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Lipidomic profile in three species of dinoflagellates (*Amphidinium carterae*, *Cystodinium* sp., and *Peridinium aciculiferum*) containing very long chain polyunsaturated fatty acids

Tomáš Řezanka [a, *], Jaromír Lukavský [b], Linda Nedbalová [c], Karel Sigler [a]

[a] Institute of Microbiology, Vídeňská 1083, 142 20 Prague, Czech Republic
[b] Institute of Botany, Academy of Sciences of the Czech Republic, Biorefinery Res. Centre of Competence, Dukelská 135, 379 82 Třeboň, Czech Republic
[c] Charles University, Faculty of Science, Department of Ecology, Viničná 7, 128 44 Prague, Czech Republic

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ABSTRACT

This study describes the identification of very long chain polyunsaturated fatty acids (VLCPUFAs) in three strains of dinoflagellates (*Amphidinium carterae*, *Cystodinium* sp., and *Peridinium aciculiferum*). The strains were cultivated and their lipidomic profiles were obtained by high resolution mass spectrometry with the aid of positive and negative electrospray ionization (ESI) mode by Orbitrap apparatus. Hydrophilic interaction liquid chromatography (HILIC/ESI) was used to separate major lipid classes of the three genera of dinoflagellates by neutral loss scan showing the ion $[M + H\text{-}28{:}8]^+$, where 28:8 was octacosaoctaenoic acid, and by precursor ion scanning of ions at *m/z* 407, which was an ion corresponding to the structure of acyl of 28:8 acid ($C_{27}H_{39}COO^-$). Based on these analyzes, it was found that out of more than a dozen lipid classes present in the total lipids, only two classes of neutral lipids, i.e. major triacylglycerols and minor diacylglycerols contain VLCPUFAs. In polar lipids, VLCPUFAs were identified only in phosphatidic acid (PA) and phosphatidyl choline (PC) or in their lyso-forms (LPA and LPC). Further analysis of individual lipid classes by reversed-phase high-performance liquid chromatography (RP-HPLC) showed the presence of triacylglycerols (TAGs) containing VLCPUFAs, i.e. molecular species of the *sn*-28:7/28:8/28:8, *sn*-26:7/28:7/28:8, or *sn*-26:7/28:8/28:8 types. These TAGs are the longest and most unsaturated TAGs isolated from a natural source that have yet been synthesized. In the case of PA and PC, tandem MS identified *sn*-28:8/16:0-PA and *sn*-28:8/16:0-PC and the corresponding lyso-forms (28:8-LPC and 28:8-LPA). All these results indicate that TAGs containing VLCPUFAs are biosynthesized in dinoflagellates in the same manner as in higher eukaryotic organisms, which means that the PA, after conversion to DAG, serves as a precursor in the biosynthesis of other phospholipids, e.g. PC, and, after further acylation, also of TAG.

1. Introduction

Dinoflagellates are primitive unicellular eukaryotic protists with some prokaryotic features in the ultrastructure of their chromosomes, which lack typical histones and nucleosomes (Herzog and Soyer, 1981; Rizzo, 1991). Photosynthetic forms are often mixotrophic (Stoecker, 1999) and can occur in all aquatic environments – freshwater, brackish and marine. Their usual storage product is starch, but some types also produce lipids (Van den Hoek et al., 1996). Some dinoflagellates have the ability to produce toxic substances. Their excessive growth in the sea or brackish water can give rise to very toxic algal blooms called red tides (Hallegraeff, 1993).

Very long chain polyunsaturated fatty acids (VLCPUFAs), i.e. fatty acids (FAs) with more than 22 carbon atoms are found in nature quite often, but with some exceptions in minor concentrations (Rezanka, 1989; Rezanka and Sigler, 2009). VLCPUFAs (e.g. 24:4n-6, 24:5n-6, 28:4n-6, 30:5n-6, 30:6n-3, 32:6n-3, 34:6n-3, etc.) were found mainly in mammalian tissues and organs (Poulos, 1995), where they are always substituted at position *sn*-1 of phosphatidylcholine (PC), while the *sn*-2 position is enriched with saturated, monounsaturated and polyunsaturated fatty acids with <24 carbon atoms. They were also identified in fish and in fish

* Corresponding author.
E-mail address: rezanka@biomed.cas.cz (T. Řezanka).

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eating animals, e.g. ringed seal, Baltic herring, and roughscale sole (Fukuda and Ando, 2011; Kakela et al., 1995; Linko and Karinkanta, 1970; Ota et al., 1994).

In addition to the occurrence in mammals and fishes VLCPUFAs were also found in invertebrates – in brittle stars and jellyfish (Mansour et al., 2005a; Svetashev and Kharlamenko, 2015; Takagi et al., 1986) or in the freshwater crustacean *Bathynella natans* , where a FA has been identified as tetratriacontanonaenoic acid (34:9n-6) and it was present in diacyl PC with 16:0, 18:0 and 16:1 as the other acyl chains (Rezanka and Dembitsky, 2004).

VLCPUFAs were found primarily in freshwater and marine dinoflagellates, see Table 1S (Supplements). These planktonic organisms serve as food for many marine animals and they form the basis of the food chains. Their VLCPUFAs are common in phospholipids (Leblond and Chapman, 2000; Leblond et al., 2003) and exceptionally also in other classes of lipids, see below. Both PC and diacylglycerylcarboxy-*N*-hydroxymethylcholine (DGCC) isolated from freshwater dinoflagellate *Peridinium aciculiferum* contain molecular species 46:15, which means that one of the two acids must have at least 24 carbon atoms or more and simultaneously either 7 or 8 double bonds (Flaim et al., 2014).

HPLC/ESI-MS and ^{13}C-NMR were used to determine that docosahexaenoic acid (DHA)-containing single cell oil (DHASCO) isolated from the marine species *Crypthecodinium cohnii* contains over 45 molecular species of TAGs with DHA (22:6n-3) as the major fatty acid. Twenty two TAGs contained one DHA, 12 TAGs contained two DHA, and tridocosahexaenoin was also present. The molecular weights of the TAGs ranged from 710 to 1182 Da. They were assigned to correspond to DHA/10:0/10:0 to DHA/28:8/28:8, the latter TAG being the most unsaturated triacylglycerol ever isolated (Obeng et al., 2005).

We analyzed the lipids of 3 strains of dinoflagellates; using liquid chromatography–mass spectrometry (LC-MS) analysis we identified different classes of lipids, predominantly triacylglycerols and phospholipids, and hundreds of different molecular species, and performed tandem MS analysis of regioisomers of many of these lipids. We found that VLCPUFA were located at position *sn*-1(3) of triacylglycerols and at position *sn*-1 of phosphatidylcholine.

2. Results and discussion

The use of dinoflagellates as model organisms is attractive for studies of VLCPUFA content because there are already studies, see below, which describe traces of VLCPUFAs in dinoflagellates, see also Table 1S, but a detailed description of the various classes of lipids isolated from them has not yet been published.

