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Lipidomic analysis of *Botryococcus* (Trebouxiophyceae, Chlorophyta) - Identification of lipid classes containing very long chain fatty acids by offline two-dimensional LC-tandem MS

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ABSTRACT

Very long chain fatty acids (VLCFAs) were identified in four strains of the green alga *Botryococcus braunii* (Trebouxiophyceae). The algae contained a series of monoenoic fatty acids up to triacontenoic acid and further VLCFAs in amounts around 1% of total fatty acids. The separation of lipid classes using hydrophilic interaction chromatography revealed that the most abundant VLCFAs (28:2, 28:1 and 28:0) were contained in neutral lipids (triacylglycerols and/or diacylglycerols) and in phospholipids (phosphatidic acid and/or phosphatidylcholine). Using non-aqueous reversed-phase liquid chromatography tandem mass spectrometry (NARP-LC/MS^2) of the appropriate collected fractions, molecular species of triacylglycerols containing one or two VLCFAs were described and phosphatidylcholines containing VLCFAs were separated for the first time. Because the presence of *Botryosphaerella sudetica* (Chlorophyceae) as contaminant of *Botryococcus braunii* strain Droop 1950/807-1 placed some doubts on the results of previous studies, a strain of this green alga of was also analyzed. In contrast to *Botryococcus*, C16, a substantially lower proportion of C18 polyunsaturated fatty acids and no VLCFAs were detected in *Botryosphaerella*.

1. Introduction

The genus *Botryococcus* (Trebouxiophyceae, Chlorophyta) comprises colonial freshwater algae that are found mainly in the plankton of temperate and tropical lakes. The colonies are composed of radially arranged cells embedded in a tough mucous envelope. When nutrients are abundant, they can form superficial blooms, which were shown to be toxic to other microorganisms and fish (Lee, 2008). These algae can biosynthesize large amounts of lipids, whose content often exceeds 50% dry matter (Metzger and Largeau, 2005). According to the content of hydrocarbons, the best studied species *Botryococcus braunii* is sometimes divided into three races: A, B, and L. This division is based on the content and type of hydrocarbons. Race A produces n-alkadienes and triene hydrocarbons, race B produces triterpenoid hydrocarbons (botryococcenes) and methylated squalenes, whereas race L produces tetraterpenoid hydrocarbon (lycopadiene) (Metzger et al., 1989). Unlike the content of hydrocarbons, the knowledge of the content of triacylglycerols (TAGs) and polar lipids (phospho- and glycolipids) is scarce. Donot et al. (2016) reported on the contents of hydrocarbons in *B. braunii* race A, which reaches 0.9% of solids while the content of TAGs was found to exceed 7% of solids. According to Metzger and Largeau (2005) the lipid content amounts to 62% dry weight while the content of TAGs amounts to 2–6% of solids in race A, races B and L lacking TAGs. According to Metzger et al. (1989) the content of TAGs in race A varies from 0 to 6.4% of dry biomass.

When the total lipids were separated and identified, they always included common lipids usually found in algae. Besides the already mentioned hydrocarbons (HC) they included TAGs, diacylglycerols (DAGs), monoacylglycerols (MAGs), sterols, alcohols, free fatty acids (FFAs), and sterol esters (SE) which represent neutral lipids

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(Kalacheva et al., 2002; MacDougall et al., 2011; Donot et al., 2016). Polar lipids, i.e. phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulphoquinovosyl diacylglycerols (SQDG), diacylglyceryltrimethylhomoserine (DGTS) and cardiolipin (CL) were also described (MacDougall et al., 2011).

Total fatty acids (FAs), including the very long chain fatty acids (VLCFAs), i.e. FA with more than 22 carbon atoms in a straight chain, have also been identified. Douglas et al. (1969) analyzed the biomass of *B. braunii* and identified saturated and monounsaturated FAs from C14 to C28, including linoleic and octacosadienoic acids. According to another study (Metzger et al., 1989) the major FAs were oleic acid (67.1% of total FAs) and an unusually high content of octacosenoic acid (28:1) (9.6% of total FAs) was found. Volova et al. (2003) and Kalacheva et al. (2002) identified a total of 35 FAs including 16:2, 16:3, and 18:3ω3 (α-linolenic acid). Monounsaturated FAs with more than 18 carbon atoms had always a double bond in position ω9, as evidenced by mass spectrometry of dimethyl disulfide adducts (Volova et al., 2003). A possible problem with FA analysis in *Botryococcus* arises from the fact that the commonly used strain of *B. braunii* (CCAP 807/1) was contaminated by another green alga, *Botryosphaerella sudetica* (Chlorophyceae). The members of the class Chlorophyceae have a FA profile that differs substantially from that of *Botryococcus* that belongs to Trebouxiophyceae (Rezanka et al., 2017b).

Analysis of TAGs in *B. braunii* revealed, apart from the common FAs (Donot et al., 2013), i.e. 16:1ω7, 18:1ω9, and 18:2ω6, also less common 26:1, 28:1, 28:2, 30:1 and 30:2 (Boyd et al., 2012) or 16:3, 24:1 and 28:0 FAs (MacDougall et al., 2011). According to our current knowledge, only few studies addressed the identification and possibly semi-quantitation of TAGs. Above all, Boyd et al. (2012) identified ten TAGs, majority representation being reported for the common TAG triolein, as well as the completely unique 28:1/18:1/18:1 (sn-1/sn-2/sn-3). Furthermore Donot et al. (2013) qualitatively determined three TAGs (16:0/16:0/18:1, 18:1/18:1/16:0, and 16:0/16:0/18:2). The most extensive article about TAGs in *B. braunii* was published by MacDougall et al. (2011), who identified dozens of TAGs, many of them containing one or two VLCFAs (28:1/28:1/18:1, 28:2/28:2/18:1, 28:1/28:2/18:1 and others). To our current knowledge, molecular species of phospholipids were not analyzed.

The most common method for lipid separation is high performance liquid chromatography (HPLC). Using hydrophilic interaction chromatography (HILIC), lipids are divided into classes according to their different polar head groups. In the case of non-aqueous reversed-phase (NARP) liquid chromatography, the lipids are divided according to their hydrophobicity, which depends essentially on the length of the chains, the number of double bonds, or their position and geometric configuration (*E* versus *Z*). If some of the methods are used alone, lipids often co-elute and the information on minor components is lost. Therefore, the two-dimensional LC method is lately beginning to be used. The two-dimensional LC/mass spectrometry (2D-LC/MS), or better tandem MS (2D-LC/MS^2), allows for separating lipids using two separation mechanisms. Lipid fractions from the first dimension are transferred to the second dimension in an off-line or on-line mode. In the off-line mode, the compounds from the first separation are manually collected and after concentration are separated and identified in the second dimension of 2D-LC. It should be noted that the off-line method is time consuming and manually demanding. In the online 2D-LC method, the eluate from the first dimension is automatically collected and further analysis proceeds in one of two modes, either a comprehensive or so-called heart-cutting one. In the case of the comprehensive mode, all the separated compounds in the first mode are further analyzed in the second mode. In the heart-cutting mode, only selected groups of compounds are analyzed which are further separated on the second column (in the second mode). This method is advantageous for targeted analysis. There are several articles dealing with the advantages and disadvantages of 2D-LC (Bang and Moon, 2013; Dugo et al., 2013; Cifkova et al., 2013; Ovcacikova et al., 2016; Wang et al., 2016).

