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Rapid screening of very long-chain fatty acids from microorganisms

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ABSTRACT

The analysis of triacylglycerols and phospholipids - phosphatidylcholines allowed the use of shotgun lipidomics to identify very long-chain fatty acids and very long-chain polyunsaturated fatty acids in microalgae. These fatty acids were determined in triacylglycerols by positive electrospray ionization of neutral loss scans of different fatty acids, e.g. 24:0, 24:1ω9, 24:6ω3, 26:0, 26:1ω9, 28:0, 28:1ω9, 28:2ω6, and 28:8ω3. Likewise, very long-chain fatty acids in phosphatidylcholines were identified by negative electrospray ionization mass spectrometry in the selected ion-monitoring of the two most important ions (R_1COO^- and R_2COO^-). The limit of detection was determined at 10 nmol/L (~11 pg/μL) in triacylglycerols and 8.6 nmoles/L (~8 pg/μL) in phosphatidylcholines. The use of liquid chromatography–mass spectrometry is suitable for very long-chain polyunsaturated fatty acids with up to 8 double bonds due to the time of analysis as well as for reasons of lower thermal stability of polyunsaturated fatty acids towards saturated fatty acids, but gas chromatography–mass spectrometry is better suited for the analysis of saturated very long-chain fatty acids.

1. Introduction

Fatty acids (FA) are classified according to the length of the carbon chain and the number of double bonds. Long-chain FA (LCFA) have chain lengths with C11-C20, of which LCFA C16 and C18 are the most common types of FAs occurring in nature. FA longer than C22, are called very long-chain FA (VLCFA) and are less frequent than LCFA. VLCFA with $C \geq 26$ are often subclassified as ultra-long-chain FA (ULCFA) and are found throughout the protozoan, plant and animal kingdoms, i.e. from the algae and higher (flowering) plants through sponges to specialized mammalian tissues (brain, retina, etc.). They are also a common part of fungi and bacteria [1]. The boundaries of chain lengths are sometimes even more shifted; see, for example [2], where VLCFA are defined as fatty acids with 24–28 carbon atoms and ULCFA as fatty acids with chains of 30 to 38 carbon atoms.

Unfortunately, their representation is very low, usually below 1% of total FA. Therefore, apart from specialized mammalian organs (retina, brain, meibomian glands, etc.), sponges or wax esters from the plant cuticle, their analysis is rarely described.

Additionally, as already reported by Byrdwell [3], most of the flame ionization detectors (FID) have a lower sensitivity than ESI-MS, and in many cases, due to low thermal stability, the stationary phase of the capillary column the FAs are not eluted from this column. Also except for a few cases (e.g. saturated even numbered VLCFA), they are not commercially available as standards and are often overlooked by the operator.

Below are listed some of the most commonly used methods for qualitative and quantitative analysis of VLCFA (VLCPUFA). Gas chromatography has been used for the qualitative identification and quantitative determination of fatty acid methyl esters (FAME) for more than 50 years. It is routinely used with FAME obtained from all possible sources, from bacteria to mammals (humans) [4–8].

Triacylglycerols have been selected primarily because they are the most abundant simple glycerolipids, whereas the phospholipids, mainly and phosphatidylcholines are present in all eukaryotic cells, i.e. from algae to flowering plants. It is also known

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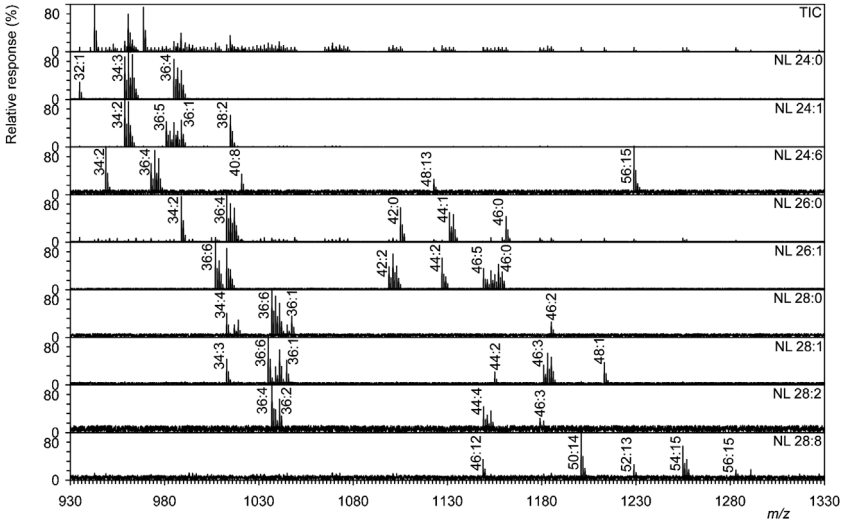

Fig. 1. Shotgun analysis of standard mixture of TAG from algae. TIC - total ion current, NL- neutral loss of appropriate fatty acids, i.e. 24:0, 24:1, 24:6, 26:0, 26:1, 28:0, 28:1, 28:2, and 28:8. Labels of the clusters with the carbon number and the number of double bonds are shown. Positive ESI-MS, the peaks were labeled X:Y, where X is the sum of the carbon number in the remaining acyls and Y is the number of double bond(s).

that VLCFAs are most common in PCs, whether in plants (e.g. [9,10]) or animals [11,12].

Table 1S (Supplements) summarizes the separation conditions commonly used for VLCFA (VLCPUFA) using gas chromatography (GC). It can be seen that retention time (tR) of conventional FAMEs ranged in tens of minutes, which is illustrated by the tR of 22:6 FAME. A number of studies have attempted to shorten the time needed for sample analysis [4]. These experiments were mostly limited to FAMEs up to C22. By using a shorter capillary column, usually 10 m in length and smaller I.D., i.e. film thickness of about 0.1 mm, it was possible to shorten tR to the order of minutes, see Table 1S. Unfortunately, these analyzes were not performed for VLCFA (VLCPUFA), where tR on normal size columns is about 1 h, see again Table 1S.

In the case of VLCFA or VLCPUFA separated and identified by HPLC (Table 2S), the situation is similar as in GC. This table summarizes some analyses given as a representative sample. In addition to the usual retention times, which are in the order of tens of minutes, examples are given where tR for the commonly occurring and frequently analyzed 22:6 FA is in the order of minutes. Unfortunately, HPLC is much more vulnerable to the fact that tR is heavily dependent on effective carbon number (ECN), where ECN = acyl carbon number (ACN) - 2 × double bond (2 × DB). Therefore, 22:6 has a similar tR as decanoic acid. This fact was fully manifested, e.g., in human plasma analysis, where 22:6 had tR 6.5 min, whereas 28:1 had 49.2 min [13]. Another option is to use a mass spectrometer without prior separation by GC–MS or LC–MS, called shotgun MS.