2.1. Analysis of VLCPUFAs

The first part of the analysis included determination of total FAs after extraction from cells (for the names of strains see Experimental), saponification, esterification and identification by gas chromatography–mass spectrometry (GC-MS), see Table 1. This table shows that VLCPUFAs (28:8n-3, 28:7n-6, 26:7n-3) were present in a very similar amount in all examined dinoflagellates. The content of 24:6n-3 was more variable. The results indicate that even-numbered FAs from C_{14} to C_{28} are present and have zero to eight double bonds. The three major acids were palmitic (16:0), octadecatetraenoic (stearidonic acid, 18:4) and octadecapentaenoic (18:5); their relative proportions were above 10% of total FAs. Relatively abundant were also two PUFAs, eicosapentaenoic and docosahexaenoic acids. Other FAs, including VLCPUFAs were found in minor amounts. Table 1 also gives the average of three replicated cultivations including standard deviation (±S.D.). Table 1S provides a comparison of VLCPUFAs taken from the literature with our results. As seen in this table, which contains data on VLCPUFAs in 33 strains that we analyzed, these fatty acids have been identified in dozens of different strains. Like Volkman et al. (1998), we believe that their presence in dinoflagellates is an important taxonomic trait. Whether VLCPUFAs have been mentioned in various studies of dinoflagellates depends, at least in our experience, substantially on the experience of the instrument operator. In layman's terms, even if there are VLCPUFAs present, they are often not identified only because the analysis is completed before these acids are eluted from the column.

Table 1
Fatty acid content (%) of total lipids from three genera of dinoflagellates.

Fatty acid[a]	*Amphidinium carterae*	*Cystodinium* sp.	*Peridinium aciculiferum*
14:0	1.0 ± 0.3[b]	1.3 ± 0.3	3.4 ± 0.6
15:0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
16:0	16.5 ± 1.2	14.0 ± 1.4	14.8 ± 1.2
16:1n-7	1.5 ± 0.4	3.8 ± 0.5	1.2 ± 0.3
18:0	2.0 ± 0.5	0.1 ± 0.1	1.3 ± 0.4
18:1n-9	1.2 ± 0.4	2.0 ± 0.6	1.4 ± 0.4
18:2n-6	1.9 ± 0.6	2.1 ± 0.5	1.8 ± 0.3
18:3n-3	0.1 ± 0.1	0.5 ± 0.2	2.5 ± 0.5
18:4n-3	19.8 ± 1.7	18.8 ± 1.5	16.9 ± 0.9
18:5n-3	27.0 ± 2.1	33.5 ± 2.2	32.0 ± 1.0
20:0	0.5 ± 0.3	0.1 ± 0.1	0.0 ± 0.0
20:5n-3	14.0 ± 0.9	3.6 ± 0.7	9.5 ± 1.1
22:0	0.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
22:6n-3	12.1 ± 0.7	19.2 ± 1.6	13.3 ± 1.4
24:6n-3	0.8 ± 0.3	0.1 ± 0.1	0.0 ± 0.0
26:7n-3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
28:7n-6	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
28:8n-3	0.8 ± 0.2	0.5 ± 0.2	1.3 ± 0.3

[a] Shorthand notations - the number before the column specifies the number of carbon atoms and that after the column 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] Data are presented as means ± S.D. from 3 cultivations.

2.2. Separation and identification of lipid classes

Identification of lipids, whether nonpolar (TAG or DAG) or polar, predominantly phospholipids, see Fig. 1, was carried out by two methods. Fig. 1 shows the separation of the standards analyzed by HILIC/ESI including total ion current (TIC) of total lipids from the dinoflagellate *Cystodinium* sp., and also the two methods of

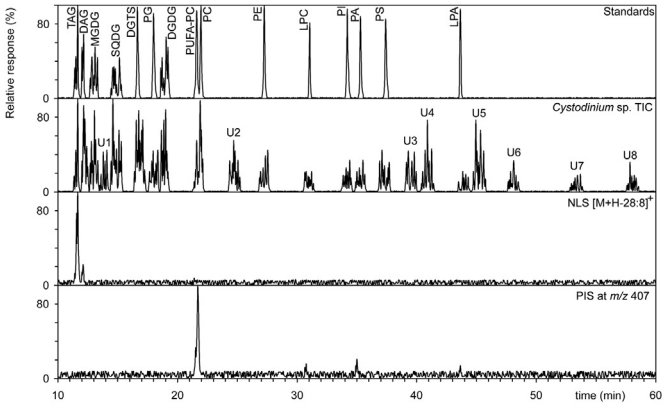

Fig. 1. High performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS) separation of standards, major lipid classes of the dinoflagellate *Cystodinium* sp., neutral loss scan (NLS) and precursor ion scanning (PIS) for $[M + H\text{-}28:8]^+$ and *m/z* 407, respectively. For experimental conditions, see "Materials and Methods". Abbreviations are explained in the text.

detection - neutral loss scan (NLS) and product ion scan (PIS). In the case of neutral lipids and phospholipids comprising VLCPUFA, we also made further identification by tandem MS analysis for the NLS value of 857.5 Da $[M + NH_4\text{-}408\text{-}NH_3]^+$, i.e. the presence of 28:8n-3 acid, and PIS in negative mode for the ion at *m/z* 407.3, which is $C_{27}H_{39}COO^-$, i.e. also 28:8n-3 acid. In the positive mode, i.e. from 10 to 60 min, neutral lipids were monitored in the range of 200–1500 Da and in the negative mode in the range of 100–1300 Da. As seen in Fig. 1, VLCPUFAs were identified only in two neutral lipids (TAGs and DAGs) by NLS and in four phospholipids, i.e. PA and PC and their lyso-forms (LPA and LPC), by PIS.

In positive ion mode (when the spraying nozzle is kept at a positive potential), the charging generally occurs via protonation (sometimes metalation, i.e. Li^+, Na^+, K^+, etc., or also NH_4^+) while in negative ion mode (when the spraying nozzle is kept at a negative potential) charging occurs via deprotonation of the analyte.

Separation and identification of individual lipid classes was carried out in high-resolution positive and negative mode and they were identified mainly based on the precursor and neutral loss scanning, which give characteristic fragments (Vitova et al., 2016), especially for phospho- and glycolipids. In order to obtain a comprehensive lipid profile obtained by extraction of the 3 strains of dinoflagellates, we used a series of ion precursors and neutral loss scan in positive and/or negative modes. From all these data it is clear that the total lipids isolated from each of the mentioned strains of dinoflagellates contain hundreds of molecular species. Of all these molecular species, we focused primarily on those containing at least one VLCPUFA. It can be seen that out of the many identified classes of lipids based on NLS and PIS, the VLCPUFAs are present in only 6 classes of lipids, two neutral lipids – TAG and DAG and four phospholipids (PC, PA, LPC and LPA), which fully corresponds with the generally accepted theory of TAG biosynthesis (Cagliari et al., 2011).

Dinoflagellates produce a variety of classes of lipids, e.g. triacylglycerols, phospholipids, galactolipids, and betaine lipids (Flaim et al., 2014). The lipids separated by TLC and determined in two *Symbiodinium* strains included DGDG, SQDG, PC, PE, cardiolipin (CL), MGDG, and PG, including five unknown lipid classes. Three galactolipids, i.e. MGDG, DGDG and SQDG accounted for more than half of the polar lipids, while PC only for 19%. Unfortunately, none of the 12 classes including the five unidentified ones was demonstrated to contain VLCPUFAs (Awai et al., 2012).