The main objective of this work was to perform complex lipidomic analysis, including molecular species identification of lipids containing VLCFA, in four *B. braunii* strains using LC/electrospray (ESI)-tandem MS and compare their FA profiles and molecular species of TAG and PCs by LC/ESI tandem MS, which has not yet been described. Another objective was to analyze the composition of the major lipid classes of another green alga, *Botryosphaerella sudetica*, which was isolated as a contaminant from a strain of *B. braunii*.

2. Results and discussion

Green alga *B. braunii* is an excellent model organism for the examination of VLCFAs, and its content of these acids has been described several times (Douglas et al., 1969; Metzger et al., 1989; Volova et al., 2003; Kalacheva et al., 2002).

Unfortunately, a detailed description of VLCFAs content in individual lipid classes as well as the molecular species analysis of these classes has not yet been fully described in the literature.

2.1. Analysis of VLCFAs

We determined the total fatty acid content in four strains of *B. braunii* and in one strain of *Botryosphaerella sudetica* (Table 1). The table also gives the average of three replicated analyses including standard deviation (±S.D.). The analysis of fatty acids in the form of methyl esters, including VLCFA analysis, is routinely carried out. However, it should be noted at this point that it is always necessary to select a suitable temperature gradient and a sufficiently long analysis time when analyzing VLCFAs by GC-MS. Various FAs are found depending on the input material, i.e. a suitable strain of alga. In our strains (*B. braunii* and *Botryosphaerella sudetica*), palmitic (16:0), palmitoleic (16:1ω7), stearic (18:0), oleic (18:1ω9), linoleic acid (18:2ω6), α-linolenic acids (18:3ω3) but also C16 PUFAs (16:2ω6, 16:3ω4 and 16:4ω3) were found as the major FAs. In addition, odd-numbered C15 and C17 FAs and, in *B. braunii*, a series of monoenoic ω9 FAs up to 21-triacontenoic acid including further VLCFAs such as 28:0, 28:2 and others were identified in amounts around 1% of total FAs. The differences in the fatty acid content of *B. braunii* and *Botryosphaerella sudetica* are best illustrated in Table 1. The main difference between the two genera is seen to be the fact that *B. braunii* does not produce any PUFAs except linoleic acid, whereas *Botryosphaerella sudetica* does not produce VLCFAs.

The analysis of FAs in *B. braunii* has been dealt with in many studies. One of the first papers (Douglas et al., 1969) described the presence of VLCFAs, namely 26:1, 28:1, 28:2, etc., and also Metzger et al. (1989) identified even-numbered monoenoic FAs up to 28:1 in TAGs. Kalacheva et al. (2002) reported on the presence of 16:3 and 18:3ω3 FAs in the strain *B. braunii* Kutz No LB 807/1 Droop 1950 H-252 = CCAP 807/1 (related to our strain CCALA 220). In this strain, the sum of PUFAs reached almost half of total FAs whereas VLCFAs were found only in trace amounts. By contrast, in field samples of *B. braunii* collected by the same authors from the Lake Shira (Siberia), PUFAs were not found at all but VLCFAs were present up to 32:1ω9. Boyd et al. (2012) described the presence of VLCFAs (28:1, 28:2, 30:2) only in DAGs and TAGs, but not in DGDG, which

Table 1
Fatty acid content (%) of total lipids from two strains of *Botryococcus braunii* and *Botryosphaerella sudetica*.

Fatty acid	*B. braunii* CCALA 220	±S.D.[b]	*B. braunii* CCALA 778	±S.D.	*B. braunii* CCALA 835	±S.D.	*B.braunii* CCALA 933	±S.D.	*Botryosphaerella sudetica* CCALA 780	±S.D.
14:0[a]	3.1	0.05	2.7	0.03	1.8	0.09	1.8	0.09	1.1	0.07
14:1	0.2	0.01	0.2	0.02	0.2	0.03	0.2	0.03	0.8	0.05
br-15:0	0.5	0.02	0.0	0.00	0.5	0.04	0.5	0.02	0.0	0.00
15:0	0.5	0.03	0.0	0.00	0.2	0.03	0.2	0.05	0.1	0.01
16:0	24.5	0.15	29.7	0.16	21.6	0.19	19.6	0.18	19.3	0.17
16:1ω-7	15.4	0.12	17.3	0.14	20.0	0.22	21.8	0.16	1.5	0.06
16:1ω-9	0.3	0.02	0.3	0.02	0.0	0.00	0.0	0.00	0.2	0.02
16:2ω-6	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.3	0.02
16:3ω-4	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	2.8	0.08
16:4ω-3	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	7.9	0.13
br-17:0	0.3	0.02	0.5	0.04	0.7	0.05	0.9	0.08	0.2	0.03
17:1	0.6	0.04	0.3	0.04	0.4	0.06	0.5	0.06	0.3	0.05
17:0	1.3	0.07	0.9	0.06	0.5	0.04	0.7	0.07	0.6	0.04
18:0	16.4	0.14	10.2	0.13	12.7	0.13	11.2	0.13	1.4	0.07
18:1ω-9	17.6	0.11	16.7	0.18	19.5	0.20	21.5	0.14	12.7	0.16
18:1ω-7	3.6	0.06	2.6	0.05	3.3	0.06	4.4	0.09	0.8	0.05
18:2ω-6	7.7	0.08	9.0	0.11	6.5	0.08	5.8	0.07	21.7	0.21
18:3ω-3	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	17.5	0.12
18:3ω-6	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	1.3	0.09
18:4ω-3	0.0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	9.0	0.10
20:0	0.3	0.04	0.5	0.03	0.9	0.05	0.7	0.06	0.3	0.03
20:1ω-9	0.2	0.03	0.3	0.04	0.4	0.03	0.5	0.04	0.2	0.04
22:0	0.0	0.00	0.0	0.00	0.4	0.05	0.4	0.03	0.0	0.00
22:1ω-9	0.6	0.05	0.2	0.03	1.2	0.08	0.9	0.05	0.0	0.00
24:0	0.2	0.03	0.5	0.04	0.0	0.00	0.0	0.00	0.0	0.00
24:1ω-9	0.9	0.08	1.0	0.06	0.5	0.04	0.6	0.04	0.0	0.00
26:1ω-9	1.3	0.06	0.3	0.02	0.5	0.06	0.8	0.07	0.0	0.00
28:2	1.0	0.07	0.8	0.04	1.6	0.07	1.3	0.09	0.0	0.00
28:1ω-9	1.1	0.08	1.7	0.06	1.4	0.09	1.8	0.08	0.0	0.00
28:0	1.3	0.09	2.7	0.07	2.4	0.08	1.8	0.11	0.0	0.00
30:2	0.0	0.00	0.0	0.00	0.6	0.05	0.4	0.06	0.0	0.00
30:1ω-9	0.8	0.05	1.4	0.08	1.8	0.07	1.2	0.07	0.0	0.00
30:0	0.3	0.02	0.2	0.02	0.4	0.03	0.5	0.02	0.0	0.00