Tandem MS, neutral loss (NL) scanning and precursor ion scanning (PIS) or multiple PIS (MPIS) can also be used for fatty acid analysis. An important advantage of shotgun lipidomics over LC–MS analysis is that during a direct infusion, the mass spectrum displays the protonated molecules of the individual molecular species of the lipid classes at constant concentrations, allowing the scanning of precursor-ion scans and/or neutral loss scans. The entire experiment is based on the principle that most lipid types are a combination of several building blocks such as glycerol, sphingoid bases, polar head groups and fatty acids. One possible platform for the use of shotgun lipidomics is the identification and semi-quantification of individual lipid molecules by means of a multidimensional shotgun lipidomics. For two dimensional MS, the

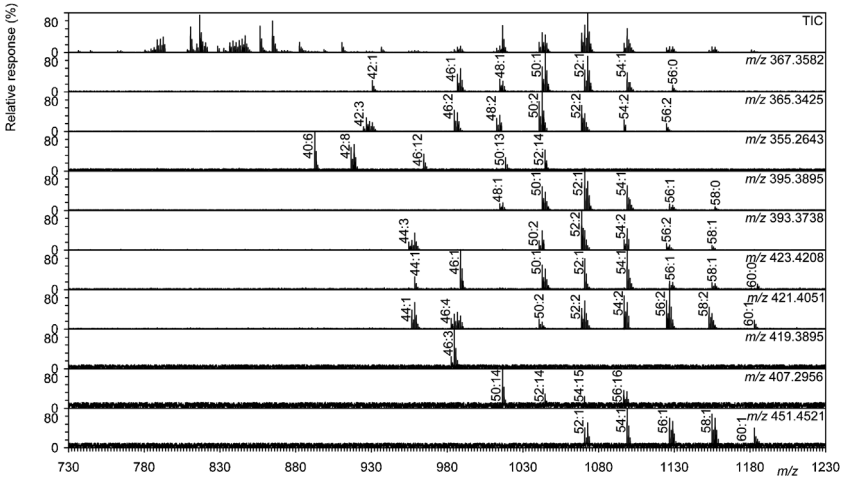

Fig. 2. Shotgun analysis of standard mixture of PC from algae. TIC - total ion current. SIM - selected ion monitoring at *m/z* 367.3582 (24:0), *m/z* 365.3425 (24:1), *m/z* 355.2643 (24:6), *m/z* 395.3895 (26:0), *m/z* 393.3738 (26:1), *m/z* 423.4208 (28:0), *m/z* 421.4051 (28:1), *m/z* 419.3895 (28:2), *m/z* 407.2956 (28:8), *m/z* 451.4521 (30:0). Labels of the clusters with the carbon number and the number of double bonds are shown. Negative ESI-MS, the peaks were labeled X:Y, where X is the sum of the carbon number in the remaining acyls and Y is the number of double bond(s).

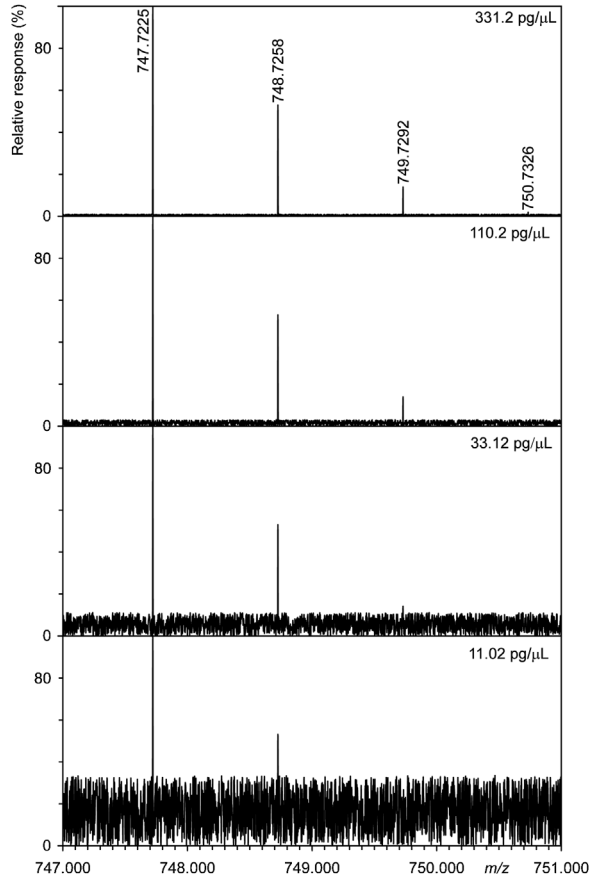

Fig. 3. Limit of detection of TAG (23:0/23:0/23:0) based on neutral loss of RCOOH + NH_3 from $[M+NH_4]^+$ at *m*/*z* 747.7225. Isotopic peaks containing the ^{13}C isotope are also shown.

x-axis in the first dimension is represented by protonated molecule values; in the second dimension, it is formed by mass values corresponding to the neutral fragment loss or the values of the observed fragment ions. In the case of positive ESI-MS, for example in TAG, neutral loss of the fatty acids corresponding to the appropriate acyl chains makes it possible to detect acyl chains bound to the glycerol backbone.

Thus, we demonstrate the use of LC–MS and shotgun analysis to identify VLCFA or VLCPUFA in microorganisms, where the main criterion was the time of analysis. This time in our case did not exceed 6 min, unlike commonly published studies, where either GC–MS or LC–MS lasts up to 1 h or vice versa, in the case of phospholipids, e.g. PE, PS, PI, PA, etc., it is possible to use SIM or PIS (better MPIS) in the negative ESI-MS to detect different classes of phospholipids.

2. Experimental

2.1. Chemical and standards

Both, 23:0/23:0-PC (1,2-ditricosanoyl-*sn*-glycero-3-phosphocholine), 1,2,3-tritricosanoylglycerol, and all other chemicals (standards PUFA No. 1 and PUFA No. 3) were purchased from Sigma-Aldrich (Prague, CR).