Betaine lipids such as diacylglycerylcarboxy-N-hydroxymethylcholine (DGCC) were found to contain highly unsaturated molecular species of the types 44:15- and 46:15-DGCC (Flaim et al., 2014) or 44:12-DGCC (Leblond et al., 2015) while diacylglyceryltrimethyl-homoserine (DGTS) was found to contain only saturated and monoenoic species, e.g. 32:1-DGTS (Leblond et al., 2015).

Both 28:7 and 28:8 acids were found only in the fraction of phospholipids, not in glycolipids (Leblond and Dahmen, 2012) such as MGDG, DGDG, and SQDG, which, on the contrary, contained 18:4 and 18:5 with the major molecular species 18:4/18:5-MGDG and 18:4/18:5-DGDG (Leblond et al., 2015). These findings indicate that the C28 fatty acids are located and probably synthesized in the cytoplasm or in an organelle other than the chloroplast, whereas the C_{18} fatty acids – 18:4n-3 and 18:5n-3 - are glycolipid constituents apparently synthesized within the chloroplast. The function(s) of these C_{28} fatty acids as components of phospholipids in cellular membranes is currently unknown (Leblond and Chapman, 2000).

Generally speaking, the VLCPUFA are most represented in phospholipids and are missing in glycolipids and triacylglycerols (Leblond et al., 2003) but there are exceptions, see below.

2.3. Identification of simple lipids (glycerolipids)

2.3.1. Identification of TAGs

Briefly, nonaqueous reversed-phase HPLC (NARP-HPLC) analysis of TAG was performed on 2 columns with the RP-18 phase connected in series. We thus achieved a sufficient number of theoretical plates for the separation of molecular species, though the retention time was about 90 min. We managed to separate unusual molecular species of TAG such as, e.g., 28:8/28:8/22:6 and 28:8/24:6/22:6 which have never been separated, see Table 2. In TAGs containing PUFAs and also VLCPUFAs, we observed again (Rezanka et al., 2016) the phenomenon that the elution order is determined not only by the equivalent carbon number (ECN) (which is defined as ECN = CN – (2 × DB) where CN is the number of carbon atoms and DB is the number of double bonds), but also by the structure of the TAG. For instance, molecular species 26:7/28:8/28:8 with ECN = 36 (retention time 33.2 min) is eluted from the column before the molecular species 18:5/18:5/16:1 with ECN = 30 (retention time 33.7 min). We believe that this is due to the steric effects in which a solvated TAG interacts with the stationary phase.

Fig. 2 shows the analysis of total TAGs – total ion current and NLS for two ions, 28:8n-3 acid (M-408 Da), i.e. $[M + NH_4\text{-}28:8\ acid\text{-}NH_3]^+$, abbreviated as $[M + H\text{-}28:8]^+$, and 24:6n-3 (M-356 Da), i.e. $[M + NH_4\text{-}24:6\ acid\text{-}NH_3]^+$, abbreviated as $[M + H\text{-}24:6]^+$. According to the literature (Baiocchi et al., 2015; Mottram and Evershed, 1996; Nagy et al., 2013) diacylglycerol ions $[M + H\text{-}FA]^+$ are the most abundant ions in positive tandem ESI (MS/MS-ESI^+). We also fully confirmed this, see Fig. 3a,b,c, where both ions of diacylglycerol's type, i.e. $[M + H\text{-}FA_1]^+$ and $[M + H\text{-}FA_2]^+$ at *m/z* 855.6285 and at *m/z* 775.5665 are the major ions in the spectrum of the *sn*-22:6/28:8/28:8 TAG. The spectrum also features an ion at *m/z* 1165.8582, the structure of which is $[M + H\text{-}H_2O]^+$. Given the fact, the loss of H_2O can be explained by the proximity of unsaturation to the carbonyl oxygen, because for methylene-interrupted unsaturated VLCPUFA the first double bond is at C4. This is close enough to the carbonyl to induce tautomeric bond shifting, providing stabilization for dehydration. This indicates that the ester carbonyl may have at least some ability to undergo some "keto↔enol" shift (tautomerism) in the mass spectrometer source. It is well known that the hydroxyl group from the enol form is readily lost by dehydration and that double bonds are prone to this rearrangement. Even in this case, the well-known rule "the loss of fatty acid in the *sn*-2 position is less favorable in comparison with the *sn*-1 and *sn*-3 position" (Byrdwell, 2005; Mottram, 2005) has been confirmed when we identified TAGs with two unusual VLCPUFA. It should be noted that position *sn*-1(3), i.e. a primary hydroxyl on glycerol backbone, is preferentially esterified by VLCPUFA.

Furthermore, the spectrum features ions of four types, i.e. $[RCO\text{-}H_2O]^+$, $[RCO]^+$, $[RCO{+}74\text{-}H_2O]^+$, and $[RCO{+}74]^+$. This allowed us to at least partially determine the regioisomers of the TAG molecule having two different FA. Other TAGs, i.e. 26:7/28:8/28:8 (Fig. 3b) and 28:7/28:8/28:8 (Fig. 3c) were similarly identified. The structures of appropriate ions, their abundances, and mass of ions are shown in Tables 2S, 3S, and 4S.

Table 5S gives the content of the TAGs that were identified in all three species of cultivated dinoflagellates. It clearly shows that, like the content of total FAs, the content of TAGs in individual genera of dinoflagellates did not differ. Major TAGs such as 18:5/18:5/20:5, 18:5/20:5/22:6, or 16:0/18:5/22:6 contained the same major FAs and PUFAs that have been found in total lipids. The content of TAGs which contained VLCPUFAs was obviously minor but still measurable. The major TAG (at 0.19–0.25%) was 22:6/28:8/28:8 while the TAG 28:7/28:8/28:8 containing the longest and the most unsaturated VPLPUFAs was represented in an amount of from 0.08 to 0.18% of total TAGs.

Table 2
High-performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS) of the TAGs from the dinoflagellate *Cystodinium* sp., and two neutral loss scan (NLS) - $[M + H\text{-}24:6]^+$, and $[M + H\text{-}28:8]^+$, retention time (tR, min), acyl carbon number (ACN) number of double bond(s) (DB), effective carbon number (ECN), total ion current (TIC) and relative quantities (%) of molecular species of TAGs.

