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Effect of salinity on the fatty acid and triacylglycerol composition of five haptophyte algae from the genera *Coccolithophora*, *Isochrysis* and *Prymnesium* determined by LC-MS/APCI

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ABSTRACT

Non-aqueous reversed-phase high-performance liquid chromatography (NARP-HPLC) with atmospheric pressure chemical ionization (APCI) was used for separation of triacylglycerols from five strains of haptophyte algae (genera *Coccolithophora*, *Isochrysis*, and *Prymnesium*). This study describes the separation and identification of C18 polyunsaturated triacylglycerols containing stearidonic and octadecapentaenoic fatty acids, including their regioisomers. Salinity affects the proportion of saturated and unsaturated fatty acids. The biosynthesis of C18 polyunsaturated triacylglycerols was found to be very stereospecific and to depend on the salinity of cultivation media, asymmetric regioisomers predominating at low salinity (*sn*-OpOpSt and/or PoStSt) and symmetric ones at high salinity (*sn*-OpStOp and or StPoSt).

1. Introduction

We studied the effect of salinity on the contents and proportions of fatty acids (FAs) and triacylglycerols (TAGs) in microalgae that are potential producers of two C18 PUFAs, octadecatetraenoic (stearidonic, St) acid and octadecapentaenoic acid (Op). These include algae from the genera *Coccolithophora*, *Isochrysis*, and *Prymnesium* that all belong taxonomically to the phyllum Haptophyta. Haptophyte algae are a relatively small group of unicellular microorganisms that have a characteristic cell organization with two flagella and a multifunctional filamentous organelle called haptonema that is unique to this group. Marine species are ecologically very important, forming periodically algal blooms and playing an important role in the global biogeochemical cycles and in climate. The best known haptophyte species is *Emiliania huxleyi* that is covered with calcareous scales and belong to the most widespread primary organisms in the open water of the World's Ocean (Van den Hoek et al., 1996).

Many microalgae are promising for biotechnological applications due to high lipid content and rapid growth. The proportion of n-3 PUFAs, especially 18:4, 20:5, 22:5 and 22:6 FAs in the chrysophyte alga *Boekelovia hooglandii* (Fujii et al., 2001) rose with increasing NaCl concentrations from 50 to 800 mM (2.9–46.4‰). Conversely, the content of 20:5n-3 acid in the eustigmatophycean alga *Nannochloropsis oculata* did not change significantly in a salt concentration range from 15 g/L to 55 g/L (15–55‰) (Gu et al., 2012). The content of α-linolenic acid in two freshwater green algae, *Chlamydomonas mexicana* and *Scenedesmus obliquus*, grown under salt stress was found to be almost unchanged at NaCl concentration ranging from 0 to 100 mM (0–5.8‰) (Salama et al., 2013). In one of the most halotolerant algae, *Dunaliella salina*, β-ketoacyl-coenzyme A synthase is a salt-inducible enzyme that may play, jointly with fatty acid desaturases, a role in adapting intracellular membranes to salinity. Microsomal plant β-ketoacyl-coenzyme A (CoA) synthases catalyze the condensation of malonyl-CoA with acyl-CoA, the first and rate-limiting step in fatty acid elongation. The content of 18:4n-3 increased with increasing salt concentration from 0.5 M to 3.5 M (29–203‰) (Azachi et al., 2002). The content of saturated and monounsaturated FAs in cultures of *Dunaliella* sp., a green microalga from an Antarctic hypersaline lake,

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increased with increasing salt concentration from 0.4 M to 4 M (23.2–232‰) NaCl while the content of PUFAs decreased; hence n-3 PUFAs show a negative correlation with salt concentration (Xu and Beardall, 1997). As seen from the above examples, the effect of NaCl concentration in the culture medium on the production of PUFAs is ambiguous. We therefore attempted to clarify this effect in microalgae. We selected strains that are potential producers of two C18 PUFAs, octadecatetraenoic (stearidonic) acid and octadecapentaenoic acid according to the study of Lang et al. (2011). These include algae of the genera *Coccolithophora*, *Isochrysis*, and *Prymnesium* that all belong taxonomically among haptophytes.

Stearidonic acid (6,9,12,15-octadecatetraenoic or 18:4n-3) is occasionally found as a minor component in plants (especially viper bugloss (*Echium vulgare*), blackcurrant (*Ribes nigrum*), borage (*Borago officinalis*), evening primrose (*Oenothera biennis*), etc.) (Lisa et al., 2009), and it occurs in algae and fish oils; 3,6,9,12,15-octadecapentaenoic acid or 18:5n-3 is a significant component of cellular lipids in dinoflagellates and other classes (Haptophyceae or Chrysophyceae), and it can enter the marine food chain from this source (Lang et al., 2011).

Both acids have significant pharmacological effects. Stearidonic acid may play a role in suppressing inflammation (Guichardant et al., 1993) while octadecapentaenoic acid causes abnormal development in embryos and larvae of the sea urchin *Paracentrotus lividus* (Sellem et al., 2000).

The first step in the biosynthesis of eicosapentaenoic and docosahexaenoic acids in the human body is 18:4n-3. However, due to the low activity or limited availability of Δ6-desaturase this acid should be delivered in food to increase the content of eicosapentaenoic and docosahexaenoic acids in the body (Plourde and Cunnane, 2007).

In algae these PUFAs are present in bound form, usually as either polar lipids (phospholipids and glycolipids) or neutral lipids – TAGs. Analysis of the TAGs is usually performed either by direct sample inlet to a mass spectrometer or by connection of a liquid chromatograph to a mass spectrometer (LC/MS). The glycerol backbone is esterified with three FAs. Depending on their structure, TAGs may occur also as regioisomers and/or enantiomers. Regioisomers can be separated on a sufficiently efficient column, usually with a reverse phase, enantiomers can be separated only on a column with a chiral phase. Separated regioisomers can be identified according to the ratio of abundances of $[M\text{-}RCOOH]^+$ ions using a soft ionization mass spectrometry. Enantiomers cannot be identified in this way because they have identical mass spectra. The only possibility of identification consists in comparing the retention time of a standard (often very difficult to obtain) with the retention time of the analyte.

TAGs containing stearidonic acid are very rarely analyzed (Lisa and Holcapek, 2008; Lisa et al., 2009) in view of the limited number of natural resources. To our knowledge, TAGs containing octadecapentaenoic acid have not yet been analyzed.

We therefore focused on two research areas, i.e. finding algal species and culture conditions suitable for enhanced production of TAGs containing C18 PUFAs and analysis of these TAGs by LC-MS/APCI on both reverse phase and chiral phase columns.

2. Results and discussion

2.1. Analysis of the FA composition using GC-MS

The FA relative content was characterized by GC-MS of FAME after esterification on a capillary column with a polar stationary phase (see Experimental). Individual methyl esters were identified both on the basis of identity of retention times with commercially available standards and also by the electron impact mass spectra.

Methyl esters of PUFAs give molecular ions with very low abundance. Characteristic for these esters are diagnostic ions at 150 or 108 Da (n-6 or n-3 unsaturation) which determine the tentative position of the double bonds (Fellenberg et al., 1987). Based on both retention times (Rezanka et al., 2012a) and mass spectra we tentatively identified the following series of C18 PUFAs: 18:3n-3, 18:4n-3, and 18:5n-3. To fully confirm these structures, we measured the mass spectra of picolinyl esters of the PUFAs (Figs. 1S, 2S and 3S, i.e. Supplements), which clearly corroborated the above proposed structures. The mass spectra of picolinyl esters of α-linolenic (Fig. 1S) and stearidonic (Fig. 2S) acids have been well described (Christie, 2016; Rezanka et al., 2012b), so we focused only on describing the mass spectra of the derivative of allZ-3,6,9,12,15-octadecapentaenoic acid (Fig. 3S). Double bond in position 15 is located by the 26 Da gap between *m/z* 310 and 336, double bond in position 12 by the gap between *m/z* 270 and 296. One can more easily identify 40 Da gaps for a double bond and an adjacent methylene group, i.e. 4 gaps from *m/z* 176 to 216 to 256 to 296 to 336. We did not identify the position of the first double bond, i.e. Δ^3 because the mass spectrum provides no information for position 3, i.e. ions at *m/z* 151 and *m/z* 164 are not present in the mass spectrum, giving neither a 26 Da gap nor 40 Da gap, and containing no diagnostic ions.

When using drastic derivatization conditions, the acid is isomerized to 2,6,9,12,15-octadecapentaenoic acid. However, its picolinyl derivative has a totally different mass spectrum with a dominant ion at *m/z* 177, see the web pages of Christie (Christie, 2016).

Based on all the above facts we can assume that the identified acid is 3,6,9,12,15-octadecapentaenoic acid (18:5n-3).