The mixture of natural triacylglycerols and also natural phosphatidylcholines was obtained by mixing at a ratio of 1:1:1 the previously prepared lipid classes from *Amphidinium carterae* [14], *Botryococcus braunii* [15], and *Balbiania investiens* [16].

Table 1
Observed *m*/*z* values, molecular species and relative concentrations (%) of TAG identified in alga *Tetraedron minimum*.

m/z	mol. spec.	%
934.8797	24:0/16:1/16:0	1.22
958.8797	24:1/18:2/16:0	1.75
958.8797	24:0/18:3/16:0	0.74
960.8954	24:1/18:1/16:0	2.97
960.8954	24:0/18:2/16:0	3.64
960.8954	24:0/18:1/16:1	1.83
962.9110	24:0/18:1/16:0	6.16
980.8641	24:1/18:2/18:3	0.33
982.8797	24:1/18:2/18:2	1.56
984.8954	24:1/18:1/18:2	2.63
984.8954	24:0/18:2/18:2	3.22
984.8954	24:0/18:1/18:3	1.13
986.9106	24:1/18:1/18:1	4.47
986.9110	24:1/18:2/18:0	0.60
986.9110	24:0/18:3/18:0	5.74
986.9110	24:0/18:2/18:1	4.36
988.9267	26:0/18:2/16:0	3.01
988.9267	24:1/18:1/18:0	1.16
988.9267	24:0/18:2/18:0	1.42
988.9267	24:0/18:1/18:1	1.90
990.9428	24:1/18:0/18:0	0.31
1006.8783	26:1/18:3/18:3	0.06
1008.8959	26:1/18:2/18:3	0.33
1010.9097	26:1/18:1/18:3	0.55
1012.9267	26:0/18:2/18:2	2.66
1012.9267	26:0/18:1/18:3	0.93
1012.9268	28:0/18:3/16:1	0.15
1014.9410	26:1/18:1/18:1	4.47
1014.9423	26:0/18:2/18:1	4.51
1014.9423	24:1/18:1/20:1	0.09
1016.9573	28:0/18:1/16:1	1.20
1016.9580	26:0/18:1/18:1	7.66
1018.9727	28:0/18:1/16:0	4.04
1034.9104	28:1/18:3/18:3	0.12
1036.9253	28:0/18:3/18:3	0.09
1038.9409	28:1/18:2/18:2	2.88
1038.9416	28:0/18:3/18:2	0.43
1040.9567	28:0/18:3/18:1	0.74
1042.9721	28:1/18:1/18:1	8.30
1044.9877	28:0/18:1/18:1	6.06
1047.0054	28:1/18:0/18:0	0.56
1049.0211	28:0/18:0/18:0	0.42
1073.0211	30:0/18:1/18:1	0.38
1099.0367	26:1/26:1/16:1	0.01
1101.0524	26:1/26:0/16:1	0.01
1103.0680	26:1/26:0/16:0	0.07
1105.0837	26:0/26:0/16:0	0.12
1127.0680	26:1/26:1/18:1	0.06
1129.0837	26:1/26:0/18:1	0.10
1131.0993	26:0/26:0/18:1	0.18
1133.1150	26:0/26:0/18:0	0.04
1155.0990	28:1/26:1/18:1	0.12
1157.1150	26:1/28:1/18:0	1.16
1159.1306	26:1/28:0/18:0	0.31
1161.1463	26:0/28:0/18:0	0.52
1183.1252	28:1/28:1/18:1	0.21
1185.1458	28:0/28:1/18:1	0.16
1213.1771	30:0/28:1/18:1	0.16

Further, the lipids were extracted from the algae *Desmodesmus quadricauda* and *Tetraedron minimum*, where were obtained by cultivation, see, subchapter 2.2. Cultivation.

2.2. Cultivation

The experimental organisms were obtained from the Culture Collection of Autotrophic Organisms (CCALA) in Třeboň, the Czech Republic (http://www.butbn.cas.cz/ccala/index.php). *Desmodesmus quadricauda* (formerly *Scenedesmus quadricauda*), strain CCALA 464, and *Tetraedron minimum*, strain CCALA 509 (both Chlorophyceae, Chlorophyta), were cultivated 4 days in glass

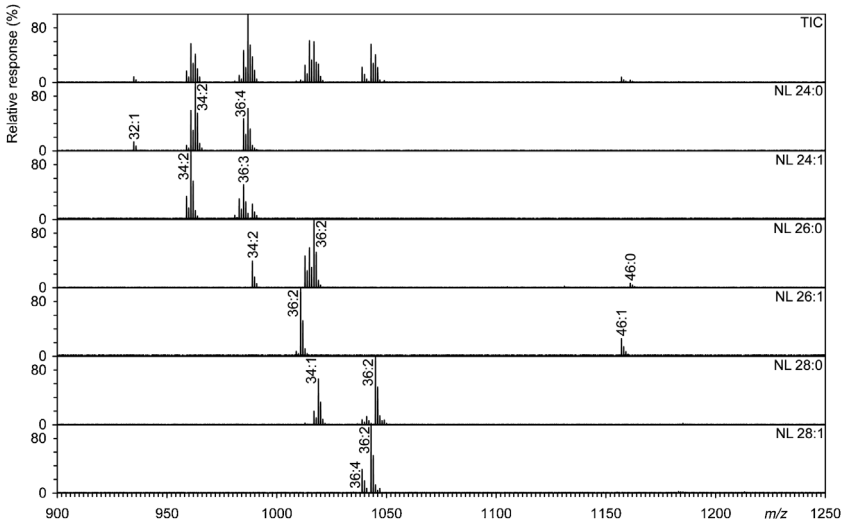

Fig. 4. Shotgun analysis of natural mixture of TAG from *Tetraedron minimum*. TIC - total ion current, NL- neutral loss of appropriate fatty acids, i.e. 24:0, 24:1, 26:0, 26:1, 28:0, and 28:1.

300 mL photobioreactors at 30 °C, continuously illuminated by a panel dimmable fluorescent lamps (OSRAM DULUX L55W/950 Daylight, Italy) at the incident light intensity 780 μmol/m^2/s (~33913 lx). The cultures were mixed by air enriched by CO_2 (2%, v/v). *D. quadricauda* and *T. minimum* were cultivated in mineral media described in [17,18].