tR	ACN	DB	ECN	$[M + NH_4]^+$	TAG	TIC	$[M\text{-}28:8]^+$ (M-407.2950)	$[M\text{-}24:6]^+$ (M-355.2637)
15.4	54	15	24	860.5955	18:5/18:5/18:5	16.10		
16.9	58	16	26	932.6768	18:5/18:5/22:6	25.61		
17.1	56	15	26	906.6612	18:5/18:5/20:5	39.24		
18.0	60	16	28	960.7081	18:5/20:5/22:6	66.21		
18.1	56	14	28	890.6424	18:5/18:5/20:4	2.40		
18.9	66	18	30	1040.7702	22:6/22:6/22:6	13.54		
19.2	64	17	30	1014.7545	20:5/22:6/22:6	25.17		
19.7	62	16	30	988.7389	20:5/20:5/22:6	29.86		
20.2	60	15	30	962.7231	20:5/20:5/20:5	8.81		
21.1	58	14	30	918.6737	20:5/20:5/18:4	11.12		
21.8	54	11	32	868.6581	18:5/18:5/18:1	4.90		
22.6	52	11	30	842.6424	18:5/18:5/16:1	3.37		
23.1	60	14	32	964.7392	20:5/20:5/20:4	2.45		
23.9	58	13	32	918.6737	18:2/18:5/22:6	3.83		
24.6	56	12	32	894.6737	16:1/18:5/22:6	3.10		
25.6	78	22	34	1200.8959	22:6/28:8/28:8	2.70	100.00	
27.1	74	20	34	1148.8646	22:6/24:6/28:8	2.38	54.34	72.96
28.8	62	14	34	992.7702	18:2/22:6/22:6	2.87		
29.3	72	19	34	1122.8490	22:6/24:6/26:7	2.25		48.00
30.1	60	13	34	966.7545	18:2/20:5/22:6	2.61		
30.7	58	12	34	940.7389	16:1/20:5/22:6	2.74		
31.8	58	12	34	922.7050	18:2/18:4/22:6	2.29		
33.2	82	23	36	1254.9429	26:7/28:8/28:8	2.06	9.23	
33.7	80	22	36	1228.9272	24:6/28:8/28:8	2.05	7.14	9.58
33.7	56	11	34	914.7230	16:0/18:5/22:6	100.00		
34.4	78	21	36	1202.9116	22:6/28:7/28:8	2.51	72.94	
34.8	56	11	34	914.7230	20:5/20:5/16:1	2.54		
35.1	56	11	34	914.7230	16:1/18:4/22:6	2.67		
35.8	54	10	34	870.6737	18:2/18:3/18:5	3.45		
36.3	64	14	36	1020.8020	18:2/22:6/24:6	2.46		88.34
36.8	62	13	36	994.7858	18:1/22:6/22:6	6.92		
37.3	62	13	36	994.7858	18:2/22:5/22:6	2.73		
37.8	60	12	36	968.7702	16:0/22:6/22:6	79.43		
38.0	60	12	36	968.7702	18:2/20:4/22:6	2.50		
38.4	58	11	36	942.7544	16:0/20:5/22:6	86.37		
38.9	58	11	36	942.7544	20:5/20:5/18:1	3.60		
39.4	58	11	36	942.7544	16:1/20:4/22:6	2.58		
40.6	58	11	36	942.7544	18:1/18:4/22:6	3.25		
41.0	56	10	36	916.7389	16:0/20:5/20:5	94.31		
41.5	56	10	36	916.7389	16:1/18:3/22:6	3.92		
42.5	56	10	36	916.7389	18:2/18:3/20:5	3.12		
42.9	56	10	36	916.7389	16:0/18:4/22:6	7.40		
43.6	54	9	36	890.7227	18:3/18:3/18:3	2.37		
44.5	54	9	36	890.7227	18:2/18:2/18:5	3.15		
45.1	84	23	38	1282.9742	28:7/28:8/28:8	1.98	9.92	
45.5	82	22	38	1256.9585	26:7/28:7/28:8	2.01	4.71	
46.1	80	21	38	1228.9272	24:6/28:7/28:8	2.04	6.27	7.67
46.3	50	7	36	820.6581	18:5/16:1/16:1	2.72		
46.5	64	13	38	1022.8180	18:2/22:6/24:5	1.81		
46.6	62	12	38	996.8015	18:1/22:5/22:6	2.31		
46.8	60	11	38	970.7858	18:1/20:4/22:6	2.71		
47.1	58	10	38	944.7702	16:0/20:4/22:6	2.97		
47.4	58	10	38	944.7702	18:2/18:2/22:6	3.24		
47.6	56	9	38	918.7544	22:6/18:3/16:0	4.70		
47.7	52	8	36	864.7076	18:4/18:4/16:0	10.43		
47.8	56	9	38	918.7544	18:2/18:2/20:5	2.88		
48.2	56	9	38	918.7544	16:1/18:2/22:6	2.52		
48.7	54	8	38	894.7545	18:2/18:3/18:3	2.27		
49.4	54	8	38	894.7545	18:5/18:1/18:2	3.88		
49.6	54	8	38	894.7545	18:4/18:2/18:2	2.44		
50.3	54	8	38	892.7381	18:1/18:4/18:3	2.89		
50.5	52	7	38	866.7229	20:5/16:1/16:1	2.34		
50.9	52	7	38	866.7238	16:0/18:2/18:5	3.07		
51.4	52	7	38	866.7238	18:5/18:1/16:1	2.33		
51.7	50	6	38	822.6737	16:0/16:1/18:5	3.42		
51.9	64	12	40	1024.8330	18:1/22:6/24:5	2.47		
52.1	62	11	40	998.8171	18:0/22:5/22:6	3.00		
52.3	60	10	40	972.8020	18:2/18:2/24:6	2.08		15.38
52.5	58	9	40	946.7858	18:1/18:2/22:6	2.22		
52.8	58	9	40	946.7858	18:2/18:2/22:5	1.89		
53.1	56	8	40	920.7702	16:1/18:2/22:5	2.14		
53.4	56	8	40	920.7702	20:4/18:2/18:2	2.09		
53.8	56	8	40	920.7702	20:4/20:4/16:0	2.42		
54.2	56	8	40	920.7702	22:6/18:1/16:1	2.50		
54.3	54	7	40	894.7540	18:2/18:2/18:3	1.95		

(*continued on next page*)

Table 2 (*continued*)