2.2. Effect of salinity on FA composition and lipid content

The relative content of FAs in strains that grew within the whole salinity range (*Coccolithophora* sp. and *Prymnesium parvum*) is given in Table 1. The results regarding the other three strains, which failed to grow over the whole range of salinity, are presented in Table 1S, that include arithmetic means from three analyses ± standard deviations values, which were added since standards of FAs are available. The tables give only those FAs that have abundance greater than 0.1% of total FAs. Therefore, for instance 22:4, one of the minor acids that is the component of only one identified TAG (see below), is not listed in the table. Table 1S shows that the optimum salinity of culture media for production of lipids was in the range from 15 to 45‰, which is close to the salinity of natural habitats of the strains. The lipid content first rises with increasing salinity and then decreases, whereas the TAG content is steadily increasing with increasing salinity. The phenomenon can be explained by the fact that high salinity levels induce the production of storage substances to survive in adverse conditions (Salama et al., 2013).

It has been found that the culture medium salinity has a significant influence on the proportion and unsaturation of FAs. Fig. 1 shows that the dependence of the relative content of saturated FA on salinity has a downward-curved shape, while that of PUFAs has an upward-curved shape with highest values at intermediate salinities regardless of which of the five strains was used for cultivation. The situation in the case of monoenoic FA is more complicated; the dependence for *Coccolithophora* sp. shows a clearly upward-curved shape whereas *Isochrysis galbana* and *Prymnesium saltans* show a downward-curved shape. With the remaining two algae the data cannot be clearly interpreted. *Isochrysis* sp. grew at only two salinity levels and no trend in monoenoic acid relative content was observed in *Prymnesium parvum*.

Isochrysis sp. (Liu and Lin, 2001) produced under optimal

Table 1
Fatty acid content (%) of triacylglycerols from *Coccolithophora* sp. and *Prymnesium parvum* cultivated at different salinities.

FA[a]		FA[b]	*Coccolithophora* sp. 7.5‰		*Coccolithophora* sp. 15.0‰	*Coccolithophora* sp. 30.0‰	*Coccolithophora* sp. 45.0‰	*Coccolithophora* sp. 60.0‰	*Prymnesium parvum* 7.5‰	*Prymnesium parvum* 15.0‰	*Prymnesium parvum* 30.0‰	*Prymnesium parvum* 45.0‰	*Prymnesium parvum* 60.0‰
14:0	M	7.5		2.6	1.4	6.3	11.0	5.8	2.3	4.3	5.4	12.4	
16:1n-7	Po	4.9		9.2	9.6	4.9	2.0	10.1	5.3	5.0	5.7	9.2	
16:0	P	23.2		13.7	15.1	21.7	26.6	15.7	8.3	7.1	13.0	17.0	
18:5n-3	Op	8.3		11.2	12.3	10.6	8.3	10.4	14.5	18.2	19.8	16.4	
18:4n-3	St	21.1		25.7	17.8	14.8	10.3	29.9	30.9	24.3	22.0	20.7	
18:3n-3	Ln	4.0		6.4	5.0	3.7	2.7	4.5	7.1	4.6	3.8	2.6	
18:2n-6	L	2.2		2.4	7.0	5.3	3.7	2.0	5.0	5.2	4.5	1.7	
18:1n-9	O	3.7		7.2	8.6	6.0	4.4	6.8	8.2	9.9	10.0	6.6	
18:0	S	12.8		4.6	2.6	12.1	19.1	4.6	4.2	3.3	3.5	4.6	
20:4n-6	A	0.2		0.3	0.5	0.4	0.4	0.1	0.2	0.3	0.2	0.2	
20:5n-3	E	3.1		4.3	5.1	2.9	3.2	0.7	1.4	2.7	1.7	1.7	
22:5n-3	Dp	2.1		3.2	4.1	3.1	2.2	2.1	3.3	5.0	3.1	1.8	
22:6n-3	D	6.9		9.2	10.9	8.2	6.1	7.2	9.3	10.1	7.3	5.1	

[a] Shorthand notations - the number before the column specifies the number of carbon atoms and that after the colon the number of double bonds; the position of the terminal double bond is denoted in the form (n-x), where n is the chain length of the fatty acid and x is the number of carbon atoms from the last double bond, assuming that all the other double bonds are methylene-interrupted.
[b] Abbreviations of fatty acids.

conditions (salinity 32‰) over 4 mg of 22:6n-3 per liter of culture. An examination of the effect of NaCl on its production in the of range 8–32‰ showed no significant trend, with slightly higher values at 32‰ salinity. The alga *Boekelovia hooglandii* was cultivated at salinity levels of 50, 200, 400 and 800 mM NaCl (i.e. 2.9, 11.6, 23.2, and 46.4‰). Myristic acid was produced the least at 11.6‰ salinity while production of eicosapentaenoic acid at this salinity peaked.

By contrast, the FA relative content *vs.* salinity dependence in *Dunaliella* sp. isolated from an Antarctic hypersaline lake (Xu and Beardall, 1997) and grown in a culture medium with salinity in the range from 23.2‰ to 232‰ is unclear. The authors argue that saturated and monounsaturated acids exhibit a positive correlation with NaCl level whereas PUFAs have a negative correlation with salinity.

There was no clear effect of salinity on FA composition in the alga *Nannochloropsis oculata* (Gu et al., 2012). For instance, the proportion of palmitic acid is about 25% at the culture medium salinity of 15–45 g NaCl/L (15–45‰); the proportion of EPA was about 22% at a salinity of 15–55 g NaCl/L (15–55‰).

The alga *Nitzschia laevis* produced high amounts of EPA (Chen et al., 2008); when the NaCl concentration was increased from 10 to 20‰ the unsaturated FAs increased, while at 30‰ the proportion of these acids decreased.

It was found that in two algae, *Chlamydomonas mexicana* and *Scenedesmus obliquus* growing in the presence of 0–100 mM NaCl (0–5.8‰) in the culture medium (Salama et al., 2013), the proportion of α-linolenic acid (18:3n-3) was practically unchanged (27.3% at 0 mM and 28.2% at 100 mM) regardless of the salt concentration in the medium. The situation was similar with other acids.

A change in salinity in the range of 4–300‰ did not result in changes in the relative content of 20:5n-3 and other FAs in the lipids of *Chlorella saccharophila* (Teshima et al., 1983).

2.3. Analysis of TAGs by NARP-HPLC/APCI-MS

Fig. 2 shows the chromatographic separation by NARP-HPLC with APCI of total ion current in the interval from 200 to 1000 Da. The model compounds were the total TAGs from *Coccolithophora* sp. cultivated at 30‰ salinity. Nearly 170 molecular species were separated and identified; Fig. 2 shows neutral loss scans (NLS) for $[M + NH_4\text{-}R_{1(3)}COOH\text{-}NH_3]^+$ and $[M + NH_4\text{-}R_2COOH\text{-}NH_3]^+$, i.e. loss of 275 Da (18:4) and 273 Da (18:5), respectively. In this way we identified all the TAGs containing at least one of the above acids. However, the separation of all the peaks was not fully to the base line, although we had a previous good experience with two columns in series that reached efficiencies of up to 1040 plates/1 cm. The method used for the identification was thus tandem MS, which enabled us to identify all the molecular species of TAGs with abundance greater than 0.1%. Preliminary characterization of the eluted TAGs was based on the equivalent carbon number (ECN) values, which are calculated as ECN = ACN-2xDB (ACN = acyl carbon number; DB = number of double bond(s)) (Rezanka et al., 2011, 2012b) although some so-called critical TAG pairs have the same ECN (ECN = 30) e.g. StStSt and OpOpPo. Although it is known (Dermaux et al., 1999) that the retention times of TAGs having the same ECN increase with decreasing number of double bonds, see e.g. the TAG pairs StStL and StStPo, or StSL and PLLn that we analyzed, this rule does not apply for TAGs containing PUFAs (Solaesa et al., 2014). The identification thus requires LC-MS analysis, the method of choice being NARP-HPLC/APCI-MS^2.

The positive APCI mass spectrum of TAGs usually contains a few abundant ions, e.g. the mass spectra (Fig. 3) of four TAGs having RT

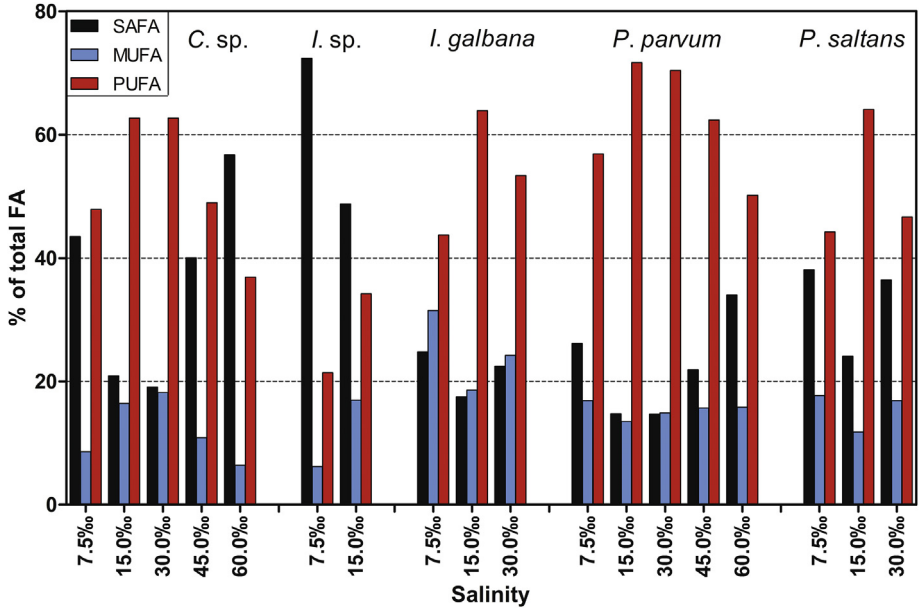

Fig. 1. Dependence of the proportion of saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids on salinity in the five algal strains.