[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.

however contained PUFAs, i.e. 16:3 and 18:3. Vazquez-Duhalt and Greppin (1987) found 16:2, 16:3, 18:3 and others but no VLCFAs in *Botryococcus sudeticus* (now *Botryosphaerella sudetica*, Silva, 1970). The strain of *Botryosphaerella sudetica* was obtained as a subisolate from the strain *B. braunii* CCAP 807/1 and designated UTEX 2629 (= CCALA 780) (Senousy et al., 2004).

The above publications (Kalacheva et al., 2002; MacDougall et al., 2011) and our Table 1 confirm that the original strain of *B. braunii* CCAP 807/1 (isolated by Droop in 1950) was a mixture of at least two species. This strain was isolated by Droop in 1950, deposited to the Cambridge Culture Collection of Algae and Protozoa (currently Culture Collection of Algae and Protozoa, Dunstaffnage, Scotland) and is currently maintained in different collections including CCALA (as CCALA 220). Both literature data and our cultivations and FAME analyses document that *B. braunii* does not produce 16:2, 16:3, 16:4, 18:3 and 18:4, and produces a smaller amount of linoleic acid in comparison with *Botryosphaerella sudetica*. The morphological difference between the two genera is clear, see Figs. 1S and 2S (Supplements). Moreover, the genus *Botryococcus* belongs to the class Trebouxiophyceae, while *Botryosphaerella* to the class Chlorophyceae (Guiry and Guiry, 2017). Because they are phylogenetically distant, they contain different FAs; i.e. *Botryosphaerella* belongs to the same class as *Scenedesmus* (*Desmodesmus*), i.e. one of the most commonly cultivated algae, which contains C16 PUFAs (Rezanka et al., 2017b). Volova et al. (2003) found in the original strain of *B. braunii* (CCAP 807/1) C16 PUFAs and 18:3; however, field samples of this alga did not contain these acids but contained VLCFAs, again confirming that the original strain *B. braunii* CCAP 807/1 was not unialgal.

2.2. Separation and identification of lipid classes

Fig. 1 shows the separation of total lipids from *B. braunii* and *Botryosphaerella sudetica* strains and commercially available standards. HILIC was used to separate the lipids by class. Two detection methods, i.e. neutral loss scan (NLS) and precursor-ion scan (PIS) were used in addition to the total ion current (TIC). Since the analysis of total FAs showed that one of the most abundant VLCFAs was 28:1, we used the ions of the type $[M + NH_4\text{-}422.4\text{-}NH_3]^+$ for the NLS and the ion at *m/z* 421.4 (ion $C_{27}H_{53}COO^-$) for the PIS. Individual lipid classes were analyzed in both positive (neutral lipids, range 200–1500 Da) and negative ion (phospholipids) ESI mode in the range of 200–2000 Da. Fig. 1 further shows that 28:1 was identified mainly in TAGs and DAGs as neutral lipids and in PCs as representatives of phospholipids. Its presence in PA and lyso forms of phospholipids (LPAs, LPCs) was less important. Therefore, in the next analysis, we focused on the identification of VLCFAs only in TAGs and PCs, with the remaining lipid classes (DAGs, PAs, LPCs and LPAs) being only slightly represented. Both PIS and NLS-based methods (see Table 1S) were used to identify individual lipid classes in both positive and negative ion ESI mode (Table 2). Their identities has also been confirmed by comparison of retention times of lipid classes with retention times obtained by using commercially available standards (see Fig. 2).

Although the *B. braunii* lipids have been analyzed relatively often, most of the papers are dedicated to the separation and identification of non-polar (neutral) lipids, for example HC, TAGs, DAGs and MAGs (Donot et al., 2016). Kalacheva et al. (2002) identified polar lipids as well as DAGs, sterols, alcohols, FFAs, TAGs, SE

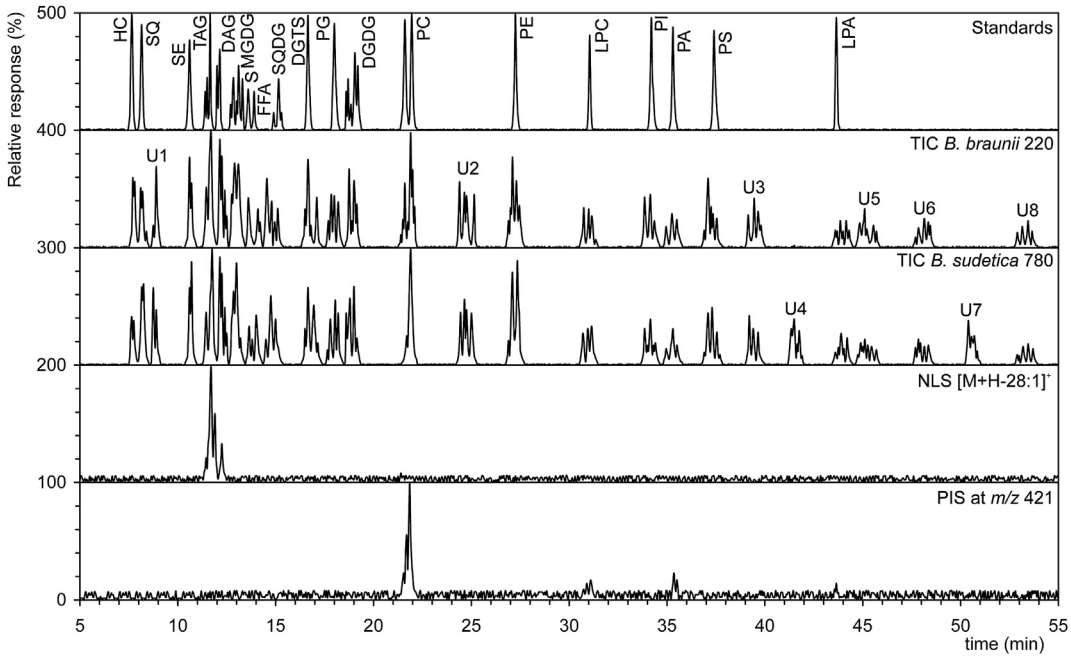

Fig. 1. HPLC/ESI-MS separation of standards (bottom) and major lipid classes of the two green algae (*Botryococcus braunii* CCALA 220 and *Botryosphaerella sudetica* CCALA 780). Precursor-ion scan (PIS) at *m/z* 421.4 (ion $[C_{27}H_{53}COO]^-$) and neutral loss scan (NLS) of the ions $[M + NH_4\text{-}422.4\text{-}NH_3]^+$.

