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Lipidomic profiling of snow algae by ESI-MS and silver-LC/APCI-MS

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ABSTRACT

The main analytical benefit of this study is the development of methods enabling a rapid determination of total lipids of algae by lipidomic analysis and detailed identification and quantification of a complex mixture of natural TAGs by silver-LC/APCI-MS and NARP-LC/APCI-MS. Both types of chromatography can readily identify, both qualitatively and semiquantitatively, triacylglycerols containing 16:3 and 16:4 acids in the molecule. We conclude that the genus *Chloromonas* is a major producer of C16 PUFAs mostly contained in TAGs. Since more detailed studies in this field have been stymied by the shortage of 16:3 and 16:4 FAs, we decided to study the alga *Chloromonas* as a potential biotechnological source of C16 PUFAs.

Introduction

Snow algae grow in the alpine or polar regions worldwide. In addition to the biosynthesis of pigments, polyols, sugars and lipids generally performed by photosynthesizing organisms they developed a number of features such as mucilage sheaths, motile stages and spore formation, which enable them to adapt to the harsh snow environment (Hoham and Duval, 2001). At low temperatures, a major role in avoiding membrane rigidization is played by unsaturation of the fatty acids in membrane lipids (Morgan-Kiss et al., 2006). Moreover, the protective role of polyunsaturated fatty acids (PUFAs) against the damaging effect of high light intensity and UV radiation obviously helps the organism to survive and adapt in extreme environments (Whitelam and Codd, 1986).

The snow alga *Chloromonas pichinchae* has a complex life cycle, which includes both asexual and sexual reproduction and formation of resting stages. The flagellates and immature zygotes are responsible for a green coloration of snow (Hoham, 1975). Studies of the optimum temperatures of snow algae of the genus *Chloromonas* showed that, regardless of whether acclimation experiments were made, the algae grew best at temperatures around 5 °C. However, the authors examined only growth (cells number) and the data on FAs or lipids in *Chloromonas* from snow are scarce (Hoham et al., 2008; Rezanka et al., 2008).

Snow algae had to develop a number of adaptive strategies to cope with cold environments. A major adaptation influencing their growth and photosynthesis is the maintenance of membrane fluidity at low temperatures. While the importance of changes in fatty acid content has been recognized, the role of lipids in the metabolism of cold-adapted microorganisms is largely unexplored (Morgan-Kiss et al., 2006).

A few studies have dealt with fatty acids in the genus *Chloromonas*. In our previous work, we analyzed the snow alga *C. brevispina* (Rezanka et al., 2008). By using GC–MS we have identified a relatively high amount of C16 unsaturated FAs, the major being 16:2 (7%), 16:3 (15.5%), and 16:4 (9.5% of total FAs). Spijkerman et al. (2012) identified C16 PUFAs, principally 16:4, in the strains of *Chloromonas nivalis* (CCCryo005-99), and the tentatively designated *C. rostafinskii* (CCCryo010-99). Extensive data were published in the study of Lang et al. (2011), which described the contents of individual FAs in 22 species of the genus *Chloromonas* (the comparison with our data is in Table 1). The highest content of 16:4, more than 30% of total FAs, was found in *C. rosae*. The taxonomy of the genera *Chloromonas* and *Chlamydomonas* is constantly evolving (Proschold et al., 2001) and brings about constant changes in the classification; we therefore also mention the lipids of *Chlamydomonas*.

One of the first papers describing the content of polar lipids in genus *Chlamydomonas* was the study of Giroud et al. (1988), who analyzed polar lipids (MGDG, DGDG, SQDG, DGTS, PG, PE, and PI) from *C. reinhardtii*. As the major acids in all studied lipids were identified trienoic and tetraenoic C16 and C18 FAs,

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Table 1
FA content in algal samples under study, identified by means of GC–MS of FAMEs and its comparison with literature data.