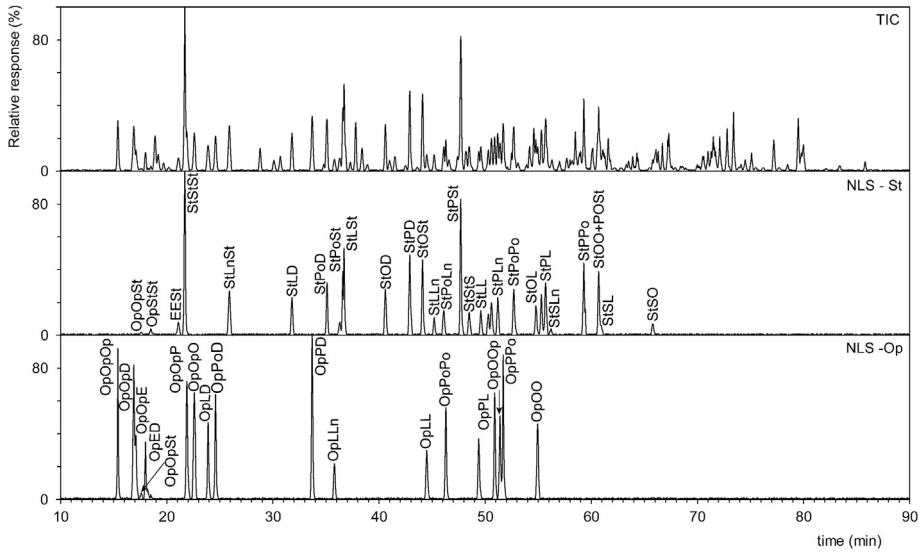

Fig. 2. NARP-LC/APCI-MS separation of TAGs of the alga *Coccolithophora* sp., cultivated at 30‰ salinity. For experimental conditions see Material and Methods. Abbreviations are explained in the text.

15.4, 17.6, 18.5, and 21.8 min, i.e. OpOpOp, OpOpSt, OpStSt, and StStSt. The spectra clearly show that abundant ions of OpStSt are ions $[M + NH_4]^+$ at *m/z* 882.7, $[M + NH_4\text{-RCOOH-}NH_3]^+$ at *m/z* 591.4 (StSt), *m/z* 589.4 (OpSt), $[RCO+74]^+$ at *m/z* 333.2, $[St]^+$ and *m/z* 331.2 $[Op]^+$ and $[RCO]^+$ at *m/z* 259.2 (St) and *m/z* 257.2 (Op). TAG were quantified based on total ion current, i.e. the sum of the abundances of ions in the range of 200–1000 Da.

Fig. 4 is a cutout from LC-MS between 15 and 20 min. While some peaks in Fig. 2 are poorly identifiable, in Fig. 4 all peaks are at least partially separated. This holds, e.g., for the TAG pair of OpOpE (Fig. 4S) and OpOpD (Fig. 5S). Even though the separation has not taken place to the base line, the tandem mass spectra of both TAGs, see Figs. 4S and 5S, clearly indicate that the proposed structure, including the determination of the majority regioisomers *sn*-OpOpE and *sn*-OpOpD, is correct. The presence of *sn*-OpOpE has been demonstrated based on the ratio of abundances of ions at *m/z* 587.4 and at *m/z* 615.4 ($[OpOp]^+$ and/or $[OpE]^+$) in a ratio of 73:100. In the case of molecular species *sn*-OpOpD the relevant ions were at *m/z* 587.4 and at *m/z* 641.5 ($[OpOp]^+$ and/or $[OpD]^+$) in the ratio of 75:100, which is in both cases indicative of the presence of asymmetric TAGs.Under the chromatographic conditions used no separation of regioisomers has taken place and the regioisomers were thus determined on the basis of ratios of diacylglycerol ions, e.g. ($[OpOp]^+$ and $[OpE]^+$ on the basis of the universally accepted rule on preferential cleavage of the secondary acyl glycerol backbone of hydroxyl (Byrdwell, 2005; Mottram, 2005). When analyzing TAGs the ±S.D. has not been determined due to the commercial unavailability of individual molecular species. This rule applies regardless of the type of instrument and also regardless of the type of the analyzed TAG, see e.g. (Holcapek et al., 2010); who analyzed

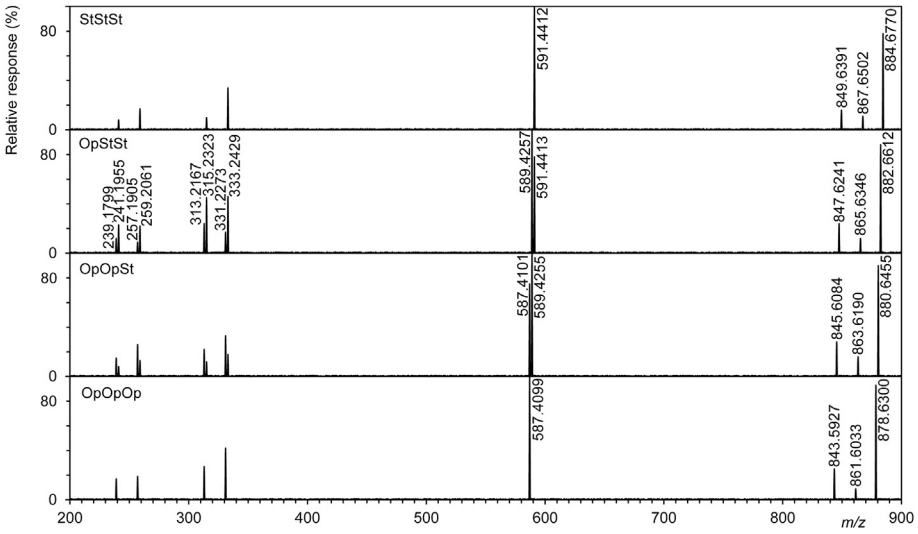

Fig. 3. Positive high resolution APCI mass spectra of TAGs (OpOpOp, OpOpSt, OpStSt, and StStSt).

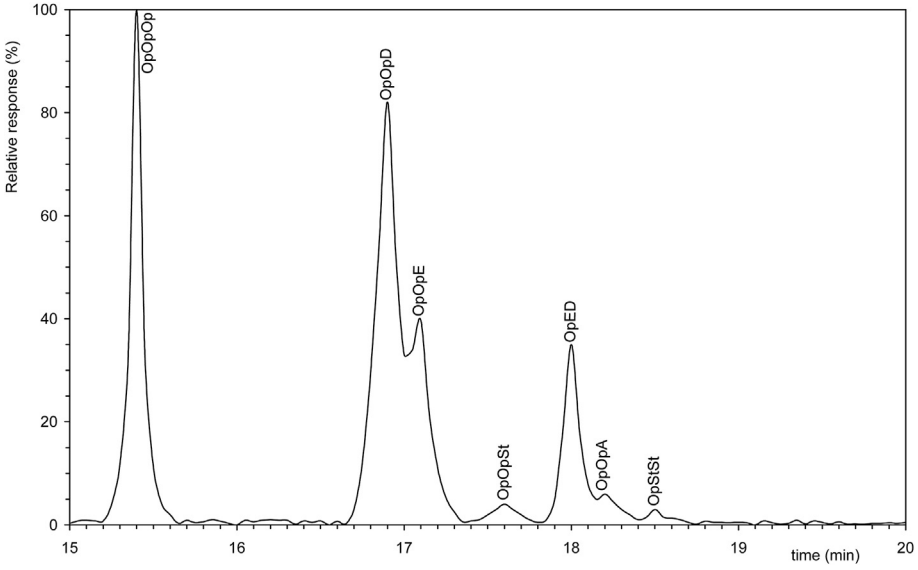

Fig. 4. A 15-to-20 min cutout from LC-MS of the alga *Coccolithophora* sp. cultivated at 30‰ salinity.

tens of TAGs with 1–8 double bonds on five structurally different mass spectrometers, e.g. Orbitrap, QqTOF, ion trap, single and triple quadrupole.