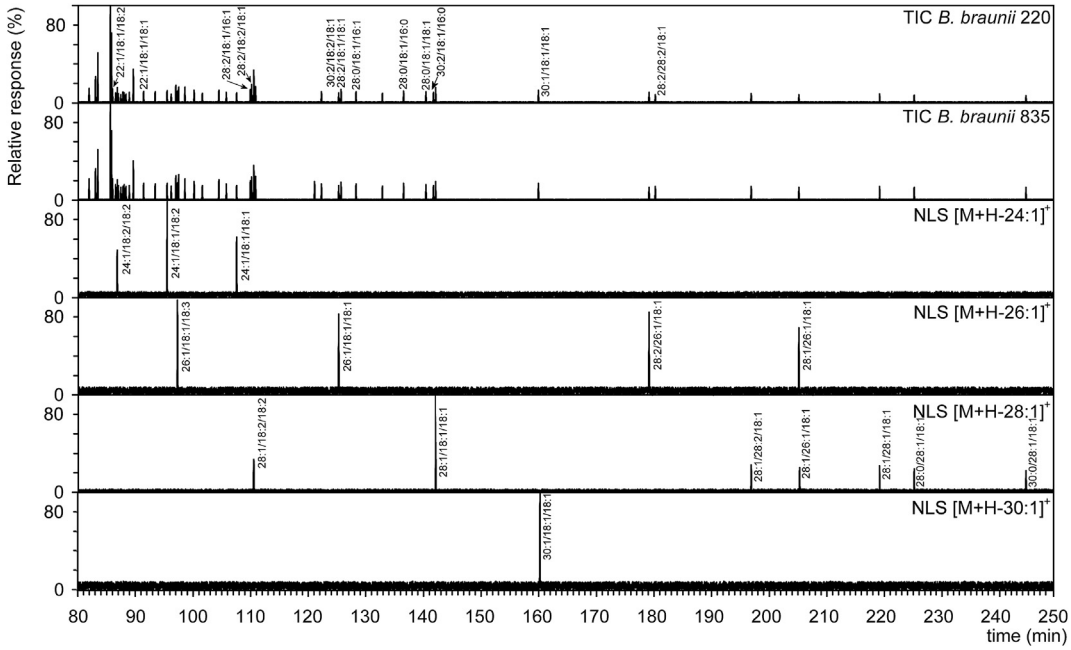

Fig. 2. Reversed phase liquid chromatography/electrospray ionization-mass spectrometry (RP-LC/ESI-MS) chromatogram from 80 to 250 min of total ion current (TIC) of the TAGs from the two strains of *Botryococcus braunii* (CCALA 220 and CCALA 835), and four NLS - $[M + H\text{-}24:1]^+$, $[M + H\text{-}26:1]^+$, $[M + H\text{-}28:1]^+$, and $[M + H\text{-}30:1]^+$.

and HC. Moutel et al. (2016) identified also PC, CL, SQDG, MGDG, DGDG, PG and PE. Essentially, only MacDougall et al. (2011) identified DGDG, SQDG, PG, MGDG, DGTS, and PC using LC-high resolution MS. Unfortunately, they only analyzed their presence without the lipid class quantification and without identifying molecular species of polar lipids. They specified only molecular species of TAGs, see below.

Boyd et al. (2012) were the only ones identifying the molecular species of glycolipids, i.e. DGDG, the glycolipid containing no VLCFAs but containing PUFAs, e.g., 16:3 and 18:3. This supports the theory that the VLCFAs are located and probably synthesized in the cytoplasm or in an organelle other than the chloroplast, whereas the C16 and C18 PUFA are glycolipid constituents apparently synthesized within the chloroplast. The function(s) of these fatty acids as components of phospholipids in cellular membranes is currently unknown. Generally speaking, the VLCFAs are most represented in phospholipids and sometimes in TAGs and are missing in glycolipids (Rezanka et al., 2017a). The authors have focused only on

Table 2
Major lipid classes of the strains *Botryococcus braunii* CCALA 220 and *Botryosphaerella sudetica* CCALA 780.

Lipid classes	Abbreviations	CCALA 220	±S.D.[a]	CCALA 780	±S.D.
Hydrocarbons	HC	3.8	0.2	4.9	0.3
Squalene	SQ	4.2	0.3	5.9	0.2
Unknown 1	U1	2.3	0.1	3.1	0.2
Sterol esters	SE	9.4	0.2	8.4	0.2
Triacylglycerols	TAG	8.3	0.1	9.1	0.1
Diacylglycerols	DAG	9.5	0.1	9.3	0.1
Monogalactosyldiacylglycerols	MGDG	7.7	0.1	7.5	0.1
Free fatty acids	FFA	0.4	0.2	0.5	0.3
Sterols	S	2.6	0.2	5.0	0.2
Sulfoquinovosyl diacylglycerols	SQDG	1.3	0.1	1.5	0.3
Diacylglycerylhydroxymethyltrimethylalanine/ Diacylglyceryltrimethylhomoserine	DGTS/DGTA	9.1	0.2	8.9	0.2
Phosphatidylglycerol	PG	5.3	0.3	4.0	0.2
Digalactosyldiacylglycerols	DGDG	1.8	0.1	2.6	0.1
Phosphatidylcholines	PC	2.7	0.2	1.9	0.2
Phosphatidyl sulfocholine	PSC	0.1	0.1	0.1	0.1
Unknown 2	U2	5.3	0.2	5.6	0.3
Phosphatidylethanolamines	PE	4.3	0.3	1.3	0.2
Lysophosphatidylcholines	LPC	4.0	0.2	2.6	0.3
Phosphatidylinositols	PI	5.3	0.2	4.6	0.3
Phosphatidic acids	PA	1.3	0.2	2.3	0.2
Phosphatidylserines	PS	3.5	0.3	3.7	0.2
Unknown 3	U3	1.7	0.4	1.9	0.5
Unknown 4	U4	0	0	2.1	0.1
Lysophosphatidic acids	LPA	2.8	0.3	1.3	0.1
Unknown 5	U5	2.1	0.1	1.1	0.2
Unknown 6	U6	0.4	0.1	0.7	0.2
Unknown 7	U7	0	0	1.0	0.1
Unknown 8	U8	1.5	0.1	2.0	0.1

[a] Arithmetic means from three cultivations ± standard deviations.

neutral lipids, mostly HC and related compounds (botryals, botryococcenes) while TAGs have been identified only exceptionally, see below. On the other hand, this narrow focus can be understood as it is always directed to the use of algae as a potential source of biofuel. It is, however, very striking that, for example, Kalacheva et al. (2002) stated that total polar lipids were responsible for more than half of total lipids, whereas hydrocarbons in the genus *Botryococcus* made up only about 10% of total lipids. *B. braunii* is unique because this alga contains a wide range of molecular species of TAGs (MacDougall et al., 2011). Such a wide chain length interval in TAGs has not been described in other algae, yet. MacDougall et al. (2011) described molecular species of only TAGs, not polar lipids (phospho- and glycolipids).