Fatty acid	Abbreviation	Systematic name	BUD_FIT	MEANDRY	UDOLI	Lang et al. (2011)[a]
Myristic	M	14:0	0.8	1.3	0.7	0.4
Palmitic	P	16:0	12.5	11.2	14.9	13.0
Palmitoleic	Po	9–16:1	3.1	5.3	8.5	4.1
Palmitlinoleic	Pl	7,10–16:2	2.8	3.1	3.4	2.6
Palmitlinolenic	Pn	7,10,13–16:3	10.1	6.7	8.2	3.3
Hexadecatetraenoic	Pm[b]	4,7,10,13–16:4	18.7	16.2	15.6	15.3
Stearic	S	18:0	1.8	1.9	2.1	3.7
Oleic	O	9–18:1	11.3	17.1	13.3	9.4
Vaccenic	V	11–18:1	0.7	0.9	1.4	6.9
Linoleic	L	9,12–18:2	3.6	5.5	3.2	6.4
α-Linolenic	Ln	9,12,15–18:3	25.7	23.6	22.9	27.1
Stearidonic	St	6,9,12,15–18:4	8.9	7.2	5.8	7.6

[a] Mean content of individual FAs in 22 *Chloromonas* species studied by Lang et al. (2011).
[b] According to Danielewicz et al. (2011).

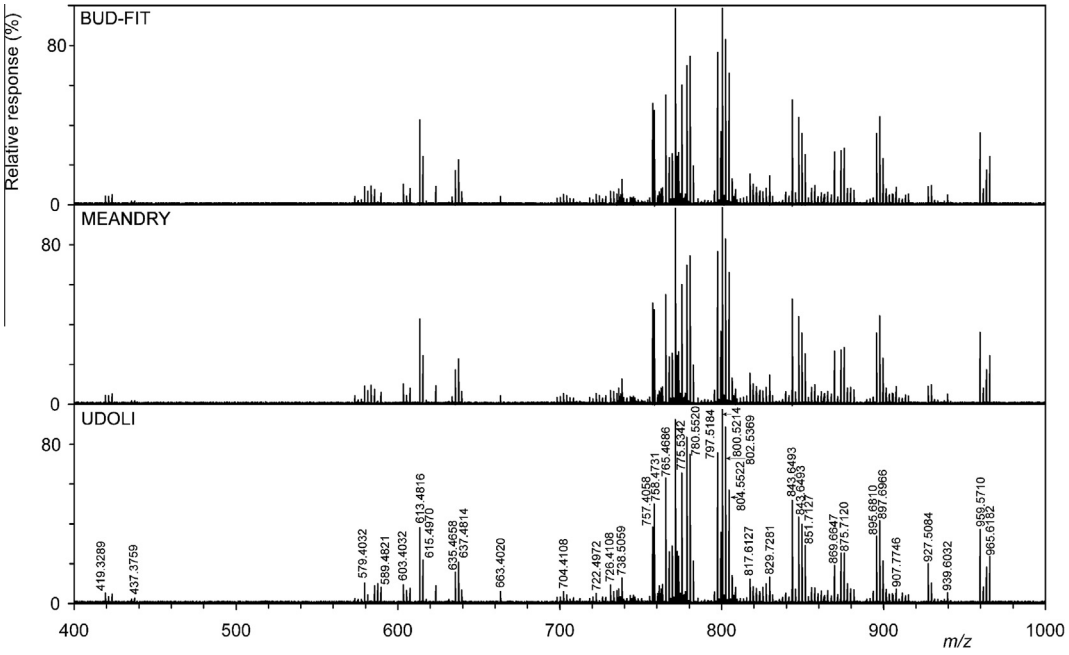

Fig. 1. Mass spectrum (positive ion mode) of total lipids from snow alga *C. pichinchae*, acquired by high mass resolution ESI-MS. Numbers correspond to the accurate mass values.

i.e. 4,7,10,13–16:4 or 9,12,15–18:3, with lower amounts of 5,9,12,15–18:4 and 7,10,13–16:3. The authors demonstrated a high stereospecificity of MGDG having FAs bound in positions 1 and 2. For example, 96% of C18 acids was bound in position 1 of DGDG, whereas up to 97% of C16 FAs was bound in the 2-position.

Several tens of molecular species of lipids (MGDG, DGDG, SQDG, DGTS, PG, PE, and PI) from the snow alga *Chlamydomonas nivalis* cultivated under salt stress or in response to nitrate or phosphate deprivation were identified by ultra-high performance liquid chromatography-mass spectrometry (UHPLC/MS) e.g. (Lu et al., 2012a, 2013). Similar polar lipids were identified by UHPLC/MS in the exponential phase of growth of the snow alga *Chlamydomonas nivalis* cultivated under NaCl stress (Lu et al., 2012b).