Vegetable oils containing PUFAs having 18 carbon atoms are the most complex mixtures of TAGs in the plant kingdom. Only fish oils can compete with them in complexity, i.e. in the number of TAG molecular species. According to the literature data, the analysis of complex vegetable oils, e.g. evening primrose, black currant, borage, etc., requires a column with a high efficiency C18 reverse phase, or better two or more columns connected in series. Separations of TAGs containing stearidonic acid are very rarely described since the starting material – suitable vegetable oil – is not routinely analyzed and the analysis is not a trivial matter.

Chromatography on two RP-18 columns connected in series (Czaplicki et al., 2009) identified 32 TAGs from the viper bugloss oil, the most abundant TAG being LnStSt. Separation of, e.g., α-Lnα-LnSt and α-Lnγ-LnSt was without problems. However, it is unclear whether some critical pairs have been separated from each other since evaporative light-scattering detector was used.

A study (Lisa et al., 2009) described the separation of C18 PUFA-containing TAGs (α-Ln, γ-Ln, and St) from hemp oil. Regioisomers were identified using a quadrupole mass spectrometer and based on the rule that a cleavage of FA from the *sn*-2 position on the glycerol skeleton is less preferred than the *sn*-1/3 positions and it was found that the *sn*-2 position is preferentially esterified by unsaturated FA. The most complex vegetable oil identified in this study was blackcurrant oil.

It is widely claimed that the retention time of TAGs having the same ECN value increases with decreasing number of double bonds (Czaplicki et al., 2009; Lisa et al., 2009). However, in many cases, this is not true - the following articles (Hori et al., 1994; Solaesa et al., 2014; Rezanka et al., 2012b) may serve as examples. On columns with C18 reverse phase, four TAGs (EEE, EED, EDD, and DDD) having an ECN = 30 were found to elute when using acetone-acetonitrile mixture, in the sequence EEE, EED, EDD, and DDD (Hori et al., 1994; Solaesa et al., 2014; Rezanka et al., 2012b),

whereas in another study the sequence was DDD, EDD, EED, and EEE (Perona and Ruiz-Gutierrez, 1999). The same applies to other TAGs, e.g. the pair LLE and LLD (Mu and Hoy, 2000), both of which have ECN = 38, or the four TAGs StStSt, StStE, StEE, and EEE with ECN = 30 (Solaesa et al., 2014). None of the published papers explains this discrepancy. We believe that with TAGs having more than ~10 double bonds, the large number of such bonds tends to distort ("curl") acyl chains, leading to the interaction of Π electrons of the double bonds with the molecules of the solvent to form complexes that are transient but for some time constant.

2.4. Effect of salinity on the relative content of TAGs

LC-MS was used for the analysis of all samples obtained by culturing the algae *Coccolithophora* sp., *Isochrysis* sp., *Isochrysis galbana*, *Prymnesium parvum* and *Prymnesium saltans* in media of five different salinities (Table 2), see also the results in Table 2S. Unfortunately, only two algae (*Coccolithophora* sp. and *Prymnesium parvum*) were able to grow at 7.5, 15, 30, 45, and 60‰ salinity. *Isochrysis* sp. grew only at 7.5 and 15‰ and *Isochrysis galbana* and *Prymnesium saltans* just under 30‰, i.e. up to the average salinity of the sea.

With increasing salinity of culture medium the representation of TAGs increases from 15% of the total TAGs in *Nannochloropsis salina* cultured at 22 PSU (Practical Salinity Unit, i.e. 22‰) to more than 35% TAGs at 58 PSU (Bartley et al., 2013).

Unfortunately, in many cases the relevant studies (Ho et al., 2014) concerned only the effect of culture medium salinity on the content of total lipids. Still, as found e.g. in the alga *Chlamydomonas*, the lipid content per total dry weight at NaCl concentration of 50‰ is higher than at 5, 20, and 35‰ NaCl.

The lipid content was examined in the green alga *Monoraphidium dybowskii* grown in a two-step cultivation, i.e. at different NaCl concentrations and in different growth phases. It was the highest at 20‰ (Yang et al., 2015).

Two green algae, *Chlamydomonas mexicana* and *Scenedesmus obliquus*, were investigated as to the effects of salinity (0.3, 0.4, 1.2, 1.9, 3.5, and 6.2‰) on the lipid content and it was found that the highest lipid content (37 and 34%) was determined at a salinity of 25 mM NaCl (1.45‰) (Salama et al., 2013).

The only study addressing the impact of salinity on the proportion of TAG molecular species in photosynthetic organisms has been performed on cotton seeds (Smaoui and Cherif, 2000). Cotton was watered with water containing various salt levels (0, 50, 100, and 150 mM NaCl, i.e. 0–8.7‰) and oil seeds 3, 4, 5 and 6 weeks after flowering were analyzed by NARP-HPLC. A total of 10 TAGs were identified. The major TAG was PLL, which alone has exceeded 20% of total TAGs. The relative content of the two most abundant TAGs, i.e. PLL and LLL, decreased with increasing soil salinity, the decrease being drastic when the plants were watered with 150 mM (8.7‰) NaCl. Conversely, the proportion of TAGs containing oleic acid increased. On the basis of published data (their Table 2) it can be said that this experiment fully confirmed the trend based on the analysis of FAs and was a good starting point for our work.

Fig. 5 shows the dependence of the number of double bonds in TAGs from *P. parvum* and *Coccolithophora* sp. on culture medium salinity. It is apparent that although the two algae taxonomically belong to different orders, they have similar maxima of TAG representation. For both algae, the maximum production of saturated TAGs takes place at NaCl concentration of 60‰ in the culture medium. Conversely, the maximum proportion of TAGs containing PUFAs (i.e. mainly St and Op) is observed at lower salinities – 7.5–30‰. As regards the chain length, Table 2, and Fig. 6 clearly show that regardless of the culture medium salinity the major FAs are always acids with 18 carbon atoms (ACN = 54). The influence of salinity on the proportion of C20 and C22 PUFA can be illustrated for the value of ACN = 56. According to Fig. 1, with increasing salinity the proportion of saturated FA decreases then increases. Saturated FA and PUFA exert effects that act against each other. For instance, as illustrated by PStD, the effect of a change in the content of palmitic acid is compensated by the change of two PUFA (St and D) and therefore the content of this TAG in *Coccolithophora* sp. at increasing salinities is highly variable (3.03, 2.29, 2.12, 2.46, 2.11% of total TAG).

Therefore, we henceforth pay attention to only three FAs (St, Op and Po) and only to those TAGs that contain these three acids. Fig. 7 shows the influence of salinity on the proportion of four TAGs (OpOpOp, OpOpSt, OpStSt and StStSt) in two algae, i.e. *Coccolithophora* sp. and *P. parvum*. The production of individual TAGs is clearly seen to differ at different salinities. The highest production of StStSt in *Coccolithophora* sp. and *P. parvum* takes place at 15‰ NaCl, while the optimum production of OpOpOp in *P. parvum* was at 45‰ NaCl in the medium, the proportion of this TAG being comparable to that of the three remaining TAGs. In both cases, the highest production of TAGs with Op and St was at 15‰.

In further analysis we focused on the relative content of regioisomers of 4 TAGs containing St and Op (*sn*-OpOpSt, *sn*-OpStOp and *sn*-OpStSt, *sn*-StOpSt), i.e. symmetric and asymmetric TAGs. Fig. 8 shows the representation of the individual regioisomers detected based on abundance ratios of the ion type $[M + NH_4\text{-}RCOOH\text{-}NH_3]^+$ (see above), specifically the ratio of pairs of ions at *m/z* 587.4 and 589.4 and at *m/z* 589.4 and 591.4. The calculation was made based on the respective abundance of diacylglycerol ions, e.g. 587.4 Da and 589.4 Da, or 589.4 Da and 591.4 Da. Its accuracy cannot be determined because of the lack of standards, but on the basis of the data of (Holcapek et al., 2010); the accuracy for a commercially available pair of TAG, i.e. LLnLn and LnLLn determined on five different instruments is in the range of ±S.D. equal to 1 to 4, e.g. 47 ± 1, 46 ± 1, 44 ± 2, etc. Based on these ratios or the ratio of abundances one can see that the ratio of asymmetric to symmetric TAGs decreases with increasing salinity. In the case of another two pairs of regioisomers, i.e. *sn*-PoStSt and *sn*-StPoSt, and *sn*-PoPoSt and *sn*-PoStPo, we found that TAGs having 6 or 9 double bonds follow the same pattern, i.e. with increasing salinity the regioisomers change from asymmetric to symmetric (Fig. 9). We have previously observed a similar trend that was caused by changes in temperature, N-starvation, etc. (Rezanka et al., 2011, 2012a, 2015).