2.3. Identification of simple lipids (glycerolipids)

2.3.1. Identification of TAGs

Total TAGs collected manually from HILIC, see Experimental, were further separated and identified by NARP-LC/MS^2. In order to obtain sufficient resolution, two reversed phase columns were connected in series. Unfortunately, the retention time of the last of the eluting peaks exceeded 4 h. It should be noted, however, that TAGs with such long chains are usually not a common part of natural fats and oils. The identification of unusual molecular species, i.e., TAGs containing one or two VLCFAs, was performed both on the basis of chromatographic behavior and tandem MS. However, this old concept is hardly applicable to modern systems because it depends on the position of double bonds (Christie and Han, 2010). Double bonds in a diene have a different effect than in two monoenes, so you can separate these two TAGs (18: 0/18: 0/18: 2 and 18: 0/18: 1/18: 1 having tR 77.7 min and 83.4 min, respectively, see Table 5S). Certain improvements can be made by adding additional coefficients (Pocklington and Dieffenbacher, 1987, 1992). ECN can be calculated much more accurately when considering the location of double bonds. If n1, n2 and n3 are the numbers of double bonds in oleic acid, linoleic acid and linolenic acid, then ECN can be calculated according to the formula: ECN=CN-d1n1-d2n2-d3n3. The coefficients d1 to d3 can be obtained using the TAG standards; the values of 2.60, 2.35, and 2.17, respectively, are given. The formula then looks like this: ECN=CN-(2.60 n1)-(2.35 n2)-(2.17 n3)".

As previously described (Ovcacikova et al., 2016; Rezanka et al., 2017b, 2017c), the order of elution from the column does not always correspond to the given ECN. For instance, retention time peaks at 140.6 and 141.9 min were determined for two structurally different TAGs, i.e. 28:0/18:1/18:1 and 30:2/18:1/16:0, with ECN being 60 and 58 respectively.

Positive ion ESI-MS of triacylglycerols produces ammonium adducts, i.e. ions of type $[M + NH_4]^+$ as the most prominent peaks in all mass spectra. This becomes especially important in cases of chromatographic overlap of species, or identification of new or rarely reported TAGs with little unsaturation, in which MS/MS of the $[M + NH_4]^+$ precursor ion can be definitive for proof of TAG identity. This is demonstrated in Fig. 3, which shows TAGs containing VLCFAs, including nervonic (24:1), ximenic (26:1), octacosenoic (28:1), and triacontenoic (30:1) fatty acids. In ESI-MS of the TAGs containing VLCFAs were produced $[M + NH_4]^+$ ions as one of the abundant peaks. Although positive ion ESI-MS/MS was used, the $[M + H\text{-}RCOOH]^+$ fragments were identified, see Fig. 3 and Tables 2S–4S, and were used also to identify regioisomers.

Fig. 4 shows the mass spectra of two TAGs containing VLCFAs, i.e. 30:1/18:1/18:1 and 28:1/18:1/28:1; for description of ions and their abundances, see Tables 3S and 4S. As can be seen, the abundance of $[M + NH_4]^+$ ions reached 1/4 to 1/3 of that of $[M + NH_4–RCOOH–NH_3]^+$ ions abbreviated as $[M + H–RCOOH]^+$. Furthermore, ions $[RC{=}O–H_2O]^+$, $[RC{=}O]^+$, $[RC{=}O+74–H_2O]^+$ and $[RC{=}O+74]^+$ are present in tandem MS spectrum. Based on the abundance of $[M + H\text{-}RCOOH]^+$ ions, it could be identified which

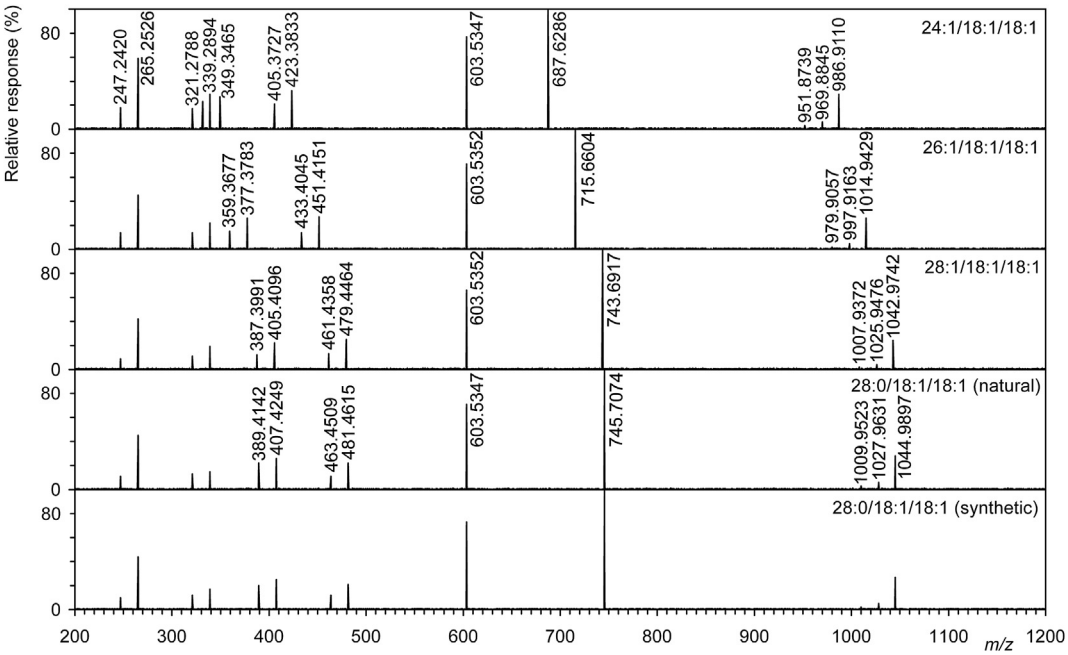

Fig. 3. Tandem mass spectra of four natural TAGs, i.e. 24:1/18:1/18:1, 26:1/18:1/18:1, 28:1/18:1/18:1, 28:0/18:1/18:1, and one tandem mass spectrum of synthetic 28:0/18:1/18:1 TAG.