UHPLC/MS was used to analyze TAGs in *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* grown in nitrogen (N)-replete or N-depleted medium. A total of 140 molecular species of TAGs were identified, but without any quantification and without the resolution of regioisomers (Liu et al., 2013).

The analysis of intact TAGs can be basically performed by using two methods, either direct sample inlet to the mass spectrometer or a connection of liquid chromatograph with mass spectrometer (Vieler et al., 2007). The former method, called lipidomics (Han et al., 2012), is based on the identification of all lipids in cells and requires only an extraction of total lipids followed by identification and quantification of lipid molecular species (Bromke et al., 2013). High-resolution mass spectrometry is predominantly used for studies of lipidomic profiling of algae. ESI linear ion trap Orbitrap mass spectrometer with either a direct inlet (Danielewicz et al., 2011; Lee et al., 2013) or with UHPLC-MS (MacDougall et al., 2011) was used to analyze TAGs from miscellaneous algae including triacylglycerols containing C16 PUFAs by different collision energy (CID) using MS^n experiments, a combination of accurate mass and diagnostic fragment ions, or by MS^2 and MS^3 analysis.

Nonaqueous reversed-phase high performance liquid chromatography (NARP-LC) represents one of the most common methods for the separation of natural TAGs. It was used to separate hundreds of TAGs (Christie, 1988; Laakso and Christie, 1991), which were then identified by MS. Silver-ion chromatography is another option for separating TAGs (Laakso and Christie, 1991).

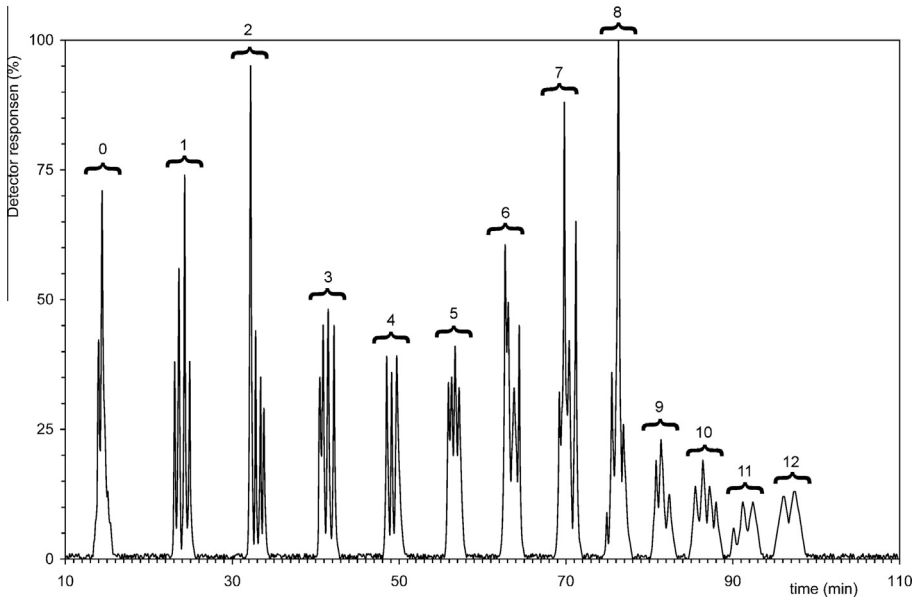

Fig. 2. Silver-LC chromatograms of the TAGs mixture from snow alga *C. pichinchae* with labeled double bonds groups (sample UDOLI).