3. Conclusion

In conclusion we would like to specify in more detail the culture conditions of the five strains of algae conducive to the production of polyenoic FAs and the TAGs containing these acids, with special reference to the two C18 PUFAs (Op and St) and one C16 PUFA (Po). In all five strains and particularly in *Coccolithophora* sp. and *P. parvum* an increase in salinity from 7.5‰ to 60‰ brings about a significant shift of FAs depending on the number of double bond(s). In both strains, the dependence of production of saturated FAs on salinity has a downward-curved shape while for PUFAs it has a upward-curved shape. The highest proportion of PUFAs was recorded at salinities of 15‰ and 30‰. The situation relating to TAGs is more complicated. TAGs containing both Op and St were investigated in most detail and it was found that the most convenient for the production of StStSt is *Coccolithophora* sp. and *P. parvum* cultured at a medium salinity of 15‰, while OpOpOp production is best in *P. parvum* growing at a medium salinity of 45‰.

Concerning the preparation of structured TAGs it was found that asymmetric TAGs are biosynthesized predominantly at lower

Table 2
Retention time (tR, min) and relative quantities (%) of molecular species of TAGs identified in algae *Coccolithophora* sp. and *Prymnesium parvum* cultivated at different salinities.

tR	TAG	*Coccolithophora* sp. 7.5‰	*Coccolithophora* sp. 15.0‰	*Coccolithophora* sp. 30.0‰	*Coccolithophora* sp. 45.0‰	*Coccolithophora* sp. 60.0‰	*Prymnesium parvum* 7.5‰	*Prymnesium parvum* 15.0‰	*Prymnesium parvum* 30.0‰	*Prymnesium parvum* 45.0‰	*Prymnesium parvum* 60.0‰
15.4	OpOpOp	0.53	0.99	1.36	1.12	0.73	0.71	1.90	4.56	5.63	3.57
16.9	OpOpD	0.44	0.83	1.20	0.87	0.54	0.49	1.23	2.54	2.10	1.13
17.1	OpOpE	0.21	0.39	0.57	0.32	0.30	0.06	0.20	0.70	0.51	0.39
17.6	OpOpSt	1.32	2.28	1.95	1.55	0.90	2.00	4.04	6.08	6.26	4.50
18.0	OpED	0.17	0.33	0.51	0.25	0.21	0.04	0.13	0.40	0.20	0.13
18.1	OpOpA	0.03	0.04	0.07	0.07	0.06	0.02	0.04	0.10	0.09	0.07
18.5	OpStSt	3.30	5.19	2.82	2.16	1.11	5.72	8.57	8.10	6.95	5.67
18.9	DDD	0.31	0.56	0.95	0.52	0.30	0.24	0.51	0.79	0.30	0.12
19.2	EDD	0.15	0.27	0.45	0.20	0.17	0.03	0.09	0.22	0.08	0.04
19.7	EED	0.07	0.13	0.22	0.08	0.09	0.01	0.02	0.07	0.02	0.02
20.2	EEE	0.03	0.06	0.11	0.03	0.06	0.00	0.00	0.02	0.01	0.01
21.1	EESt	0.20	0.35	0.35	0.13	0.16	0.03	0.06	0.15	0.06	0.07
21.8	StStSt	8.34	11.87	4.07	3.01	1.39	16.30	18.23	10.80	7.72	7.15
21.8	OpOpPo	0.32	0.83	1.06	0.53	0.19	0.69	0.71	1.27	1.64	2.01
22.6	OpOpO	0.24	0.65	0.95	0.64	0.40	0.47	1.08	2.49	2.86	1.45
23.9	LOpD	0.13	0.19	0.69	0.45	0.26	0.10	0.43	0.74	0.49	0.13
24.6	PoOpD	0.27	0.68	0.94	0.41	0.14	0.48	0.46	0.71	0.62	0.64
25.9	StLnSt	1.61	2.98	1.16	0.77	0.39	2.49	4.22	2.07	1.36	0.93
28.8	LDD	0.11	0.16	0.61	0.35	0.19	0.07	0.28	0.42	0.19	0.04
30.1	LED	0.05	0.08	0.29	0.13	0.10	0.01	0.05	0.12	0.05	0.02
30.7	PoED	0.11	0.27	0.40	0.12	0.06	0.04	0.05	0.12	0.06	0.08
31.8	LStD	0.31	0.42	1.00	0.62	0.31	0.29	0.91	0.99	0.54	0.17
33.7	POpD	1.21	1.01	1.47	1.76	1.71	0.74	0.71	1.00	1.38	1.17
34.8	EEPo	0.05	0.13	0.19	0.04	0.03	0.01	0.01	0.04	0.02	0.03
35.1	PoStD	0.66	1.55	1.36	0.57	0.17	1.36	0.97	0.95	0.68	0.81
35.8	LLnOp	0.08	0.13	0.32	0.21	0.11	0.07	0.33	0.35	0.26	0.07
36.3	LnLnSt	0.32	0.76	0.34	0.21	0.11	0.39	0.99	0.41	0.25	0.13
36.6	LStSt	0.90	1.14	1.62	1.10	0.51	1.13	2.98	2.34	1.60	0.62
36.6	PoStSt	1.97	4.27	2.21	1.01	0.29	5.55	3.16	2.25	2.02	3.20
36.8	ODD	0.17	0.44	0.75	0.39	0.23	0.23	0.45	0.78	0.40	0.15
37.3	LDpD	0.04	0.06	0.24	0.14	0.07	0.03	0.11	0.21	0.08	0.02
37.8	PDD	1.01	0.83	1.31	1.37	1.27	0.51	0.46	0.56	0.52	0.38
38.4	PED	0.47	0.40	0.62	0.50	0.69	0.06	0.08	0.16	0.13	0.14
38.9	EEO	0.04	0.10	0.17	0.05	0.07	0.01	0.02	0.07	0.03	0.02
40.6	OStD	0.50	1.21	1.22	0.70	0.37	0.92	1.48	1.85	1.19	0.58
41.0	PEE	0.22	0.19	0.30	0.20	0.37	0.02	0.02	0.05	0.04	0.06
41.5	PoLnD	0.13	0.39	0.39	0.15	0.06	0.21	0.23	0.19	0.13	0.11
42.5	LLnE	0.03	0.06	0.14	0.07	0.06	0.01	0.04	0.06	0.03	0.01
42.9	PStD	3.03	2.29	2.12	2.46	2.11	2.09	1.50	1.34	1.53	1.47
43.6	LnLnLn	0.07	0.20	0.10	0.05	0.03	0.06	0.23	0.08	0.05	0.02
44.1	OStSt	1.49	3.35	1.98	1.24	0.60	3.75	4.87	4.42	3.53	2.30
44.5	LLOp	0.05	0.06	0.45	0.29	0.16	0.03	0.24	0.39	0.31	0.05
45.2	LLnSt	0.19	0.30	0.47	0.28	0.14	0.19	0.70	0.46	0.29	0.09
46.1	PoLnSt	0.39	1.08	0.63	0.27	0.09	0.86	0.74	0.44	0.37	0.42
46.3	OpPoPo	0.19	0.68	0.83	0.25	0.06	0.67	0.27	0.36	0.49	1.14
46.6	ODpD	0.06	0.16	0.29	0.15	0.09	0.07	0.17	0.39	0.18	0.06
47.4	LLD	0.04	0.05	0.40	0.23	0.11	0.02	0.16	0.22	0.12	0.02
47.6	DLnP	0.60	0.58	0.61	0.63	0.59	0.33	0.35	0.26	0.28	0.20
47.7	StStP	9.17	6.34	3.65	3.55	3.53	8.61	4.92	3.18	4.57	5.88
47.8	LLE	0.02	0.02	0.19	0.09	0.07	0.00	0.03	0.06	0.03	0.01
48.2	PoLD	0.08	0.16	0.54	0.21	0.07	0.10	0.16	0.21	0.15	0.08
48.5	SStSt	5.08	2.15	0.62	2.47	2.54	2.55	2.51	1.50	1.25	1.61
48.7	LLnLn	0.04	0.08	0.14	0.08	0.04	0.03	0.17	0.09	0.06	0.01
49.4	OpOL	0.07	0.15	0.55	0.33	0.19	0.10	0.38	0.73	0.67	0.17
49.6	StLL	0.11	0.12	0.65	0.40	0.20	0.09	0.50	0.52	0.34	0.07