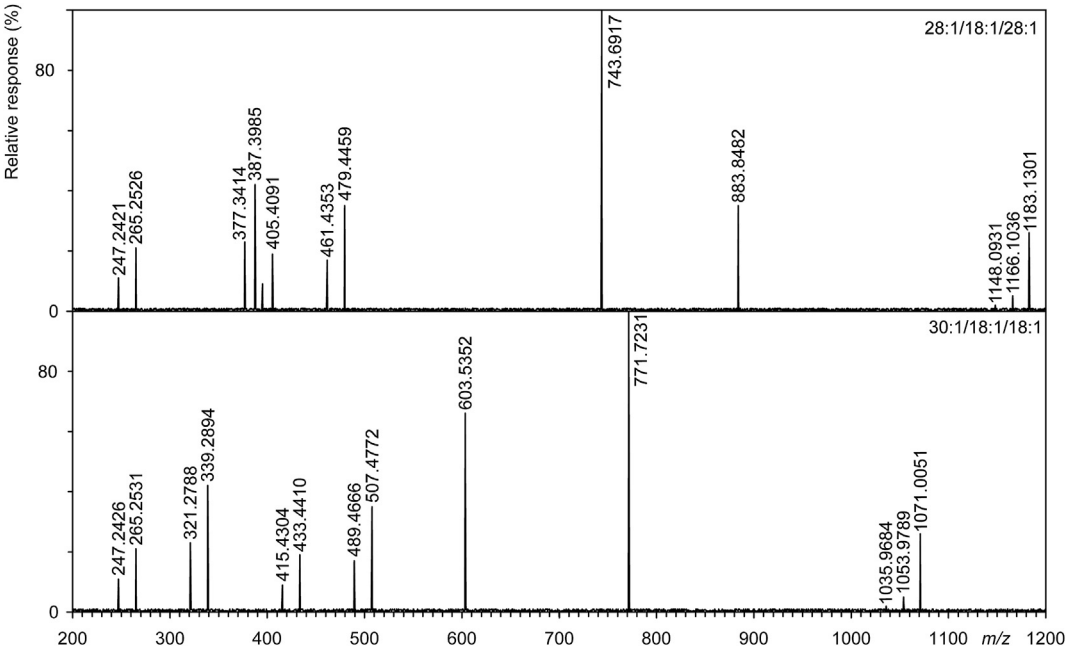

Fig. 4. Tandem mass spectra of two natural TAGs, i.e. 28:1/18:1/28:1 and 30:1/18:1/18:1.

regioisomer is involved in TAGs containing two identical FAs. Although APCI-MS has been demonstrated to be rather reliable for regioisomer identification, additional work needs to be done toward consistent characterization of regioisomers by ESI-MS/MS.

To confirm the validity of the rule stating that "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) that makes it possible to distinguish, even in TAGs containing VLCFAs, the position of the acyl on the glycerol backbone i.e. on the primary and secondary glycerol hydroxyls, from the abundance of ions $[M + H + R_{1,3}COOH]^+$ versus $[M + H + R_2COOH]^+$ we performed a stereospecific synthesis of 28:0/18:1/18:1 TAG based on commercially available 1,2-dioleoyl-phosphatidylcholine (18:1/18:1-PC), the hydrolysis of which by phospholipase C gives *sn*-1,2-dioleoyl glycerol. Subsequent reaction with montanic acid (commercially available 28:0 fatty acid) catalyzed by *N*,*N*′-dicyclohexylcarbodiimide yields *sn*-28:0/18:1/18:1, see the Supplements. Its mass spectrum (Fig. 3) shows minimal differences in abundance of all ions in the synthetic and natural TAG, thus confirming that the above rule also applies to TAGs with VLCFAs. Based on this rule, we

then identified additional TAGs, see the mass spectra for the three selected TAGs, i.e. 28:1/18:1/18:1, 26:1/18:1/18:1, and 24:1/18:1/18:1. Using the same rule, we found in the case of TAGs containing two VLCFAs, i.e. 28:2/18:1/28:2 or 28:0/18:1/28:1 that very long chain fatty acids are always bound to the primary hydroxyl of the glycerol backbone, i.e. they are in the *sn*-1 and *sn*-3 positions.

To our current knowledge, there are very few studies describing molecular species of TAGs in *B. braunii*. Metzger et al. (1989), besides conventional TAGs usually contained in vegetable oil, i.e. linoleoyl-dioleoyl-glycerol (LOO), linoleoyl-palmitoyl-oleoyl-glycerol LPO, triolein (OOO), palmitoyl-dioleoyl-glycerol (POO), stearoyl-dioleoyl-glycerol (SOO), described a TAG containing 18:1/18:1/28:1 that formed ~10% of total TAGs. More than 20 years later, Boyd et al. (2012) identified triolein as the major TAG using ultra-performance LC/ESI-MS (UPLC/ESI-MS) in positive ion mode. Other less abundant but unusual TAGs contained VLCFAs, e.g. 28:2/18:1/18:1 and 28:1/18:1/18:1. A complex analysis performed by MacDougall et al. (2011) identified in *B. braunii* dozens of TAGs, including those containing VLCFAs, e.g. 28:1/28:1/18:1, 28:2/28:1/18:1, 28:2/28:2/18:1 and many more, unfortunately without defining the origin of algal strain (collection name and number). However, they also identified TAGs containing C16 and C18 PUFAs, e.g. 16:4/18:2/18:2, 18:1/18:1/16: 3, 18:1/18:4/18:3 and others. Their analysis represents, at least to our current knowledge, the most complex study of the molecular species of triacylglycerols in the genus *Botryococcus*. Unfortunately, as already noted in the part dealing with fatty acid composition, the original *B. braunii* Kuetzing, strain CCAP 807/1 (Droop 1950/807-1) was a mixture of at least two algae, now called *B. braunii* and *Botryosphaerella sudetica*. Although the authors did not indicate the origin of their strain, we believe that it was the strain Droop 1950/807-1, which also corresponds to the composition of the TAGs. For comparison, if we add up the TAGs listed in our Table 5S, in columns marked *B. braunii* and *Botryosphaerella sudetica*, the results are very similar to those shown in Fig. 4 of the study by MacDougall et al. (2011).