tR	ACN	DB	ECN	$[M + NH_4]^+$	TAG	TIC	$[M-28:8]^+$ (M-407.2950)	$[M-24:6]^+$ (M-355.2637)
54.6	54	7	40	894.7540	22:6/16:1/16:0	4.28		
54.8	54	7	40	876.7207	18:5/18:1/18:1	2.64		
55.0	56	8	40	920.7702	20:5/18:1/18:2	2.45		
55.6	54	7	40	894.7540	16:0/18:2/20:5	2.98		
55.7	52	6	40	868.7391	16:0/18:2/18:4	2.36		
55.8	54	7	40	894.7540	18:1/18:3/18:3	2.48		
56.3	54	7	40	894.7540	20:5/18:1/16:1	2.79		
57.0	52	6	40	868.7391	16:1/18:2/18:3	2.30		
57.4	60	9	42	974.8172	18:1/18:2/24:6	2.12		23.04
57.8	58	8	42	948.8015	16:0/18:2/24:6	2.53		100.00
58.0	52	6	40	868.7391	18:3/18:3/16:0	1.92		
58.1	58	8	42	948.8015	18:1/18:2/22:5	2.20		
58.2	56	7	42	922.7853	20:5/18:1/18:1	2.43		
58.3	54	6	42	896.7698	18:1/18:2/18:3	2.02		
58.5	56	7	42	922.7853	16:0/18:1/22:6	8.94		
58.6	56	7	42	922.7853	16:0/18:2/22:5	2.83		
59.0	52	6	40	868.7378	20:5/16:1/16:0	3.78		
59.2	50	5	40	842.7232	16:1/16:1/18:3	2.35		
59.6	50	5	40	842.7232	14:0/18:2/18:3	2.48		
60.2	48	4	40	816.7076	14:0/16:1/18:3	2.58		
60.6	54	6	42	896.7702	18:4/18:1/18:1	3.16		
60.7	52	5	42	870.7535	16:0/18:1/18:4	5.68		
60.8	54	6	42	896.7698	18:2/18:2/18:2	2.19		
60.9	52	5	42	870.7535	16:0/18:2/18:3	3.77		
61.0	50	4	42	844.7388	16:0/16:1/18:3	2.81		
61.1	50	4	42	844.7388	16:1/16:1/18:2	2.28		
61.2	54	6	42	896.7698	16:0/18:2/20:4	2.57		
61.3	52	5	42	870.7535	16:1/18:2/18:2	2.23		
61.6	52	5	42	870.7535	16:0/16:0/20:5	64.52		
61.8	52	5	42	870.7535	18:1/18:3/16:1	2.46		
61.9	50	4	42	844.7388	18:3/18:1/14:0	2.78		
62.2	50	4	42	844.7388	14:0/18:2/18:2	2.03		
62.7	60	8	44	976.8328	18:1/18:1/24:6	2.18		34.38
62.8	48	3	42	818.7229	18:2/16:1/14:0	2.46		
63.0	60	8	44	976.8328	18:1/20:1/22:6	2.07		
63.3	58	7	44	950.8172	18:0/18:1/22:6	2.86		
63.5	54	5	44	898.7856	18:1/18:1/18:3	2.62		
63.7	58	7	44	950.8172	18:0/18:2/22:5	2.16		
63.9	56	6	44	924.8009	16:0/18:1/22:5	3.28		
64.0	48	3	42	818.7229	14:0/16:0/18:3	2.75		
64.1	54	5	44	898.7856	18:0/18:2/18:3	2.32		
64.2	46	2	42	792.7073	16:1/16:1/14:0	2.56		
64.3	52	4	44	872.7692	18:3/18:1/16:0	3.39		
64.4	54	5	44	898.7856	18:1/18:2/18:2	1.75		
64.5	52	4	44	872.7693	18:3/18:0/16:1	2.39		
65.0	54	5	44	898.7856	16:0/18:1/20:4	2.82		
65.5	54	5	44	898.7858	18:0/18:1/18:4	2.99		
65.8	52	4	44	872.7693	16:1/18:1/18:2	1.85		
66.0	50	3	44	846.7545	16:1/16:1/18:1	2.45		
66.3	52	4	44	872.7693	16:0/18:2/18:2	3.40		
66.7	50	3	44	846.7545	16:0/16:1/18:2	3.72		
67.2	50	3	44	846.7545	16:0/16:0/18:3	6.41		
67.3	48	2	44	820.7386	16:1/16:1/16:0	2.21		
67.5	48	2	44	820.7386	18:1/16:1/14:0	2.69		
67.6	48	2	44	820.7386	14:0/16:0/18:2	2.91		
67.9	48	2	44	820.7386	14:0/16:1/18:1	2.76		
68.0	58	6	46	952.8333	16:0/18:1/24:5	2.55		
68.4	46	1	44	794.7230	16:1/16:0/14:0	2.63		
68.6	56	5	46	926.8164	16:0/18:0/22:5	2.24		
68.9	56	5	46	926.8164	16:0/18:1/22:4	2.11		
69.3	56	5	46	926.8164	18:0/18:1/20:4	2.17		
70.2	54	4	46	900.8004	18:0/18:2/18:2	2.26		
70.6	54	4	46	900.8004	18:1/18:1/18:2	2.49		
71.0	52	3	46	874.7850	16:1/18:1/18:1	2.59		
71.6	52	3	46	874.7850	18:1/18:2/16:0	2.10		
71.7	52	3	46	874.7850	18:1/18:1/16:1	2.60		
71.9	50	2	46	848.7693	18:1/18:1/14:0	3.01		
72.1	50	2	46	848.7693	18:1/16:1/16:0	2.84		
72.8	50	2	46	848.7693	16:0/16:0/18:2	3.05		
73.4	48	1	46	822.7541	16:0/16:0/16:1	5.93		
74.1	48	1	46	822.7541	18:1/16:0/14:0	2.94		
74.5	46	0	46	796.7389	14:0/16:0/16:0	7.02		
75.1	54	3	48	902.8160	18:1/18:1/18:1	2.80		
76.2	54	3	48	902.8160	18:0/18:1/18:2	2.41		
77.2	52	2	48	876.8009	16:0/18:1/18:1	2.85		
78.5	52	2	48	876.8009	16:0/18:0/18:2	2.92		
79.5	50	1	48	850.7853	18:1/16:0/16:0	3.22		
80.0	48	0	48	824.7696	16:0/16:0/16:0	4.40		
83.4	54	2	50	904.8322	18:1/18:1/18:0	2.68		
85.8	52	1	50	878.8171	16:0/18:0/18:1	1.67		

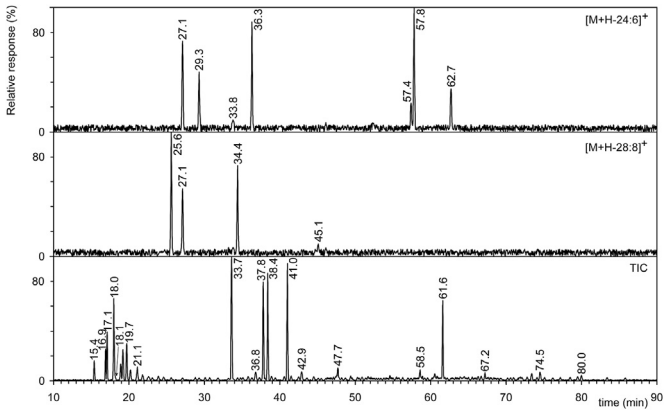

Fig. 2. Reverse phase liquid chromatography/electrospray ionization-mass spectrometry (RP-LC/ESI-MS) chromatogram of total ion current (TIC) of the TAGs from the dinoflagellate *Cystodinium* sp., and two NLS - $[M + H\text{-}24:6]^+$, and $[M + H\text{-}28:8]^+$. The identities of the TAGs are given in Table 2.

TAGs containing two C_{28} – solely monoenoic and/or dienoic acids – plus oleic acid have so far been identified in the green alga *Botryococcus braunii* (MacDougall et al., 2011). A report (Obeng et al., 2005) on DHASCO oil isolated from the marine alga *Crypthecodinium cohnii* containing over 45 of TAG species ranging from 22:6/10:0/10:0 to 22:6/28:8/28:8 has appeared only at a symposium more than 10 years ago. The 22:6 acid was a major component of TAGs, and although regioisomers were determined by ^{13}C-NMR, the result is not entirely clear. Even these authors did not identify 28:7/28:8/28:8 or 26:7/28:7/28:8 or 26:7/28:8/28:8. The TAGs that we identified, i.e. TAGs containing at least two C_{28} acids together with C_{14}-C_{22} PUFAs, are the longest and most unsaturated TAGs isolated from a natural source. According to our current knowledge they have not yet been obtained by organic synthesis.