2.3. Isolation of lipids

The extraction procedure was based on the method of Bligh and Dyer [19], except that 2-propanol was exchanged for methanol, since 2-propanol does not serve as a substrate for phospholipases [20]. The alcohol-water mixture was cooled, one part 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 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

2.4. Preparation of lipids and/or FAME

Total lipid extracts were applied to Sep-Pak Vac Silica cartridge 35cc (Waters; with 10 g of normal-phase silica), and TAG were subsequently eluted from the cartridge with 40 mL of chloroform-methanol (9:1) and the TAGs were dissolved in acetonitrile-2-propanol-hexane mixture (2:2:1, v/v/v) and aqueous 1 mM ammonium acetate was added before TAG shotgun analysis. Further, polar lipids were eluted from the cartridge by a mixture of 50 mL of methanol acidified with 0.1% of formic acid. The eluate of polar lipids was evaporated and phosphatidylcholines were dissolved in the mixture of acetonitrile-2-propanol (1:1, v/v), which was injected to mass spectrometer.

The lipids (both TAG and polar lipids) (~5 mg) were saponified overnight in 10% KOH-MeOH at room temperature. A fatty acid fraction obtained from saponification was partitioned between alkali solution (pH 9) and Et_2O to remove basic and neutral components. The aqueous phase, containing fatty acids, was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using CH_2N_2 [21].

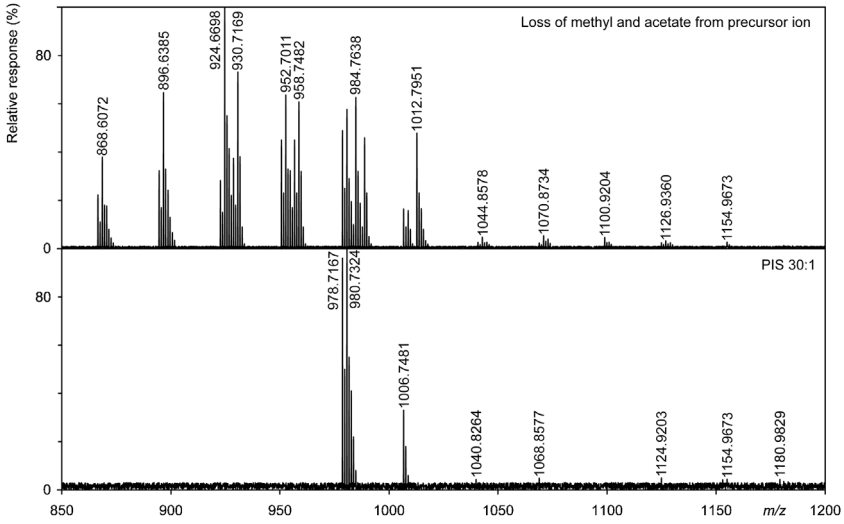

Fig. 5. Shotgun analysis of natural mixture of PC from *Desmodesmus quadricauda*. Loss of methyl and acetate from precursor ion, and precursor ion scan at *m*/*z* 451.4521 (30:1).

2.5. GC–MS analysis of FAME

FAME were analyzed using a high performance 7890A gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with column Supelcowax 10 (Sigma-Aldrich), 10 m x0.10 mm I.D., 0.10 μm film thickness coupled to an Agilent 7000 MS/MS (Agilent) with chemical ionization (CI) source. Oven temperature was programed at oven 170 °C, 20 °C/min to 270 °C; and held at 270 °C for 0.5 min. The injection conditions were: inj. temp. 280 °C, injection 0.2 μL in the split mode (100:1), carrier gas helium at 1.5 mL/min, 0.5 min solvent delay. Positive chemical ionization (PCI; full scan mode) mass spectra were recorded as normal mode, CI reagent ammonia at 1.3 mL/min [14,22–24].

2.6. LC–MS analysis of FAME

LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, Santa Clara, CA, USA) and column Ascentis® Express C8, 10 cm ×2.1 mm ×2.7 μm (Sigma-Aldrich). This setup was used as a high efficiency column - approximately 98,000 plates m^{-1} (methyl ester of heptadecenoic acid as an internal standard was used) with a flow rate of 0.3 mL/min, an injection volume of 1 μL, and column temperature of 35 °C. The FAME were separated using a solvent gradient program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (100:0, vol/vol); linear from 0 min to 6 min of ACN/iPrOH 10:90 (vol/vol). The composition was returned to the initial conditions over 1 min. The whole HPLC flow (0.3 mL/min) was introduced into the APCI source without any splitting. 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* 50–600 were scanned with a scan time of 0.2 s. A peak threshold of 0.05% 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. Double bond positions were determined by both retention time identity with commercially obtained standards (see Experimental), i.e. PUFA No. 1 and PUFA No. 1. 3 from Sigma, Prague, Czech Republic) and on the basis of earlier analyses, see [14].

2.7. Lipidomic analysis by high-resolution electrospray ionization mass spectrometry

The high resolution hybrid mass spectrometer LTQ Orbitrap Velos (Thermo Scientific, FL, USA) was used. ESI-MS analysis was performed in the positive and negative ionization mode. MS spectra were obtained by the Fourier transform mode and were acquired with target mass resolution of R = 105,000 at *m/z* 400. The ion spray voltage was set at 3.5 kV (in the positive ionization mode) and −2.5 kV (in the negative ionization mode). The scan range of the instruments was set at *m/z* 200–1500. Both ionization modes used the following parameters: sheath gas flow, 18 arbitrary units (AU); auxiliary gas flow, 7 AU; ion source temperature, 250 °C; capillary temperature, 230 °C; capillary voltage, 50 V; and tube lens voltage, 170 V. Helium was used as a collision gas for collision-induced dissociation (CID) experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The calibration of the MS spectrometer was conducted with the use of a Pierce LTQ Orbitrap positive and/or negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA). The elemental composition of the ions of interest was determined with the use of Xcalibur 2.2 software (Thermo Scientific).