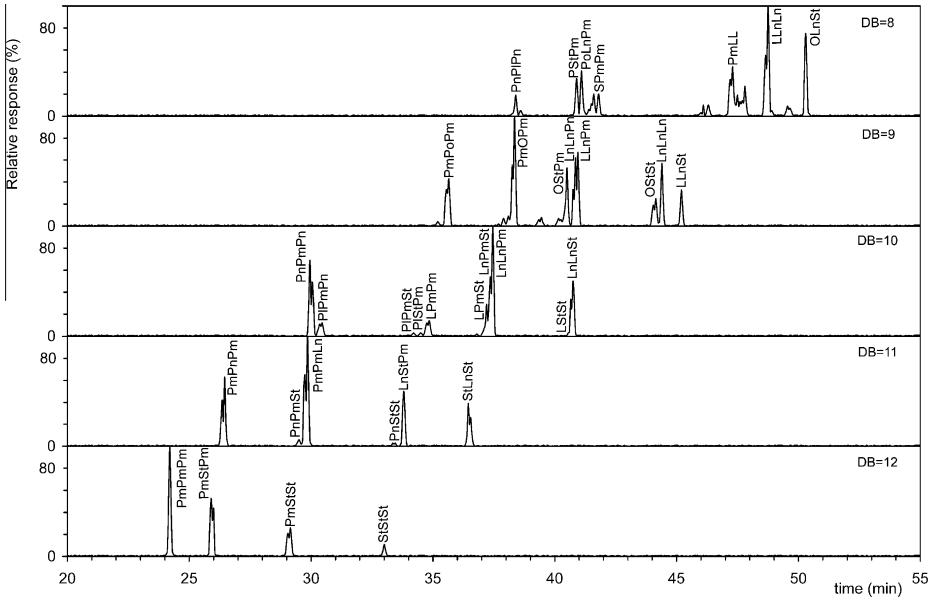

Fig. 3. Analysis of TAGs from snow alga *C. pichinchae* by NARP-HPLC/APCI-MS (sample UDOLI).

Silver-ion HPLC is often used for separating TAGs into groups according to the number of double bonds (DBs). Chromatographic silver-ion high performance liquid chromatography (silver-LC) separation is based on weak reversible complexes of Π electrons of DBs with electrons of silver ions embedded in the stationary phase during the sample elution through the chromatographic column. Double bond positional isomers or regioisomers can be also separated. The retention of TAGs increases with increasing unsaturation of TAGs (Nikolova-Damyanova, 2009).

We used an integrated approach based on both lipidomic profiling using Orbitrap high-resolution mass spectrometry and the classical method of analysis by silver-LC and NARP-LC/MS of TAGs to identify regioisomers of C16 and C18 PUFAs in three samples of the snow alga *C. pichinchae* collected in the Czech Republic (Giant Mountains and Jizera Mountains). Separation on the two different phases aids in ready identification and semiquantitation of regioisomers of triacylglycerols containing PUFAs with up to 12 double bonds in the molecule. This approach may provide valuable insights into the strategies of cold adaptation in extremophiles living in snow. We also found that the snow alga can be a biotechnologically important source of PUFAs.

Results and discussion

Lipidomic analysis by Orbitrap

Lipid extracts of the three samples of *C. pichinchae* obtained according to the methodology of Bligh and Dyer (1959) were directly analyzed by LTQ Orbitrap Velos high-resolution mass spectrometer. The results are shown in Fig. 1 and described in detail in Table 1S. We identified 330 molecular species, mostly using the LIPID MAPS (LIPID MAPS, 2013; Fahy et al., 2009). Individual molecular species were analyzed using MS prediction tools for TAGs and also for polar lipids based on high-resolution precursor

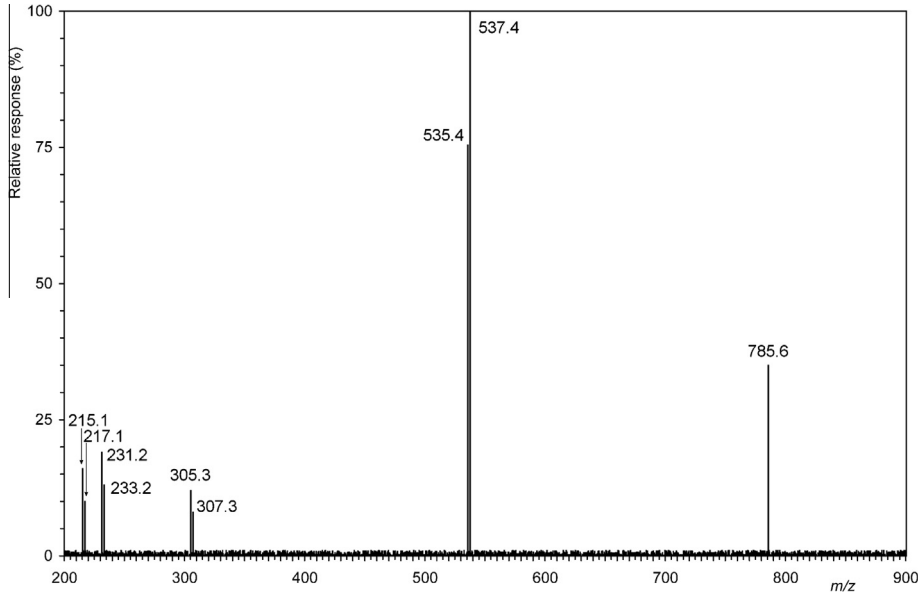

Fig. 4. Tandem mass spectrum of natural PnPmPm TAG.

