The first two canonical axes are plotted; they explained 94.5% of the variability. For FA abbreviations, see Table 1.

Our literature search did not reveal any such case and this observation thus requires further measurements in order to establish a valid hypothesis about the biosynthesis of TAGs.

3. Conclusion

The ratio of regioisomers and enantiomers of TAGs in *T. minutus* has been shown to be affected by cultivation temperature. We used the principal component analysis to detect and illustrate this effect. This approach can conceivably be used on a broader scale to identify if and to what degree the production of some compound of interest is influenced by factors such as cultivation conditions.

4. Experimental

4.1. Chemical and standards

Acetonitrile, 2-propanol, THF (tetrahydrofuran), hexane, dichloromethane, 4-dimethylaminopyridine (DMAP) EDCI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), 1,2-dieicosapentaenoyl-*sn*-glycero-3-phosphocholine, arachidonic and cis-5,8,11,14,17-eicosapentaenoic acids and phospholipase C from *Clostridium perfringens* (*Clostridium welchii*) Type I, lyophilized powder, 10–50 units/mg protein were purchased from Sigma–Aldrich (Prague, CR). Triarachidonin (5c,8c,11c,14c) and trieicosapentaenoin (5,8,11,14,17-all cis) was purchased from Larodan Fine Chemicals, Malmö, Sweden. 1,2-Diarachidonoyl-*sn*-glycero-3-phosphatidylcholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Polar Lipids, Inc., AL, USA.

4.2. Cultivation

T. minutus Lukavsky et Pribyl 2005/1 CCALA 931 was obtained from Culture Collection of Autotrophic Organisms of the Institute of Botany AS CR (http://ccala.butbn.cas.cz/index.php). Laboratory cultivation units consisted of glass cultivation flasks, which were placed in a thermostatic bath and continuously illuminated by white fluorescent light tubes (Osram L 36W/830 Lumilux, Germany). The incident light intensity was 80 μmol/m^2/s^1 photosynthetic active radiation (PAR). The light intensity was measured using a digital lux-meter PU 550 (Metra, Czech Republic) equipped with a PAR sensor calibrated according to the LI-185 quantum sensor (LI-COR, USA). Cultures were grown at 2× concentrated "Z" nutrient medium (Staub, 1961) and aerated by the mixture of air and CO_2 (2%, v/v). The volume of the algal suspension in each cultivation flask was 900 mL. Three different temperatures were used: 10, 25 a 32 °C. The cultures were cultivated 21 days. The biomass was harvested by centrifugation, frozen at −80 °C and lyophilized.

4.3. Enzymatic synthesis of 1,2-dieicosapentaenoyl-, 1,2-diarachidonoyl- and 1,2-dimyristoyl-sn-glycerols

1,2-Dieicosapentaenoyl-*sn*-glycero-3-phosphocholine, 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine were treated with phospholipase C (Type I from *C. perfringens* (*C. welchii*)) as described by Christie (1989). In brief, phospholipase C from *C. welchii* (3 mg) in 0.5 M tris(hydroxymethyl)methylamine (tris) buffer (pH 7.5; 2 mL), containing 2 mM 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-diacyl-*sn*-glycerols were 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).

The esterification of glycerol derivatives with acids was described previously (Ziegler and Berger, 1979; Halldorsson et al., 2003). Briefly, a solution of 1,2-diacyl-*sn*-glycerol (57 mg) and eicosapentaenoic or arachidonic acids (33 mg) 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 products as yellowish oil. The yields of the target *sn*-EEA and *sn*-AAE were 45 and 38 mg, respectively. Other TAGs were prepared similarly, the list of all synthesized triacylglycerols, see Table 1S.

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 35cc (Waters; with 10 grams 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-HPLC/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 min 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.

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 mg/mL in methanol) were chromatographed on two Astec cyclobond™ I 2000 DMP (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin) chiral LC columns (5 μm, 25 cm × 4.6 mm) connected in series, 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 B during 180 min, and then isocratically for 30 min, where A is hexane and B is hexane-2-propanol (99:2, v/v) mixture (Rezanka and Sigler, 2014). The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 μl, respectively. Other separation conditions were as used 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.2014.12.013.

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