In contrast to the above studies, Donot et al. (2013) did not identify any TAGs containing VLCFAs, but only TAGs and DAGs with C16 and C18 fatty acids (e.g., PPO, OOP, PPL). It is not clear from their results whether TAGs containing VLCFAs in algae lipids were present, as they also analyzed the strain *B. braunii* CCAP 807/1 that was isolated by M.R. Droop in 1950. As can be seen from the above data, all the complications arose from the fact that the original *B. braunii* Kuetzing strain Droop 1950/807-1 has spread to many collections around the world, without stringent control ensuring that there was no other alga in the inoculum. In fact, this was obviously true (Senousy et al., 2004). In conclusion, we can say that most of the published studies were conducted on a mixed culture with a variable ratio of two algae, namely *B. braunii* and *Botryosphaerella sudetica*, provided the authors used strains derived from the original strain *B. braunii* Kuetzing, strain Droop 1950/807-1. Many culture collections, such as the Culture Collection of Algae at the University of Texas at Austin (UTEX), Culture Collection of Autotrophic Organisms of the Institute of Botany CAS (CCALA), Culture Collection of Algae and Protozoa (CCAP), Culture Collection of Algae at Göttingen University (SAG), etc., still offer the *B. braunii* strain derived from the original strain.

2.3.2. Identification of PCs

Our analysis of PCs from the alga *B. braunii* algae brought about the identification of many dozens of molecular species, see Table 6S and also Fig. 5. In order to be able to identify such a large number of molecular species, we used again two reversed phase columns with relatively small sorbent particles (5 μm) connected in series to achieve the level of 85.000 theoretical plates (18:1/18:1-PC was used as a standard). Furthermore, using the mobile phase aqueous ammonium acetate-acetonitrile-2-propanol and using a gradient, we were able to identify more than 120 molecular species of PCs. Phosphatidylcholines containing VLCFAs, i.e. even-numbered FA from C24 to C30 with 0–2 double bonds, were separated for the first time.

Lipid molecular species were separated according to ECN. Although this system was used for TAGs, it is also applicable to polar lipids (Ovcacikova et al., 2016). Partial invalidity of the rule can be expected in some phospholipids, especially those that have

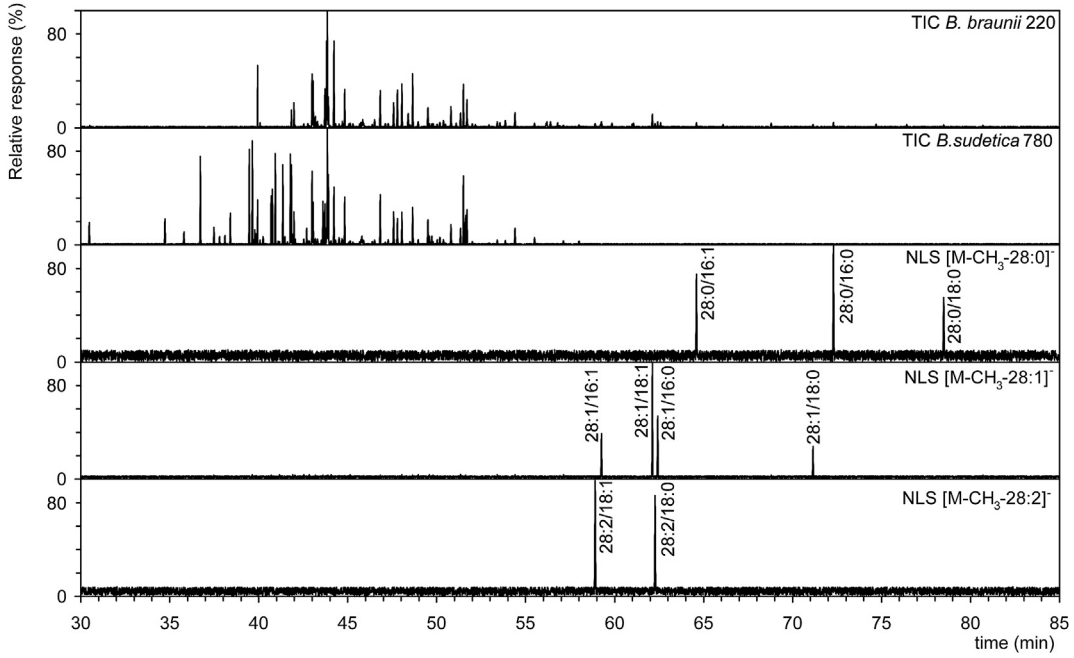

Fig. 5. Reversed phase liquid chromatography/electrospray ionization-mass spectrometry (RP-LC/ESI-MS) chromatogram of total ion current (TIC) of the PCs from the strains *Botryococcus braunii* (CCALA 220) and *Botryosphaerella sudetica* (CCALA 780), and three neutral losses of RCOOH group (28:0, 28:1, and 28:2, respectively), loss of CH_3 and acetate from precursor ion ($[M + acetate]^-$), see Table 6S.

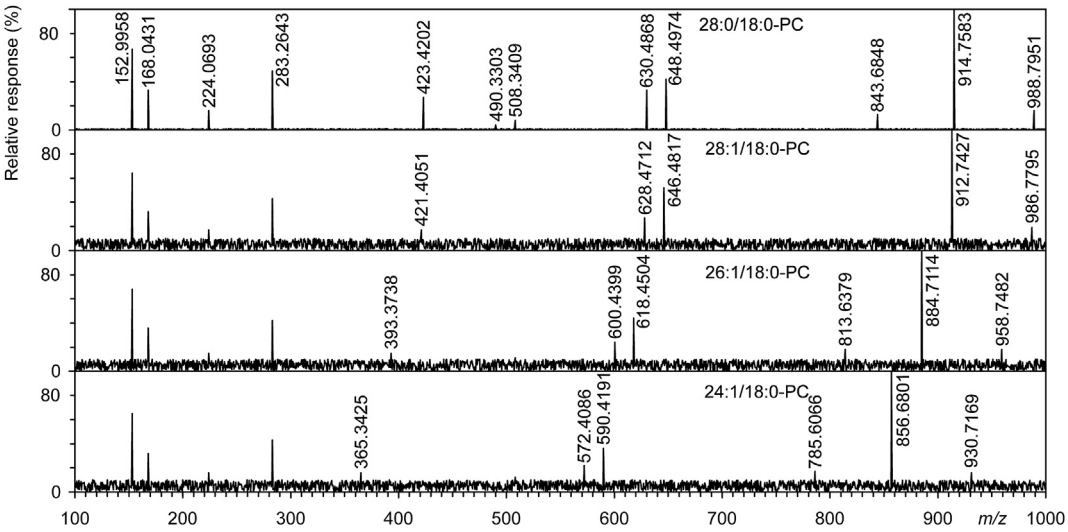

Fig. 6. Tandem mass spectra of four natural PCs, i.e. 24:1/18:0-PC, 26:1/18:0-PC, 28:1/18:0-PC, and 28:0/18:0-PC.

two completely different acyls, e.g. a combination of highly polyunsaturated and saturated fatty acyls, or chains varying in up to 10 methylenes. An example is 18:2/18:2-PC having ECN = 28 and RT 40.93 min versus 17:0/16:3-PC with ECN 27 and RT = 41.09 min, see Table 6S. Similar behavior was observed in TAGs in RP-HPLC, see above.