Table 2
Observed *m/z* values, molecular species and relative concentrations (%) of PC identified in *Desmodesmus quadricauda*.

m/z	mol. spec.	%
866.5915	22:1/16:4	1.95
868.6072	22:1/16:3	3.34
870.6229	22:1/16:2	1.56
872.6386	22:0/16:2	0.39
894.6228	24:1/16:4	2.84
896.6385	24:1/16:3	5.69
898.6542	24:1/16:2	2.13
900.6699	24:0/16:2	0.59
922.6541	26:1/16:4	2.48
924.6698	26:1/16:3	8.81
926.6855	26:1/16:2	3.65
928.7012	26:0/16:2	3.29
930.7169	24:0/18:1	6.44
950.6854	28:1/16:4	3.96
952.7011	28:1/16:3	5.60
954.7168	28:0/16:3	2.84
956.7325	28:1/16:1	3.95
958.7482	28:1/16:0	5.35
978.7167	30:1/16:4	4.31
980.7324	30:1/16:3	5.08
982.7481	30:1/16:2	1.72
984.7638	28:1/18:1	5.51
986.7795	22:1/24:0	1.66
988.7951	22:0/24:0	4.04
1006.7481	30:1/18:4	1.44
1008.7637	30:0/18:4	1.38
1012.7951	26:1/22:1	4.21
1014.8108	26:0/22:1	1.45
1016.8264	26:0/22:0	0.29
1040.8264	30:1/20:1	0.23
1042.8421	28:0/22:1	0.41
1044.8578	28:0/22:0	0.24
1068.8577	30:1/22:1	0.20
1070.8734	30:0/22:1	0.47
1072.8891	30:0/22:0	0.34
1096.8890	28:1/26:1	0.07
1098.9047	28:0/26:1	0.41
1100.9204	28:0/26:0	0.24
1124.9203	30:1/26:1	0.21
1126.9360	30:0/26:1	0.28
1128.9517	32:0/24:0	0.21
1152.9516	30:1/28:1	0.10
1154.9673	30:1/28:0	0.24
1156.9830	30:0/28:0	0.09
1180.9829	30:1/30:1	0.11
1182.9986	30:0/30:1	0.10
1185.0142	30:0/30:0	0.10

Flow Injection Analysis (FIA) was used for sample introduction into the heated ESI-MS (H-ESI-MS) ion source. Acetonitrile/water (50/50 v/v) was used at the flow rate of 150 μL/min. The chemical structure of the compounds was confirmed with the help of the spectral database LIPID MAPS Structure Database [25].

The working standard solution of TAG was diluted appropriately with mobile phase to prepare the calibration standards in the concentrations 5–1000 nmol/L corresponding to 5.51, 11.02, 33.06, 110.2, 330.6 ug/L and 1.102 mg/L for the MS detection of TAG. Similarly, as with TAG, phosphatidylcholine was also prepared the solution of calibration standards in the concentrations 5–1000 nmol/L corresponding to 4.22, 8.44, 25.32, 84.4, 253.2, and 844 μg/L.

3. Results and discussion

3.1. VLCFA and/or VLCPUFA in nature

Based on our experience with VLCFA analysis, we have prepared a mixture of natural TAG obtained from four sources, i.e. *Amphidinium carterae* [14], *Botryococcus braunii* [15], *Desmodesmus*

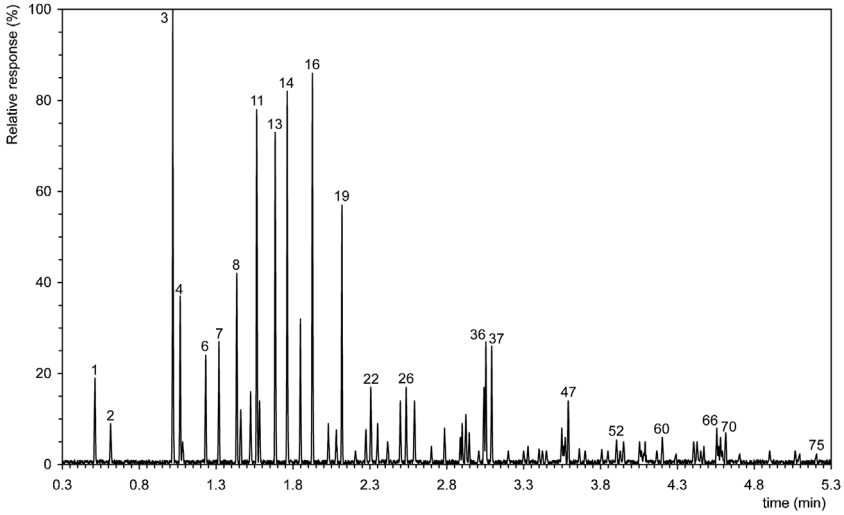

Fig. 6. GC–MS of standard mixture of FAME from algae. The peak numbers, see Table 3.

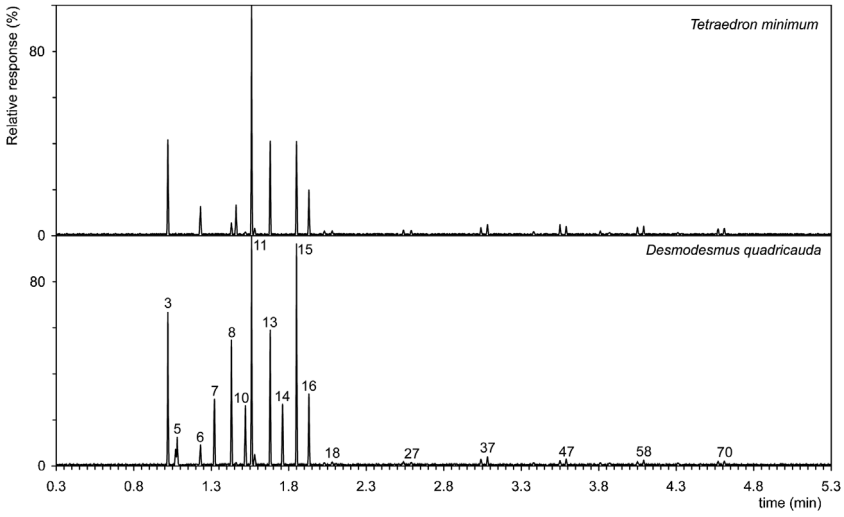

Fig. 7. GC–MS of natural mixture of FAME from two algae (*Desmodesmus quadricauda* and *Tetraedron minimum*). The peak numbers, see Table 3.

quadricauda (see Experimental), and *Pseudochantransia* sp. [16]. This mixture was prepared to obtain the widest range of VLCFA (VLCPUFA).