2.3.2. Identification of DAGs

Tandem mass spectra of DAGs differ substantially from tandem MS of TAGs. The largest difference was seen mainly in the abundance of ions of the type $[M + NH_4]^+$, $[M+H]^+$ and $[M + H\text{-}H_2O]^+$, see Table 6S. Regardless of the value of collision energy, ion $[M + H\text{-}H_2O]^+$ was always the major ion, and the abundance of ions of the type $[M + NH_4]^+$ and $[M+H]^+$ was always lower by an order of magnitude, see also the publications by Deng et al. (2016) and Mu et al. (2000). Other ions of the type $[M + NH_4\text{-RCOOH-}NH_3]^+$ and $[RCO+74\text{-}H_2O]^+$ make it possible to diagnose the structure of DAG. We identified a total of only seven DAGs (Table 3) containing one or two VLCPUFAs. The major compounds were three DAGs, 28:8/28:8, 28:7/28:8 and 18:2/24:6, which accounted for more than 2/3 of all DAGs containing VLCPUFAs. To our current knowledge, no report has as yet been published about DAGs from algae containing VLCPUFAs. The number of 7 DAGs is entirely consistent with the values reported in the literature where, for example Holcapek et al. (2003) detected in soybean oil a total of 52 TAGs, but only 5 DAGs. Similarly, Mottram et al. (1997) identified in soybean 7 DAGs.

2.4. Analysis of phospholipids in negative mode

Although the analysis of PC or LPC in negative mode is in general considered less sensitive than in the positive mode (Hsu and Turk, 2009), we performed it mainly for reasons of analysis of fatty acids in the form of $RCOO^-$, which is illustrated in Fig. 1. We identified four classes of phospholipids – PC and PA and their lyso-forms i.e. LPC and LPA. All four tandem mass spectra are shown in Fig. 4a,b,c,d and description of the ion structures is given in Tables 7S–10S.

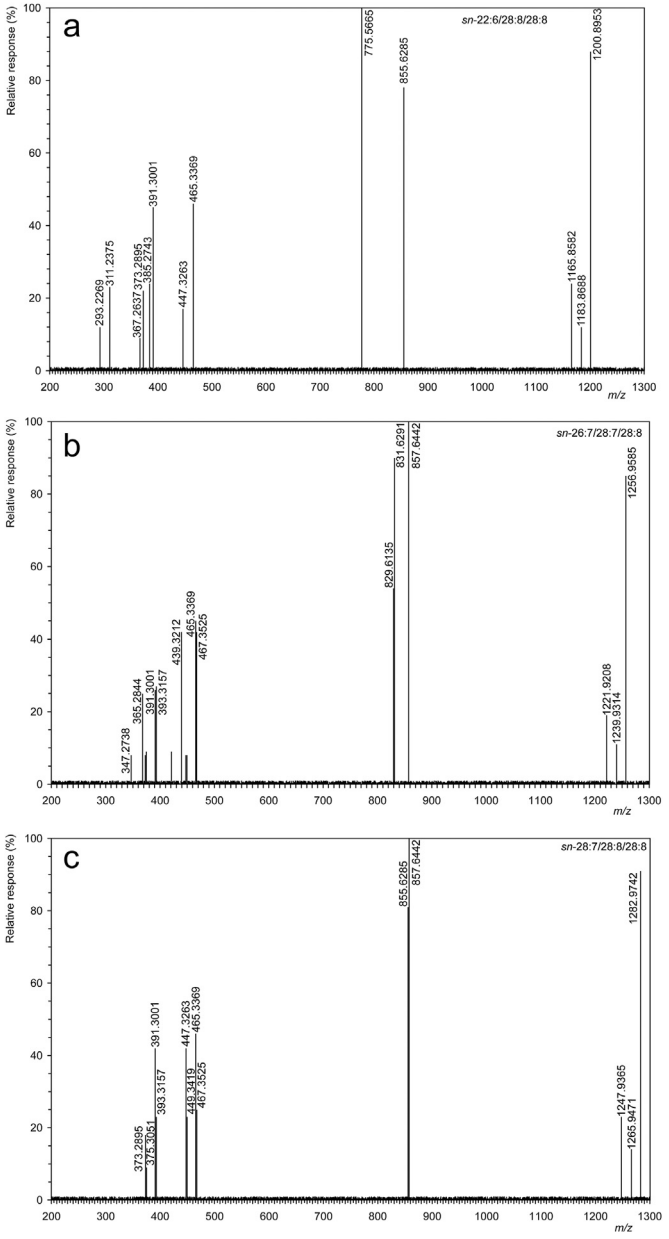

Fig. 3. a. Tandem mass spectrum of natural 22:6/28:8/28:8 TAG. b. Tandem mass spectrum of natural 26:7/28:7/28:8 TAG. c. Tandem mass spectrum of natural 28:7/28:8/28:8 TAG.

2.5. Phosphatidylcholine

Since PC contains a quaternary amino group, for its detection in negative ion mode it is necessary to spike the eluate with e.g. acetate buffer; negative ESI then produces the anion adducts $[M + CH_3COO]^-$, see Table 7S. Fig. 4a shows that the base peak in the spectrum of VLCPUFA-PC (28:8/16:0-PC) is an ion at 870.6021 Da. Upon collision-induced dissociation, anion adducts of PC ions lose both an acetate and a methyl group from the choline moiety to produce a fragment ion (denoted $[M\text{-}15]^-$) with the mass 74 Da lower than the mass of the zwitterionic form of intact PC (Hsu and Turk, 2009). The $[M\text{-}15]^-$ ion undergoes further fragmentation to yield less abundant intermediates. This includes neutral loss of ketene $[M\text{-}15\ \text{-}R'CH{=}C{=}O]^-$, a neutral loss of free FA $[M\text{-}15\ \text{-}RCOOH]^-$ and finally generation of acyl anions of FAs $[RCOO]^-$.

Table 3
Molecular species content (%) of diacylglycerols from three genera of dinoflagellates.

DAG	*Amphidinium carterae*	*Cystodinium* sp.	*Peridinium aciculiferum*
28:8/28:8	30.3	26.9	29.0
28:7/28:8	20.3	20.5	22.0
24:6/28:8	7.3	8.3	5.4
24:6/26:7	3.3	6.4	5.4
22:6/24:6	7.7	9.0	9.1
18:2/24:6	24.3	22.5	23.7
18:1/24:6	6.8	6.4	5.4

The acyl anion fragment of *sn*-2 FA was more abundant than the FA at *sn*-1 position (Chen and Subbaiah, 2013). Further confirmation of the structure of regioisomers was based on the rule that ions arising by a loss of ketene ($R_2'CH{=}C{=}O$) or fatty acid at *sn*-2 are more abundant in the product ion spectrum than the ions arising from loss of the fatty acid substituents at *sn*-1. Fig. 4a documents the tandem MS spectrum of a natural 28:8/16:0-PC. This molecular species was detected as acetate anion at *m/z* 944.6391 and its spectrum showed major peaks at *m/z* 870.6021 ($[M\text{-}15]^-$), 407.2953 ($[C_{27}H_{39}COO]^-$), and 255.2329 ($[C_{15}H_{31}COO]^-$). Other ions that were useful for determining the structure included ions at *m/z* 480.3094 [M-15 -$C_{26}H_{37}CHC{=}O]^-$ and at *m/z* 632.3719 [M-15 -$C_{14}H_{29}CHC{=}O]^-$ formed by loss of FAs as ketenes, or ions arising by neutral loss of free FA at *m/z* 462.2989 [M-15 -$C_{26}H_{37}CH_2COOH]^-$ and at *m/z* 614.3617 [M-15 -$C_{14}H_{29}CH_2COOH]^-$. The abundances of the ions 480.3094, 407.2953 and 255.2329 are given in Table 6S. Even more characteristic is an ion for PUFAs, i.e. ion at *m/z* 363.3060, resulting by loss of CO_2 from R_1COO^- ion. Other ions present in the spectrum, i.e. ions at 799.5286 Da (loss of choline and acetate from precursor ion), at 224.0690 Da (glycerophosphocholine with loss of CH_3 and H_2O), at 168.0429 Da (phosphocholine with loss of CH_3), and at 152.9961 Da (glycerol-3-phosphate ion with loss of H_2O).