Phosphatidylcholines can be analyzed in negative ion ESI mode if the eluate is spiked e.g. with acetate. MS^2 of molecular species 28:0/18:0-PC gives rise to an ion at 988.7951 Da, i.e. anion adduct $[M + CH_3COO]^-$. Furthermore, in MS^2, other diagnostic ions are present in the mass spectrum, see Fig. 6. In particular, it is a base peak at *m/z* 914.7583 ($[M + CH_3COO{-}CH_3COO{-}CH_3]^-$, more preferably $[M\text{-}CH_3]^-$), resulting from a loss of acetate and a methyl group from the choline moiety (Hsu and Turk, 2009). This ion is further fragmented to form four diagnostic ions, in which the abundance ratio is important. These are ions from which the structure of *sn*-28:0/18:0-PC can be assumed on the basis of the higher abundance of $[M{-}CH_3{-}R_2COOH]^-$ ion at *m/z* 630.4868 than the $[M{-}CH_3{-}R_1COOH]$- ion at *m/z* 490.3303 and higher abundance of $[M\text{-}R'_2CH{=}C{=}O]^-$ ion at m/z 648.4974 than the $[M{-}CH_3{-}R'_1CH{=}C{=}O]^-$ ion at *m/z* 508.3409. Further, in the mass spectrum, ions at 843.6848 Da (loss of choline and acetate from the 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) are present. In addition, among other diagnostic ions the acyl anion fragment of *sn*-2 FA at *m/z* 283.2643 ($[R_2COO]^-$) was less abundant than FA at *sn*-1 position at *m/z* 423.4202 ($[R_1COO]^-$), which fully acknowledged the *sn*-28:0/18:0-PC structure, see Chen and Subbaiah (2013).

Minority classes of lipids, i.e. DAGs, PAs, LPCs and LPAs, were identified only by HILIC-tandem MS, not after separation into individual molecular species using NARP-LC. In tandem MS, diacylglycerols always yield the major ion type $[M + H{-}H_2O]^+$, which is accompanied by minor ions $[M + NH_4]^+$ and $[M{+}H]^+$. A similar abundance of ions was described by Deng et al. (2016). Ion types $[M + NH_4\text{-RCOOH}]^-$ $[RCO{+}74\text{-}H_2O]^+$ provide information on the DAG structure. The amount of molecular species was much lower than that found in TAGs, which is consistent for instance with the data of Boyd et al. (2012) who identified 10 TAGs but only three DAGs. Only DAGs having one VLCFA were identified, which is understandable since the acid bound to position *sn*-2, i.e. to the secondary hydroxyl of TAGs, was common FA, not VLCFA.

All three classes of phospholipids were analyzed in negative ion mode of ESI. Lysophosphatidylcholine, like the aforementioned PCs, forms an ionic adduct $[M + CH_3COO]^-$ and further $[M{-}CH_3]^-$ including $[RCOO]^-$ ions, which allow further identification of acyl groups. PA and LPA then form ions of the type $[M{-}H]^-$ and ions $[M{-}H{-}RCOO]^-$, or ions resulting from the loss of ketene, i.e. ions of $[M{-}H{-}R'CH{=}C{=}O]^-$ type. All of the above types of ions allow us to identify molecular species but not their regioisomers. We attempted to identify regioisomers in PA based on the abundance of ions $[M\text{-}H\text{-}R_1COOH]^-$ and $[M\text{-}H\text{-}R_2COOH]^-$. However, since we did not have the relevant regioisomer standards, the identification of the acyls in the *sn*-1 and *sn*-2 positions was not convincing.

3. Conclusion

This study provided a complex analysis of VLCFAs in the green alga *B. braunii*, which is characterized by an unusual and extremely high lipid content. Our results show that VLCFAs are associated with TAGs and phospholipids in this species.

The use of NARP-LC/ESI-MS enabled us to not only separate, but also identify the molecular species of TAGs and PCs. We also identified their individual regioisomers (evidenced also by the organic synthesis of the regioisomer of TAG, i.e. 28:0/18:1/18:1) containing VLCFAs up to C30 (30:2, 30:1 and 30:0). We showed that VLCFAs were always ester-bound to the primary alcohol group, i.e. the *sn-1* or *sn-3* position of TAG or *sn-1*in the case of PC. This opens the possibility of using this knowledge for analyzing molecular species of lipids containing VLCFAs in higher plants, fungi, or animals.

Comparison of the *B. braunii* FA profile with that of *Botryosphaerella sudetica* isolated as a contaminant of *B. braunii* confirmed large differences related to the fact that the two algae belong to different algal classes within green algae (Chlorophyta). This further stresses the need of a careful examination of the biomass to be analyzed.

4. Experimental

4.1. Chemical and standards

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). All other chemicals were purchased from Sigma-Aldrich (Prague, CR).

4.2. Cultivation

Five strains, CCALA 220 *Botryococcus braunii* Kuetzing, strain: DROOP 1950/807-1, origin: United Kingdom, Cambridge; CCALA 778 *Botryococcus braunii* Kuetzing, strain: SANTOS 1997/97-56, origin: Portugal, Serra da Estrela, Barragem da Erva da Fome; CCALA 780 *Botryosphaerella sudetica* P.C.Silva, strain: Vazquez-Duhalt; CCALA 835 *Botryococcus braunii* Kuetzing, strain: Hegewald 1977-207, origin: Peru, Cuzco, Huaypo lake; CCALA 933 *Botryococcus* sp. strain: Cepak et Pribyl 2010/1, origin: Czech Republic, Třeboň, pool, water bloom, were obtained from the Culture Collection of Autotrophic Organisms at the Institute of Botany CAS, Třeboň, Czech Republic. They are kept as growing cultures on test tube slants at about 15 °C, under fluorescent lighting with photosynthetically active radiation (PAR) intensity of ca 40 μmol m^{-2} s^{-1}. The cultures were used to pre-cultivate the strain in a batch stationary culture in liquid Z8 medium in 2 L glass bottles containing initially 0.25 L medium. When the cultures became denser, the volume was increased to 2 L and the PAR intensity was increased to 48 μmol m^{-2} s^{-1}. Stirring of the suspension and CO_2 saturation was ensured by bubbling an excess air with 2% CO_2 introduced to the bottom of the bottle via a tube; the gaseous mixture was filtered through membrane filters (Midisart 2000, 0.2 μm PTFE Sartorius). After reaching stationary phase, the cultures were harvested by centrifugation at 2000 rpm for 15 min, frozen to −20 °C, then lyophilised and the samples were stored in aluminium foil until solvent extraction.

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 and Podojil, 1986; Vancura et al., 1987). 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 ion ESI is again in the Supplements.

4.6. Reversed phase separation of TAGs and PCs

Separation and identification of molecular species of TAGs and PCs 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 https://doi.org/10.1016/j.phytochem.2018.01.011.

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