The nine very long-chain fatty acids in Fig. 1, (24:0, 24:1, 24:6, 26:0, 26:1, 28:0, 28:1, 28:2, and 28:8), are typical for representatives of either plant or microbial origin. All previously known samples in which VLCFA were identified [9,10] contained both saturated and monounsaturated FA minimal up to C30. For this reason we chose the above-mentioned fatty acids as typical representatives of VLCFA. In the case of VLCPUFA, the situation is more complicated, so we focused only on two VLCPUFA, 24:6 and 28:8. Both are, however, commonly identified in specialized mammalian tissues. Furland et al. [26] reported the presence of VLCPUFA up to 31:5n-6 in rat testis and Takashima et al. [27] identified them in fibroblasts and the plasma of patients with peroxisomal diseases, but they are also present, for example, in dinoflagellates [28,29].

3.2. Identification of TAG and PC containing VLCFA

To identify the TAG, positive ESI-MS with the addition of ammonium acetate in mobile phase was used to identify $[M + NH_4]^+$ ions. Fig. 1 shows the total ion current (TIC) and neutral loss scans of different FA (24:0 (385.4 Da), 24:1 (383.4 Da), 24:6 (373.3 Da), 26:0 (413.4 Da), 26:1 (411.4 Da), 28:0 (441.5 Da), 28:1 (439.4 Da), 28:2 (437.4 Da), and 28:8 (425.3 Da). As noted in [15,30], FA range in the TAG was from C16 to C32 and more than 100 molecular species of TAG containing VLCFA (VLCPUFA) was identified.

In the case of a mixture of phosphatidylcholines (PC), the molecular species were analyzed by negative ESI-MS tandem MS based on acyl ions (product ion scan) and the two most important ions (R_1COO^- and R_2COO^-) by the tandem MS were identified. Moreover based on the known rule ion arising from loss of the fatty acid at sn-2 is more abundant than the ion arising from loss of the fatty acid substituent at sn-1" [31], it was possible to identify the FA in the *sn*-1 and/or *sn*-2 position. In this case, see Fig. 2, the selected-ion monitoring (SIM) chromatogram of the following FAs (SIM 24:0 at 367.3582 Da, SIM 24:1 at 365.3425 Da, SIM 24:6 at 355.2643 Da, SIM 26:0 at 395.3895 Da, 26:1 at 393.3738 Da, SIM 28:0 at 423.4208 Da, SIM 28:1 at 421.4051 Da, SIM 28:2 at 419.3895 Da, SIM 28:8 at 407.2956 Da, and SIM 30:0 451.4521 Da) was used. Again, it is quite clear that this selection completely covers the presence of relevant FAs in different organisms.

Table 3
Relative concentrations (%) of fatty acids identified in standard mixture of algae using–MS and/or LC-MS including retention times (tR) of their FAMEs.

Peak No.	tR GC	abundance	FA
1	0.51	1.78	14:0
2	0.61	0.79	14:1ω5
3	1.02	9.36	16:0
4	1.07	3.47	16:1ω9
5	1.08	0.45	16:1ω7
6	1.23	2.25	16:2ω4
7	1.32	2.50	16:3ω4
8	1.43	3.93	16:4ω4
9	1.46	1.12	16:4ω1
10	1.52	1.44	18:0
11	1.56	7.31	18:1ω9
12	1.58	1.30	18:1ω7
13	1.68	6.84	18:2ω6
14	1.76	7.68	18:3ω6
15	1.85	3.00	18:3ω3
16	1.93	8.06	18:4ω3
17	2.03	0.84	20:0
18	2.08	0.75	20:1ω9
19	2.12	5.34	18:5ω3
20	2.21	0.24	20:2ω6
21	2.27	0.77	20:3ω6
22	2.31	1.59	20:4ω6
23	2.35	0.91	20:3ω3
24	2.42	0.44	20:4ω3
25	2.50	1.31	20:5ω3
26	2.54	1.61	22:0
27	2.59	0.32	22:1ω9
28	2.70	0.33	22:2ω6
29	2.79	0.73	22:3ω6
30	2.89	0.52	22:3ω3
31	2.90	0.88	22:4ω6
32	2.92	1.03	22:5ω6
33	2.95	0.63	22:4ω3
34	3.01	0.28	22:5ω3
35	3.04	0.59	24:0
36	3.05	2.53	22:6ω3
37	3.09	2.43	24:1ω9
38	3.20	0.20	24:2ω6
39	3.30	0.26	24:3ω6
40	3.33	0.36	24:4ω6
41	3.40	0.28	24:3ω3
42	3.42	0.25	24:5ω6
43	3.45	0.30	24:4ω3
44	3.55	0.71	26:0
45	3.56	0.37	24:5ω3
46	3.57	0.44	24:6ω3
47	3.59	1.38	26:1ω9
48	3.66	0.35	24:7ω3
49	3.70	0.23	26:2ω6
50	3.81	0.22	26:3ω6
51	3.85	0.29	26:4ω6
52	3.90	0.47	26:3ω3
53	3.93	0.28	26:5ω6
54	3.95	0.49	26:4ω3
55	4.05	0.46	28:0
56	4.07	0.32	26:5ω3
57	4.08	0.27	26:6ω3
58	4.09	0.43	28:1ω9
59	4.17	0.25	26:7ω3
60	4.20	0.56	28:2ω6
61	4.29	0.12	28:3ω3
62	4.41	0.42	28:4ω6
63	4.43	0.50	28:5ω6
64	4.45	0.29	28:4ω3
65	4.47	0.34	28:6ω6
66	4.56	0.78	28:5ω3
67	4.57	0.39	30:0
68	4.58	0.53	28:6ω3
69	4.59	0.28	30:1ω9
70	4.61	0.65	28:7ω6
71	4.70	0.20	30:2ω6
72	4.90	0.32	28:8ω3
73	5.07	0.30	32:0
74	5.09	0.22	32:1ω9
75	5.20	0.14	32:2ω6

Table 3 (*Continued*)