The higher abundance of the $[M\text{-}H\text{-}R_2COOH]^-$ ion at *m/z* 614.3617 than the $[M\text{-}H\text{-}R_1COOH]^-$ ion at *m/z* 462.2989 and a higher abundance of the $[M\text{-}H\text{-}R'_2CHC{=}O]^-$ ion at *m/z* 632.3719 than the $[M\text{-}H\text{-}R'_1CHC{=}O]^-$ ion at *m/z* 480.3094 fully confirmed the structure of *sn*-28:8/16:0-PC.

2.6. Phosphatidic acid

In negative ESI, PA molecular species with 28:8 and 16:0 acids produce, in addition to an $[M\text{-}H]^-$ ion at 799.5281 Da, also other abundant ions, see Fig. 4b and Table 8S. The first of them at 409.2368 Da arises by loss of *sn*-1 acyl chain as ketene ($R_1'CH{=}C{=}O$) from $[M\text{-}H]^-$, another at 391.2253 Da by neutral loss of R_1COOH group from $[M\text{-}H]^-$. A similar loss of *sn*-2 acyl chain as ketene ($R'CH{=}C{=}O$) from $[M\text{-}H]^-$ produces an ion at 561.2990 Da and neutral loss of RCOOH at *sn*-2 position from $[M\text{-}H]^-$ yields an ion at 543.2879 Da. We determined the regioisomers of individual molecular species of PA based on the above mentioned rule on the preferential loss of FA from *sn*-2. The ion at *m/z* 407.2957 (*sn*-1 $RCOO^-$ ion) had lower abundance than the ion at *m/z* 255.2328 (*sn*-2 $RCOO^-$ ion). The higher abundance of the $[M\text{-}H\text{-}R_2COOH]^-$ ion at *m/z* 543.2879 than the $[M\text{-}H\text{-}R_1COOH]^-$ ion at *m/z* 391.2253 and a higher abundance of the $[M\text{-}H\text{-}R'_2CHC{=}O]$- ion at *m/z* 561.2990 than the $[M\text{-}H\text{-}R'_1CHC{=}O]^-$ ion at *m/z* 409.2368 fully confirmed the structure of *sn*-28:8/16:0-PA.

2.7. Lyso-phosphatidylcholine

In the negative mode lyso-PC produces the adduct ion $[M + CH_3COO]^-$ (Fig. 4c) which, after loss of methyl and acetate

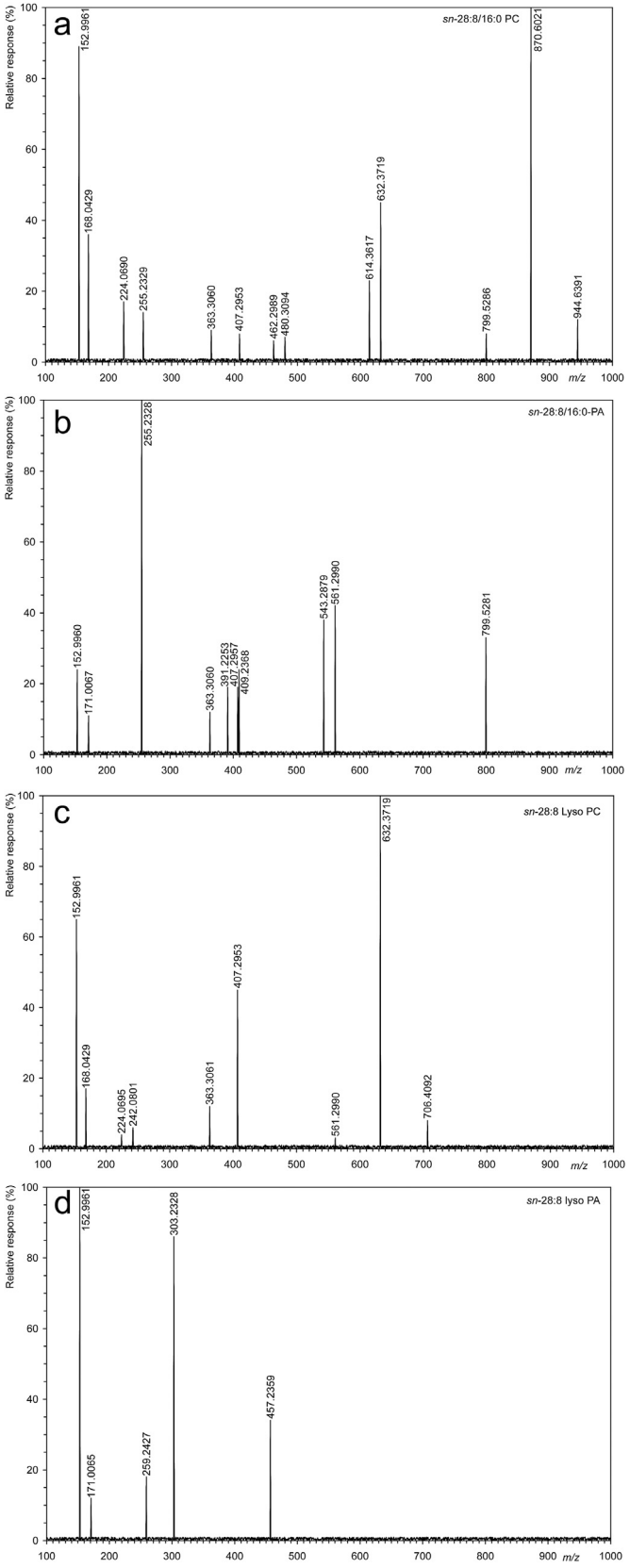

Fig. 4. a. Tandem mass spectrum of natural 28:8/16:0-PC. b. Tandem mass spectrum of natural 28:8/16:0-PA. c. Tandem mass spectrum of natural 28:8-lyso PC. d. Tandem mass spectrum of natural 28:8-lyso PA.

group, gives a base peak $[M-15]^-$. The masses of the ions are given in Fig. 4c and Table 9S. Tandem MS also features abundant type R_1COO^- ions and ions generated by loss of the neutral cyclic glycerophosphate ester. Identification or determination of whether the loss is from the *sn*-1 or *sn*-2 of lyso-PC is a relatively difficult, since both types of phospholipids have very similar structures. As described in the paper by Han and Gross (1996), lyso-PC regioisomers have in tandem MS different intensities of ions generated from $[M+Na]^+$. Unfortunately, this has not been confirmed under our conditions. We therefore cannot confirm that the lyso-PC is a 1-*sn*-acyl/2-hydroxy-PC.