50.2	PoLnLn	0.08	0.28	0.19	0.08	0.03	0.14	0.18	0.09	0.07	0.06
50.3	OStLn	0.30	0.85	0.57	0.33	0.17	0.58	1.14	0.86	0.63	0.31
50.5	EPoPo	0.08	0.27	0.35	0.08	0.03	0.06	0.03	0.06	0.05	0.13
50.6	PoLSt	0.23	0.42	0.88	0.37	0.11	0.39	0.53	0.50	0.43	0.28
50.9	PLOp	0.40	0.28	0.95	1.15	1.06	0.22	0.39	0.53	0.86	0.41
51.2	StLnP	1.77	1.60	0.99	1.12	0.96	1.32	1.15	0.62	0.81	0.76
51.4	OpOPo	0.15	0.54	0.75	0.30	0.11	0.45	0.41	0.70	0.84	0.82
51.7	PPoOp	0.86	1.01	1.30	1.07	0.59	1.03	0.41	0.51	1.08	2.09
52.1	SDpD	0.18	0.11	0.10	0.30	0.34	0.05	0.09	0.14	0.07	0.04
52.5	OLD	0.06	0.12	0.49	0.26	0.14	0.07	0.25	0.41	0.25	0.06
52.7	PoPoSt	0.47	1.55	1.20	0.35	0.07	1.89	0.56	0.48	0.54	1.43
52.8	LLDp	0.01	0.02	0.16	0.09	0.04	0.01	0.06	0.11	0.05	0.01
53.1	PoLDp	0.03	0.06	0.21	0.09	0.03	0.03	0.06	0.11	0.07	0.03
53.9	LnOLn	0.06	0.22	0.17	0.09	0.06	0.09	0.27	0.17	0.12	0.05
54.2	DOPo	0.12	0.44	0.66	0.24	0.09	0.32	0.26	0.39	0.32	0.26
54.3	LLLn	0.02	0.03	0.19	0.11	0.06	0.02	0.12	0.10	0.06	0.01
54.6	DPoP	0.72	0.83	1.15	0.83	0.44	0.72	0.27	0.29	0.41	0.66
54.8	OLSt	0.18	0.33	0.79	0.46	0.23	0.27	0.81	0.99	0.74	0.21
54.8	OpOO	0.11	0.42	0.67	0.37	0.21	0.31	0.62	1.36	1.46	0.60
55.0	EOL	0.03	0.06	0.23	0.10	0.07	0.01	0.04	0.12	0.07	0.02
55.3	PoOSt	0.36	1.21	1.08	0.42	0.13	1.28	0.85	0.93	0.93	1.04
55.6	PLE	0.16	0.11	0.40	0.34	0.43	0.02	0.04	0.09	0.09	0.06
55.7	PLSt	0.99	0.62	1.37	1.60	1.30	0.60	0.82	0.70	0.96	0.51
55.8	OLnLn	0.06	0.22	0.17	0.09	0.06	0.09	0.27	0.17	0.12	0.05
56.2	SLnSt	0.99	0.55	0.18	0.63	0.70	0.40	0.59	0.30	0.23	0.22
56.3	EOPo	0.06	0.21	0.32	0.09	0.04	0.04	0.05	0.11	0.08	0.10
57.0	PoLLn	0.05	0.11	0.26	0.10	0.03	0.07	0.13	0.10	0.08	0.04
57.7	LLPo	0.03	0.05	0.35	0.14	0.04	0.03	0.09	0.11	0.09	0.03
58.0	LnLnP	0.36	0.41	0.29	0.29	0.27	0.21	0.27	0.12	0.15	0.11
58.1	OLDp	0.02	0.05	0.19	0.11	0.06	0.02	0.09	0.21	0.11	0.02
58.2	EOO	0.05	0.17	0.29	0.11	0.09	0.03	0.07	0.22	0.14	0.07
58.3	OLLn	0.04	0.09	0.23	0.12	0.07	0.05	0.19	0.19	0.14	0.03
58.5	POD	0.55	0.65	1.04	1.01	0.93	0.49	0.41	0.55	0.71	0.48
58.6	PLDp	0.12	0.09	0.33	0.36	0.30	0.05	0.09	0.15	0.15	0.06
58.9	PoLPo	0.06	0.16	0.48	0.13	0.03	0.14	0.10	0.11	0.12	0.13
59.0	EPoP	0.34	0.40	0.55	0.30	0.24	0.08	0.05	0.08	0.11	0.24
59.2	PoPoLn	0.10	0.39	0.35	0.10	0.03	0.30	0.13	0.10	0.10	0.19
59.3	PPoSt	2.16	2.29	1.87	1.48	0.73	2.93	0.86	0.67	1.20	2.63
59.6	MLLn	0.07	0.04	0.04	0.13	0.16	0.04	0.06	0.09	0.08	0.06
59.8	SLnLn	0.20	0.14	0.06	0.17	0.20	0.07	0.14	0.06	0.04	0.03
60.1	PoPoPo	0.12	0.56	0.65	0.12	0.01	0.65	0.10	0.10	0.15	0.65
60.2	MPoLn	0.14	0.12	0.06	0.12	0.09	0.17	0.06	0.08	0.09	0.26
60.6	StOO	0.28	0.96	0.97	0.51	0.27	0.87	1.31	1.82	1.62	0.75
60.7	POSt	1.64	1.80	1.68	1.80	1.53	1.98	1.32	1.31	2.09	1.89
60.8	LLL	0.02	0.01	0.26	0.15	0.07	0.01	0.09	0.12	0.08	0.01
60.9	SLSt	0.56	0.22	0.25	0.90	0.94	0.19	0.42	0.34	0.27	0.15
60.9	PLLn	0.21	0.16	0.40	0.41	0.37	0.10	0.19	0.14	0.18	0.08
61.0	PPoLn	0.43	0.58	0.54	0.39	0.21	0.45	0.21	0.13	0.22	0.35
61.1	PoPoL	0.06	0.16	0.48	0.13	0.03	0.14	0.10	0.11	0.12	0.13
61.3	PoLL	0.03	0.05	0.35	0.14	0.04	0.03	0.09	0.11	0.09	0.03
61.6	PPE	1.52	0.58	0.86	1.29	2.86	0.12	0.07	0.11	0.23	0.42
61.8	OLnPo	0.08	0.31	0.31	0.11	0.04	0.20	0.20	0.19	0.17	0.14
61.9	LnOM	0.11	0.09	0.05	0.14	0.19	0.12	0.09	0.16	0.16	0.19
62.2	MLL	0.04	0.02	0.06	0.17	0.21	0.02	0.04	0.10	0.09	0.04
62.8	LPoM	0.08	0.05	0.08	0.16	0.11	0.08	0.05	0.09	0.11	0.17
63.3	SOD	0.31	0.23	0.19	0.57	0.67	0.15	0.21	0.26	0.20	0.14
63.5	OOLn	0.06	0.25	0.28	0.14	0.07	0.14	0.31	0.36	0.29	0.10
63.7	OLM	0.07	0.04	0.07	0.20	0.24	0.06	0.07	0.18	0.19	0.13
63.7	SLDp	0.07	0.03	0.06	0.20	0.23	0.02	0.05	0.07	0.04	0.02

(*continued on next page*)

Table 2 (*continued*)