Peak No.	tR LC	abundance	FA
1	0.33	1.08	16:4ω1
2	0.35	3.78	16:4ω4
3	0.37	2.43	16:3ω4
4	0.39	0.79	14:1ω5
5	0.54	5.12	18:5ω3
6	0.56	2.16	16:2ω4
7	0.58	1.71	14:0
8	0.62	7.73	18:4ω3
9	0.75	4.04	16:1ω9[a]
10	0.77	7.37	18:3ω3
11	0.80	2.88	18:3ω6
12	0.84	1.31	20:5ω3
13	0.99	0.41	20:4ω3
14	1.01	8.91	16:0
15	1.03	6.56	18:2ω6
16	1.05	2.49	22:6ω3
17	1.08	1.55	20:4ω6
18	1.20	0.81	20:3ω3
19	1.22	0.28	24:7ω3
20	1.23	0.61	20:3ω6
21	1.24	8.27	18:1ω9[a]
22	1.27	0.69	22:5ω3
23	1.30	0.99	22:5ω6
24	1.40	0.71	22:4ω3
25	1.43	0.83	22:4ω6
26	1.46	0.29	22:3ω6
27	1.48	0.50	24:6ω3
28	1.50	1.44	18:0
29	1.64	0.30	24:5ω3
30	1.67	0.22	24:5ω6
31	1.70	0.63	22:3ω3
32	1.72	0.23	26:7ω3
33	1.75	0.65	20:1ω9
34	1.77	0.73	22:2ω6
35	1.81	0.31	28:8ω3
36	1.84	0.24	26:6ω3
37	1.88	0.32	24:4ω3
38	1.90	0.33	24:4ω6
39	1.92	0.34	22:2ω6
40	2.05	0.67	28:7ω6
41	2.07	0.85	20:0
42	2.09	0.35	26:5ω3
43	2.11	0.17	24:3ω6
44	2.13	0.18	26:5ω6
45	2.15	0.25	24:3ω3
46	2.23	1.36	22:1ω9
47	2.29	0.51	28:6ω3
48	2.31	0.42	28:6ω6
49	2.37	0.43	26:4ω3
50	2.39	0.26	26:4ω6
51	2.41	0.36	24:2ω6
52	2.51	0.75	28:5ω3
53	2.54	0.44	28:5ω6
54	2.59	1.53	22:0
55	2.62	0.45	26:3ω3
56	2.66	0.37	26:3ω6
57	2.72	2.34	24:1ω9
58	2.79	0.46	28:4ω6[a]
59	2.90	0.38	26:2ω6
60	3.06	0.27	28:3ω3
61	3.09	1.52	24:0
62	3.22	1.39	26:1ω9
63	3.43	0.52	28:2ω6
64	3.58	0.77	26:0
65	3.64	0.13	unknown
66	3.79	0.47	28:1ω9
67	3.96	0.19	30:2ω6
68	4.19	0.48	28:0
69	4.38	0.39	30:1ω9
70	4.68	0.20	32:2ω6

Table 3 (*Continued*)

Peak No.	tR GC	abundance	FA
71	4.74	0.49	30:0
72	5.12	0.21	32:1ω9
73	5.64	0.40	32:0

[a] Failed to separate positional isomers.

3.3. Limit of detection

One of the most important steps in the whole analysis is to select suitable standards. First of all, it is that the standard should not be present in a real sample. Only compounds labeled with stable isotopes such as deuterium or ^{13}C essentially satisfy this requirement. Unfortunately, none of the commercially available compounds contain acyls longer than C22 and isotopically labeled. Accordingly, two compounds that satisfy at least the chain length condition (longer than 22 carbon atoms) were chosen as standards, i.e. 23:0/23:0/23:0-TAG (tandem MS, see Table 3S) and 23:0/23:0-PC (tandem MS, see Table 4S). These molecular species are either minor or not represented in samples [10].

A working standard solutions of both standards was prepared by dissolving the two commercially available lipids (23:0/23:0/23:0-TAG and 23:0/23:0-PC), see Subsection 2.1. The solutions containing 5 mg/10 mL of TAG and/or PC in dichloromethane-isopropanol (1:1, v/v) were prepared. The solutions were kept at −25 °C and were stable for at least 1 month. The working standard solution of TAG was diluted appropriately with mobile phase to prepare the calibration standards, see Experimental. Based on the analysis, see Fig. 3, the limit of detection (LOD) was determined. The LOD, which was determined based on signal to noise ratio (S/N = 3) of TAG was ~11 μg/L (approximately 10 nmol/L) and amount of sample which was introduced into H-ESI-MS was 11 pg of 1,2,3-tritricosanoylglycerol in 1 μL. Similarly, the LOD of the PC was 8 μg/L, which is about 8.6 nmol/L, and injected volume of 1 μL into the MS, contained ~8 pg of 1,2-ditricosanoyl-*sn*-glycero-3-phosphocholine. The value of 10 nmol/L is almost identical to the value given by [30] for TAG.

The most abundant ions in the mass spectra of all TAG are the fragment ions formed by the neutral loss of $RCOOH + NH_3$ from $[M + NH_4]^+$ [32]. In the case of a PC, one of the most abundant ion was ion ($RCOO^-$) at *m*/*z* 353.3425 [31]. Values of limit of quantification (LOQ) at S/N = 10 is ~3 times higher. The calibration curves were linear from ~300 to ~1000 μg/L for both standards (TAG and PC).

Because our analysis was primarily about demonstrating the presence of VLCFA in the sample(s), differences in the ionization efficiency caused by varying lengths of acyls were neglected.

3.4. *VLCFA in the green alga* Tetraedron *and* Desmodesmus

To confirm the above analysis, a shotgun lipidomic of two green algae was performed. As an example of the analysis applied to the natural source of VLCFA, the green algae *Tetraedron* and *Desmodesmus* were selected. Fig. 4 shows both TIC and neutral loss of six even-chain saturated and monounsaturated VLCFA from *Tetraedron minimum*. A total of 57 TAG containing VLCFA have been identified, see Table 1. Similarly, the presence of VLCFA in PC (Fig. 5) has been demonstrated in *D. quadricauda* by precursor ion scan (PIS) at *m*/*z* 451.4521 ($C_{29}H_{59}COO^-$) and loss of CH_3 and acetate from precursor ions ($[M + acetate]^-$ - 74.0368 Da). A total of 6 molecular species of PC containing 30:1 were identified, see Table 2.

Several articles that describe the contents of the FAs have already been published [28–30]. While Lang et al. [33] did not find any FAs with 21 or more carbon atoms in the *Tetraedron* genus, Duong et al. [34] identified lignoceric acid (24:0) in an amount over 3% of total FA. Furthermore, some algae, including *Chlorella, Scenedesmus, Tetraedron* and others, contained an insoluble high aliphatic biopolymer called algaenan [35–39]. During its degradation, a mixture of variously long aliphatic compounds, especially fatty acids to C28 (e.g., 28:0, 28:1), methyl ketones (31:1), fatty alcohols (30:1), etc. are formed. It can therefore be assumed that all of the aforementioned algae are capable of biosynthesizing VLCFA, i.e., the biosynthetic precursors of algaenan.