2.8. Lyso-phosphatidic acid

In the case of lyso-PA, negative ESI gives abundant ions $[M-H]^-$ and $[RCOO]^-$ (Fig. 4d and Table 10S), and a less abundant glycerol-3-phosphate ion. This may stymie the differentiation between regioisomers of 1- and 2-lysophosphatic acid by tandem MS. Again, we cannot accurately determine whether the compound is *sn*-1-acyl/2-hydroxy-PA or *sn*-1-hydroxy/2-acyl-PA.

Phosphatidylcholines having at position *sn*-1 VLCPUFAs and at position *sn*-2 saturated, monounsaturated and polyunsaturated fatty acids with <24 carbon atoms have been identified in mammalian tissues (Poulos et al., 1988; Robinson et al., 1990; Sharp et al., 1991), the FA being determined stereospecifically by phospholipase A2, which cleaves the *sn*-2 ester bond. Tandem MS has recently been successfully used (Berdeaux et al., 2010; Hama et al., 2013) to show that all diacylphospholipid molecular species in skin fibroblasts of Zellweger syndrome contained only one VLCFA in their DAG moieties and they were mostly found in the *sn*-1 position. This suggested that some acyltransferases responsible for *sn*-1 position acylation preferred certain VLCFA or that some VLCFA-selective acyltransferases possessed higher affinity for the *sn*-1 than the *sn*-2 position. In mammalian tissues, VLCPUFAs are essential components of epidermal acylceramides and important actors in skin barrier; they were also shown to be involved in sperm maturation in testis or in the retina, appear to be associated with rhodopsin in photoreceptor outer segments and are suspected of being involved in phototransduction processes. Their function in dinoflagellates is unclear.

3. Conclusions

Detailed analysis of FAs plays a very important role in identification of PUFAs, as can be shown in the work of Mansour et al. (2005b) and Mansour (2005) who analyzed some dinoflagellates (see Table 1S) cultured to stationary phase. Fatty acids 24:6n-3, 24:5n-6, 26:7n-3 and 28:8n-3 were detected in minor quantities in most strains. It is obvious that all genera mentioned in Table 1S contain at least two polyunsaturated C28 fatty acids. Identification of other FAs depends on the limit of detection (e.g. traces are less than 0.2% or 0.05% of total FAs). The detection limit for picolinyl esters of VLCPUFAs in our previous studies (Rezanka et al., 2008a,b) was 0.0001% of total FAs and thus the number of identified VCPUFAs was much higher, i.e. 67 as opposed to two to six, see above. It is thus clearly evident that the presence or absence of identified biosynthetic precursors depends on the detection limit.

We analyzed FAs and especially VLCPUFAs of total lipids of three strains of dinoflagellates (*Amphidinium carterae*, *Cystodinium* sp. and *Peridinium aciculiferum*). Further analysis of TAGs and phospholipids (PA, PC, LPA and LPC) was performed using HILIC connected with positive and negative high resolution ESI. Tandem MS, and simultaneously neutral loss scan and product ion scan was used to identify more than 150 molecular species of TAGs and it was strongly suggested that both PC and PA serve as the biosynthetic precursors of TAGs, VLCPUFAs being always esterified at primary hydroxyl of glycerol backbone. In other words, VLCPUFAs are always at the *sn*-1(3) position in TAGs and at *sn*-1 position in phospholipids. Based on these results it can be assumed that dinoflagellates biosynthesize TAGs containing VLCPUFAs in same way as higher organisms, i.e. PA and PC serve as the biosynthetic precursors. Based on the above analysis, mainly on positive high resolution tandem ESI, we identified the TAG 84:23 (*sn*-28:7/28:8/28:8), which is the most unsaturated TAG that has hitherto been described. The methods allowed us to expand the number of molecular species of triacylglycerols containing VLCPUFAs. These methods are of course not limited to dinoflagellates, but can in the future be used for other organisms including higher plants.

4. Experimental

4.1. Chemical and standards

Acetonitrile, 2-propanol, hexane and dichloromethane were purchased from Sigma-Aldrich (Prague, CR). 1,2-Dipalmitoyl-diacylglyceryltrimethylhomoserine (DGTS), di-all *cis*-4,7,10,13,16,19-docosahexaenoyl-PC, and 1,2-dioleoyl PI (ammonium salt) were purchased from Avanti Polar Lipids, Alabaster, AL, USA. DGDG, MGDG, and sulfoquinovosyldiacylglycerol (SQDG) (plant leaf lipids), cholesteryl linoleate, 1,2-di-all *cis*-4,7,10,13,16,19-docosahexaenoin, 1,2-dioleoyl- phosphatidic acid (PA), 1,2-dioleoyl-PC, 1,2-dioleoyl-PE, 1,2-dioleoyl-phosphatidylglycerol (PG) (Na-salt), tri-all *cis*-4,7,10,13,16,19-docosahexaenoin, and 1,2-dimyristoyl-phosphatidylserine (PS) (Na-salt) from Larodan (Malmö, Sweden).

4.2. Cultivation

The strains of dinoflagellates were obtained from the Culture Collection of Algae at the University of Göttingen, Germany (*Cystodinium* sp. Klebs, SAG 59.87, family Phytodiniaceae) and from the Scandinavian Culture Collection of Algae and Protozoa (*Peridinium aciculiferum* Lemmermann, SCCAP K-0985, family Peridiniaceae and *Amphidinium carterae* Hulburt, SCCAP K-0406, family Gymnodiniaceae). The strains SAG 59.87 and SCCAP K-0985 were isolated from freshwater habitats in Europe (lakes Lunzer Obersee and Erken, respectively), whereas SCCAP K-0406 is a marine strain isolated from sediment in Merimbula, New South Wales, Australia. The media used ($MiEB_{12}$, Schlosser, 1982; MWC + Se, Guillard and Lorenzen, 1972; TL30, Larsen et al., 1994) were set up according to the recommendation of the culture collections (www.epsag.uni-goettingen.de, www.sccap.dk). The cultivations were performed at 20 °C (SAG 59.87) or at 5–10 °C (SCCAP K-0985 and SCCAP K-0406) under continuous light. After reaching stationary phase, the cultures were harvested by centrifugation at 2000 rpm for 15 min, and the samples were lyophilized and stored at −18 °C.

4.3. Isolation of lipids

Isolation and pretreatment of lipids was carried out according to Bligh and Dyer (1959), with modifications as described previously (Kates and Volcani, 1996). For full description of the experiments see the Supplements.

4.4. FAMEs analysis

Analysis of fatty acids in the form of methyl esters (FAME) was described previously (Rezanka, 1993; Dembitsky and Rezanka, 2005). A complete description of all conditions of FAME analysis by GC-MS is in the Supplements.

4.5. HILIC/MS-ESI

After purification by Sep-Pak Vac Silica cartridge (Waters Gesellschaft m.b.H., Czech Republic), the lipids were identified and quantified by LC/MS. A detailed description of both positive and negative ESI is again in the Supplements.

4.6. Separation and identification of regioisomers of TAGs

Separation and identification of regioisomers of TAGs by two Hichrom HIRPB columns (Hichrom Limited, UK) in series connected with high resolution positive electrospray ion mode is described in detail in the Supplements.

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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.2017.04.012.

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