tR	TAG	*Coccolithophora* sp. 7.5‰	*Coccolithophora* sp. 15.0‰	*Coccolithophora* sp. 30.0‰	*Coccolithophora* sp. 45.0‰	*Coccolithophora* sp. 60.0‰	*Prymnesium parvum* 7.5‰	*Prymnesium parvum* 15.0‰	*Prymnesium parvum* 30.0‰	*Prymnesium parvum* 45.0‰	*Prymnesium parvum* 60.0‰
63.9	PODp	0.18	0.24	0.40	0.40	0.36	0.15	0.15	0.28	0.31	0.18
64.0	MPLn	0.64	0.17	0.09	0.49	1.03	0.27	0.09	0.12	0.21	0.46
64.1	SLLn	0.12	0.06	0.08	0.24	0.27	0.03	0.10	0.07	0.05	0.02
64.2	PoPoM	0.17	0.17	0.11	0.15	0.07	0.38	0.05	0.09	0.14	0.86
64.3	LnOP	0.33	0.46	0.48	0.47	0.43	0.31	0.31	0.26	0.38	0.25
64.4	OLL	0.02	0.04	0.32	0.17	0.09	0.02	0.14	0.22	0.16	0.02
64.5	LnSPo	0.24	0.20	0.10	0.22	0.16	0.14	0.11	0.07	0.06	0.10
65.5	POA	0.04	0.04	0.06	0.08	0.10	0.02	0.02	0.03	0.04	0.04
65.8	SOSt	0.91	0.62	0.31	1.01	1.11	0.60	0.68	0.62	0.58	0.53
66.0	PoOL	0.04	0.12	0.43	0.15	0.06	0.10	0.15	0.21	0.20	0.10
66.0	PoPoO	0.09	0.44	0.59	0.14	0.03	0.44	0.15	0.20	0.25	0.47
66.3	PLL	0.12	0.07	0.55	0.59	0.49	0.05	0.14	0.16	0.21	0.06
66.7	PPoL	0.25	0.23	0.75	0.54	0.29	0.21	0.15	0.15	0.26	0.24
67.2	PPLn	1.94	0.86	0.84	1.64	2.41	0.70	0.32	0.19	0.48	0.63
67.3	PoPoP	0.52	0.83	1.02	0.51	0.17	1.00	0.16	0.15	0.32	1.18
67.5	OPoM	0.13	0.13	0.10	0.18	0.14	0.26	0.07	0.17	0.24	0.63
67.6	MPL	0.37	0.07	0.12	0.70	1.39	0.13	0.07	0.13	0.24	0.31
67.9	MPoO	0.13	0.13	0.10	0.18	0.14	0.26	0.07	0.17	0.24	0.63
68.3	PMM	1.19	0.08	0.03	0.82	1.17	0.34	0.03	0.11	0.29	2.13
68.4	PoPM	0.78	0.25	0.16	0.64	0.77	0.58	0.07	0.13	0.31	1.58
68.6	PSDp	0.58	0.16	0.13	0.78	1.43	0.11	0.08	0.10	0.12	0.13
70.0	SLnP	1.08	0.30	0.16	0.92	1.74	0.21	0.16	0.09	0.14	0.18
70.2	SLL	0.07	0.02	0.10	0.34	0.36	0.02	0.07	0.08	0.06	0.02
70.6	OOL	0.04	0.10	0.39	0.20	0.10	0.07	0.22	0.40	0.34	0.07
70.7	SOLn	0.19	0.16	0.09	0.26	0.31	0.10	0.16	0.13	0.11	0.07
71.0	PoOO	0.07	0.35	0.53	0.17	0.06	0.30	0.23	0.39	0.43	0.34
71.5	POPo	0.40	0.65	0.91	0.61	0.33	0.68	0.24	0.28	0.55	0.85
71.6	OLP	0.19	0.18	0.67	0.66	0.57	0.15	0.22	0.29	0.44	0.17
71.9	OOM	0.10	0.11	0.09	0.23	0.29	0.18	0.11	0.33	0.41	0.45
72.8	PPL	1.09	0.33	1.17	2.33	3.30	0.32	0.23	0.21	0.57	0.42
73.0	SOPo	0.22	0.23	0.17	0.35	0.24	0.21	0.12	0.14	0.16	0.24
73.4	PPPo	2.37	1.23	1.60	2.15	1.81	1.54	0.24	0.20	0.72	2.16
73.9	SLnS	0.60	0.11	0.03	0.72	1.26	0.07	0.09	0.05	0.04	0.07
74.1	OPM	0.60	0.19	0.15	0.78	1.64	0.40	0.11	0.24	0.53	1.14
74.5	MPP	3.61	0.36	0.25	2.76	3.94	0.90	0.11	0.18	0.68	2.91
75.1	OOO	0.05	0.27	0.47	0.22	0.11	0.20	0.35	0.75	0.74	0.25
75.5	SPM	2.00	0.13	0.05	1.55	2.67	0.27	0.06	0.09	0.19	0.80
76.2	SOL	0.11	0.07	0.12	0.37	0.41	0.05	0.12	0.14	0.13	0.05
77.2	POO	0.31	0.51	0.82	0.75	0.67	0.46	0.36	0.54	0.96	0.62
77.7	SLS	0.34	0.04	0.04	0.74	1.71	0.03	0.06	0.05	0.05	0.04
78.5	PSL	0.61	0.12	0.21	1.30	2.37	0.10	0.12	0.10	0.16	0.13
79.5	OPP	1.80	0.96	1.23	2.83	3.90	1.05	0.36	0.39	1.17	1.56
80.0	PPP	1.58	1.82	2.49	2.88	4.77	2.39	0.37	0.28	1.61	3.97
81.8	PPS	6.13	0.62	0.45	3.54	5.30	0.71	0.19	0.14	0.45	1.09
82.1	SOS	0.56	0.12	0.05	0.84	2.03	0.10	0.10	0.09	0.10	0.12
83.4	OOS	0.17	0.18	0.15	0.42	0.49	0.14	0.19	0.26	0.27	0.17
85.8	PSO	1.01	0.33	0.26	1.45	2.81	0.32	0.19	0.19	0.35	0.44

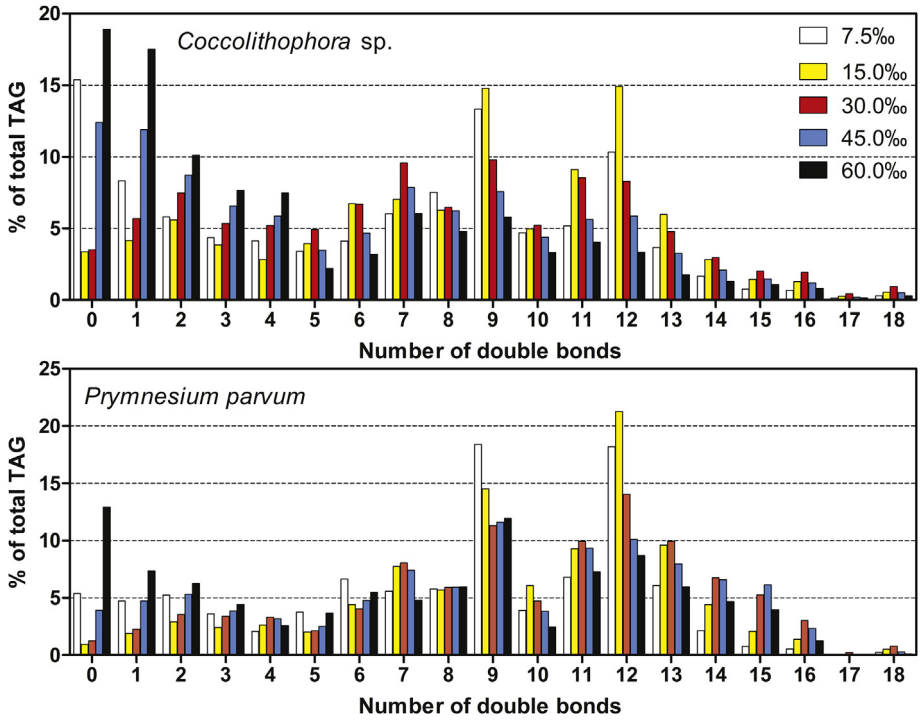

Fig. 5. Dependence of the number of double bonds in *Coccolithophora* sp. and *P. parvum* on salinity.

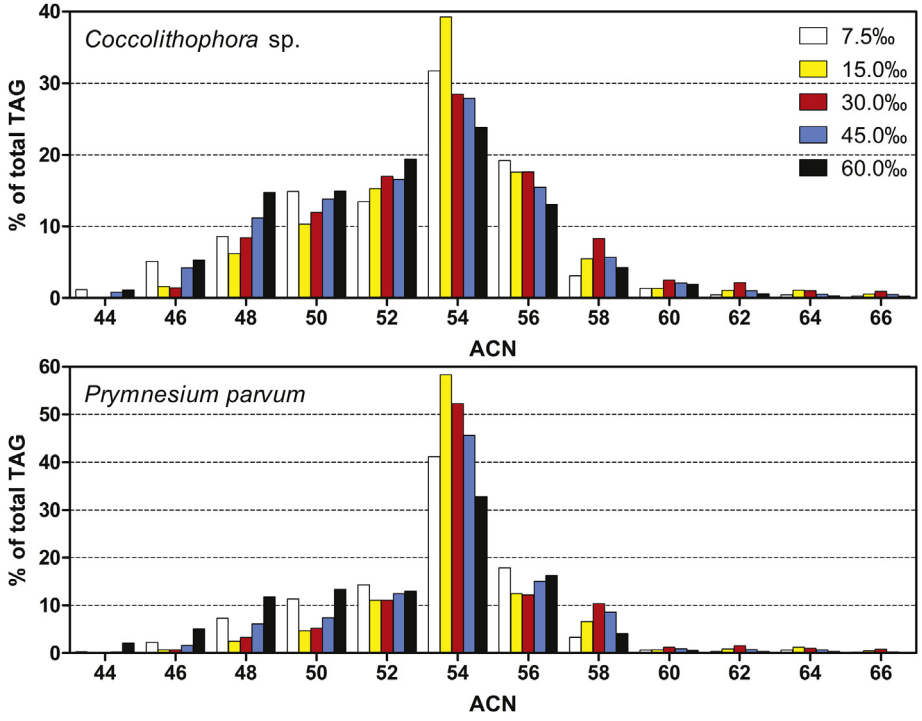

Fig. 6. Dependence of the acyl chain length in *Coccolithophora* sp. and *P. parvum* on salinity.

salinity (7.5‰) where e.g. the ratio of *sn*-OpOpSt to *sn*-OpStOp (asymmetric to symmetric TAG) decreased from 9:1 to 2:3 at 60.0‰. The situation with the other pair of TAGs (sn-OpStSt, sn-StOpSt) was similar, see Fig. 8.

With increasing salinity, two pairs of regioisomers containing palmitoleic and stearidonic acids, i.e. TAGs having 6 or 9 double bonds exhibited a similar change of the regioisomers from asymmetric to symmetric (Fig. 9).

Another important finding is that TAGs containing PUFAs with 5 and 6 double bonds do not behave in NARP-LC according to the rule "the retention time of TAG within one ECN group increases with decreasing DB number in acyl chains", but the other way around. This can be explained by the formation of a transition complex of the TAG molecules with acetonitrile in the mobile phase.