Green algae are known to produce compounds with very long-chains, whether they are FAs, alcohols or hydrocarbons. For instance, FAs up to C45 were found in *Parachlorella kessleri* (formerly *Chlorella kessleri*) [40]. MacDougall et al. [30] in his article on TAG from algae described the presence of molecular species of TAG up to 28:1/28:1/18:1 from *B. braunii* and 28:1/18:1/18:1 from *Scenedesmus obliquus*. We have also identified both molecular species see the Table 1. Also, one of our recent publications on VLCFA from algae [15] describing the lipidomic analysis of the *Botryococcus* species clearly shows that green algae, as predecessors of green plants, are able to biosynthesize VLCFA. We have attempted to summarize all the above-mentioned knowledge of the presence of VLCFA in the following figures and tables. The reason why the overwhelming majority of authors do not describe presence of VLCFA in algae probably include too narrow mass ranges (not scanning high enough masses) in infusion and chromatography experiments, relatively short retention times and not sufficiently strong elution conditions at the end of runs in chromatographic experiments.

3.5. GC–MS of FAME

To confirm the above conclusions using shotgun analysis, further analyses were performed, namely FAME separation using GC–MS and LC–MS. In the case of GC–MS (see also Table 3) a short, i.e. 10 m long capillary column with a small diameter (ID 0.1 mm) and a film thickness of only 0.1 μm was used for analysis. In contrast to commonly used electron impact ionization (EI), chemical ionization (CI) was used in GC–MS. The EI MS of docosahexaenoic acid methyl ester (22:6ω3) shows numerous low mass fragments (see, for instance, the figure in [15] and but the molecular ion of FAME is typically rarely detectable. Conversely, when using CI, the dominating peak of the GC–MS is the protonated molecule $[M+H]^+$ at *m*/*z* = 343. The other dominant peak is the ammonium adduct $[M + NH_4]^+$ at *m*/*z* = 360, where ammonia was used as reagent gas for CI. Fig. 6 shows a total ion chromatogram (TIC) from 50 to 600 Da FAME obtained from four algae. A total of 57 FAME were identified. Even though a short capillary column was used and the analysis time did not exceed 5.5 min, positional isomers were also separated from each other. This included for example 16:1ω9 versus 16:1ω7 but also 28:6ω6 vs. 28:6ω3. Of course, the separation of some peaks is not baseline one, as reported by [41,42]. These authors used capillary columns with a length of 200 m, the tR for FAME 22:6ω3 being ~80 min. For comparison with the shotgun analysis of PC and TAG from two green algae of the genus *Desmodesmus* and *Tetraedron*, also FAME were analyzed by GC–MS, see Fig. 7.

3.6. LC–MS of FAME

Another possibility to verify and confirm the presence of VLCFA (VLCPUFA) in the samples is the LC–MS method. The above-prepared methyl esters can be used for the analysis. The LC–MS of the same mixture of FAME is shown in Fig. 8.

The topic of VLCFA (VLCPUFA) analysis by HPLC (LC–MS) has been treated several times [43,44], so we will only limit ourselves to newer publications related to VLCPUFA. One of the few articles describing RP-LC of VLCPUFA from algae was published by [45]. Both on analytical and preparative C18 phase columns, the sepa-

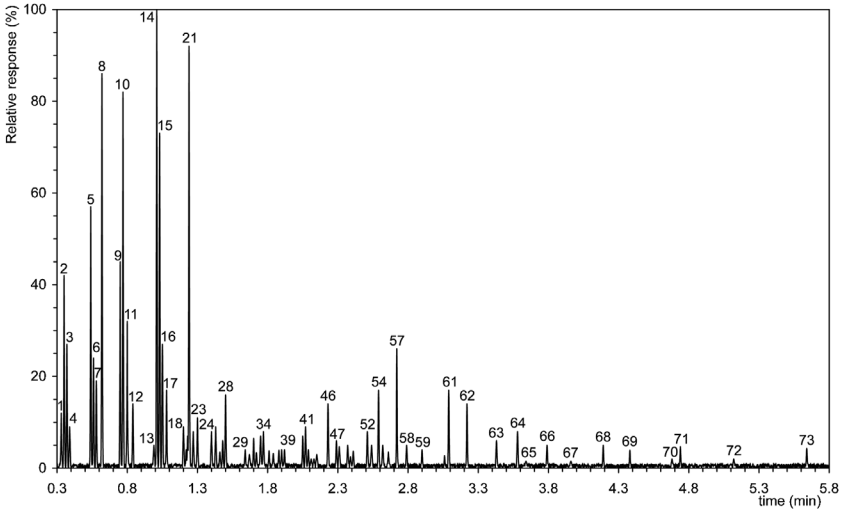

Fig. 8. LC–MS of standard mixture of FAME from algae. The peak numbers, see Table 3.

ration of 28:8ω3 from other FAME with a tR of about 18 min was described. The palmitic acid methyl ester had a tR of 22 min. From these values, it is obvious that RP-LC is particularly suitable for VLCPUFA.

VLCPUFA up to 36:6 were separated on a C18 phase column over 10 min, but it should be noted that the separation was not a baseline one with all the peaks [46]; an example is the coelution of 27:0 and 36:6 with the same tR 7 min. The authors unfortunately did not publish a chromatogram showing the TIC of the analyzed mixture. The use of the C18 reversed phase has allowed the separation of FA up to 44:13. Unfortunately again some FA have the same tR (24:1 vs. 30:6 or 36:8 vs. 34:6). The total analysis time did not exceed 15 min [27].

4. Conclusion

In conclusion, the use of LC–MS as shown in the examples above is more suitable for VLCPUFA due to the time of analysis as well as for reasons of lower thermal stability of polyunsaturated FA towards saturated FA. We believe that, in addition to the great benefit of shotgun analysis, verification of the results by GC–MS and LC–MS is still required. Based on the above data, GC–MS is better suited for the analysis of VLCFA, i.e. saturated FAs, while LC–MS is more suitable for the analysis of VLCPUFA, i.e. unsaturated FA with up to 8 double bonds. The answer to the question whether VLCFA and (or VLCPUFA are present) can be very easily and quickly proved by a shotgun lipidomic analysis. In this case, the sample is subjected to a single analysis step excluding hydrolysis and/or derivatization of FAs. Further, the analysis time one sample takes tens of seconds, which means that it is possible to analyze dozens of samples per hour. Of course, despite all the limitations and problems, we see the greatest potential in lipidomic analysis by shotgun analysis.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.460365.

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