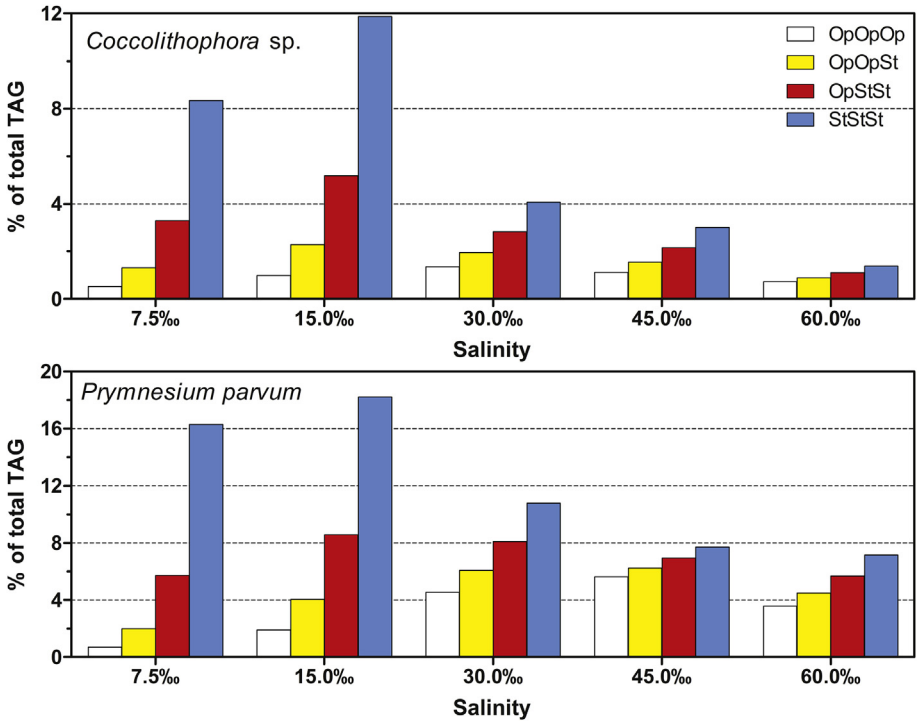

Fig. 7. Dependence of the proportion of OpOpOp, OpOpSt, OpStSt, and StStSt in *Coccolithophora* sp. and *P. parvum* on salinity.

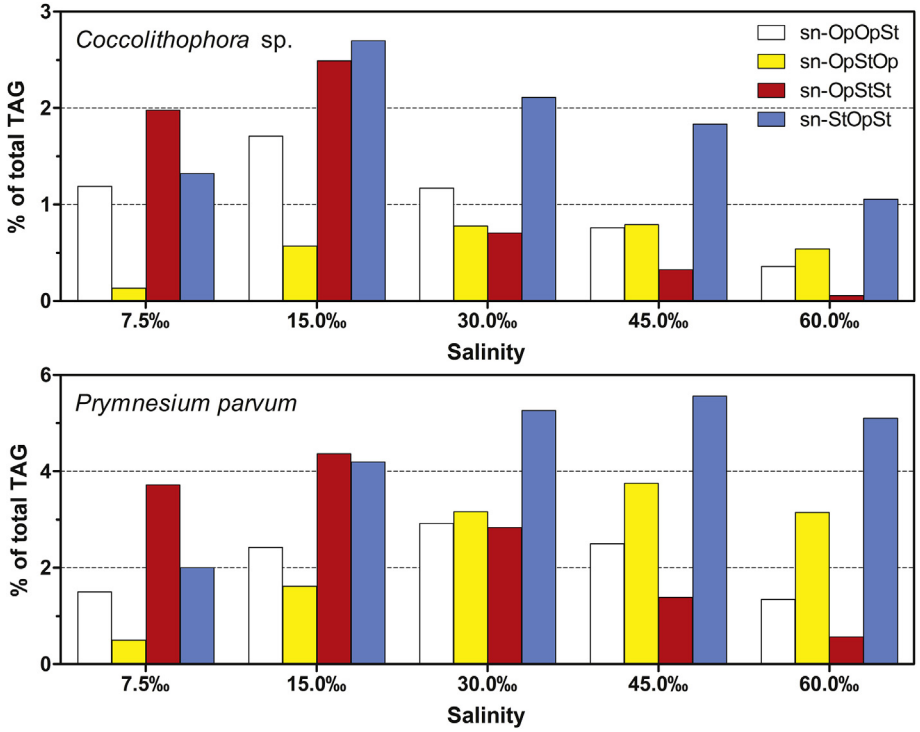

Fig. 8. Dependence of the representation of the individual regioisomers of *sn*-OpOpSt, *sn*-OpStOp and *sn*-OpStSt, *sn*-StOpSt (symmetric and asymmetric TAGs) in *Coccolithophora* sp. and *P. parvum* on salinity.

4. Experimental

4.1. Standards and instrumentation

Acetonitrile, 2-propanol, Mg turnings, THF, hexane, dichloromethane, glycerol, 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 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

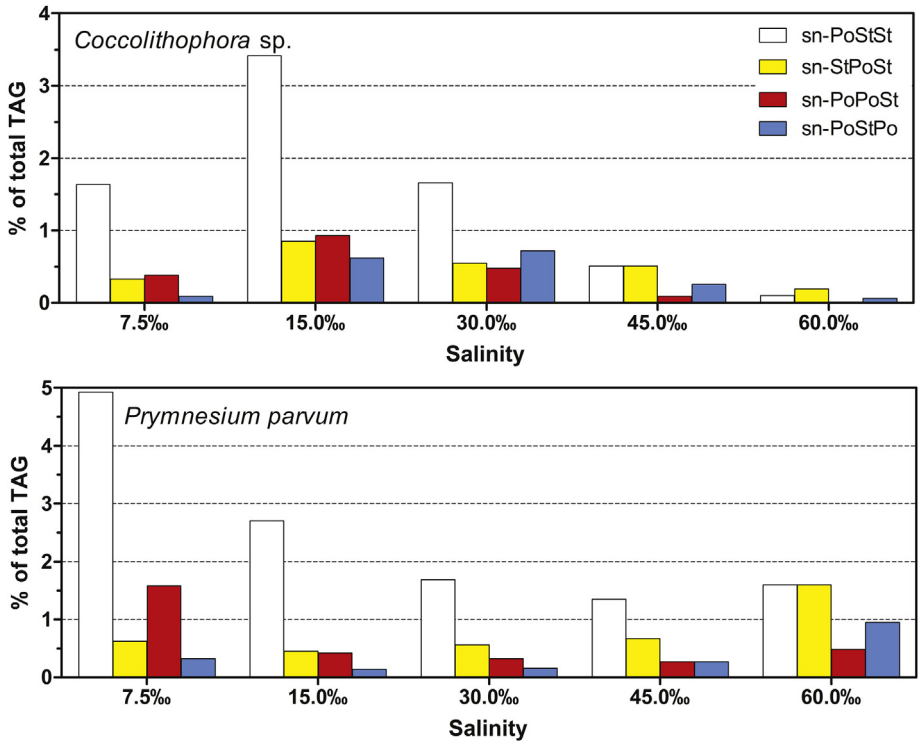

Fig. 9. Dependence of the representation of the individual regioisomers of *sn*-PoStSt, *sn*-StPoSt and *sn*-PoPoSt, *sn*-PoStPo (symmetric and asymmetric TAGs) in *Coccolithophora* sp. and *P. parvum* on salinity.

(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 (^{1}H) and 125.7 MHz (^{13}C).

4.2. Cultivation

Five strains of the haptophyte algae *Coccolithophora* sp. (SAG 912-1), *Prymnesium parvum* (SAG 127.79), *Prymnesium saltans* (SAG 18.97), *Isochrysis galbana* (SAG 13.92) and *Isochrysis* sp. (SAG 927-3) were obtained from the Culture Collection of Algae at the University of Göttingen, Germany. The strains were isolated from marine (SAG 13.92), brackish (SAG 127.79) or freshwater habitats (SAG 18.97); in case of the two other strains the salinity of original habitat was not indicated. The cultures were grown in liquid artificial seawater medium with vitamins (ASM + V) (www.epsag.uni-goettingen.de) and aerated. To assess the influence of different salinity levels, five modifications of the ASM medium were used, with 7.5‰, 15‰, 30‰, 45‰ and 60‰ salinity. The salinity level in the medium was obtained by adding the given amount of NaCl. The light was provided continuously by a cool white fluorescent tube (Standard 8W, Sylvania, USA) and the cultivation temperature was 22 °C. The volume of the algal suspension in cultivation flasks was 450 ml. After reaching stationary phase, the cultures were harvested by centrifugation at 1800 rpm for 15 min, and the samples were stored frozen until solvent extraction.

4.3. 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 from room temperature to 4 °C, one part of chloroform was added and the lipids from the lyophilized cells 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 of 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 TAG 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.

4.4. FAMEs analysis

The TAGs (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A FA fraction obtained from saponification was partitioned between alkali solution (pH 9) and diethyl ether to remove basic and neutral components. The aqueous phase, containing FAs, was acidified to pH 2 and extracted with hexane. The FA fraction was methylated using diazomethane. 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).

4.5. 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 APCI 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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.06.001.

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