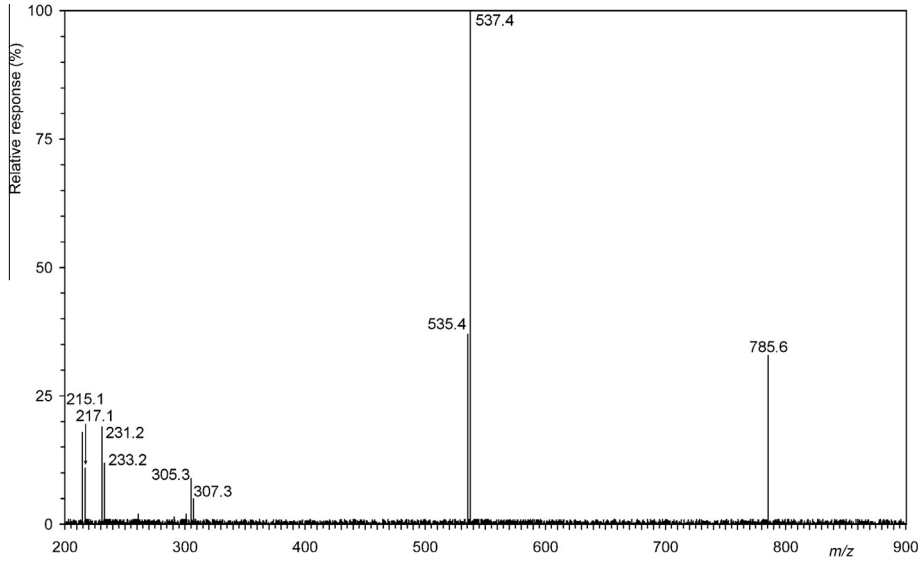

Fig. 5. Tandem mass spectrum of natural PmPnPm TAG.

ion $[M+Na]^+$ in the positive mode. It should be noted that hundreds, or even thousands, of other molecular species of lipids may be present. Indeed, as found by, e.g., Lu et al. (2012a), the snow alga *Chlamydomonas nivalis* was revealed to exhibit nearly 5000 peaks of ions in positive ESI; however, only a few tens of molecular species were identified.

Literature search (Danielewicz et al., 2011; Lee et al., 2013) and our own experience made us to choose ESI as the most effective ionization method for identification of lipid classes in snow algae. Since the focal point in our further analysis was to identify above all lipid classes that contain C16 PUFAs, i.e. both polar lipids (mostly phospholipids) and TAGs, we used the nearly universal ESI method in positive mode (see also Lee et al., 2013). Its advantage is in the possibility to use the presence of sodium ions directly in the extract; in their analyses Lee et al. (2013) supplied the extract with potassium acetate. HRMS thus allows a direct analysis of relevant lipids in the snow alga extract. One should however note that the ESI method has some limitations with regard to highly nonpolar hydrocarbons (squalene), which are not efficiently ionized and thus also detected.

The use of HRMS offers considerable advantages mainly in the range of higher *m/z* values. An example concerns, e.g., the three molecular species at *m/z* 857, i.e. TAG 16:0/16:0/18:0 at *m/z* 857.7606, PI 16:0/18:2 at *m/z* 857.5163 and SQDG 18:4/18:4 at *m/z* 857.4501. However, it does not make it possible to distinguish structural isomers; some of them (e.g. regioisomers) can be identified by tandem MS, others (e.g. positional isomers of TAGs having oleic and/or vaccenic acids in the molecule) can be identified only by using chromatographic methods.

While Lee et al. (2013) characterized 48 lipid species, we succeeded in determining several hundreds of them. However, the method does not provide an exact quantitative determination of TAGs having a higher number of double bonds in the molecule. It is known that in ESI analysis the highest response in the MS spectrum is given by saturated TAGs, while in APCI analysis it is provided by unsaturated TAGs (Byrdwell and Neff, 2002).

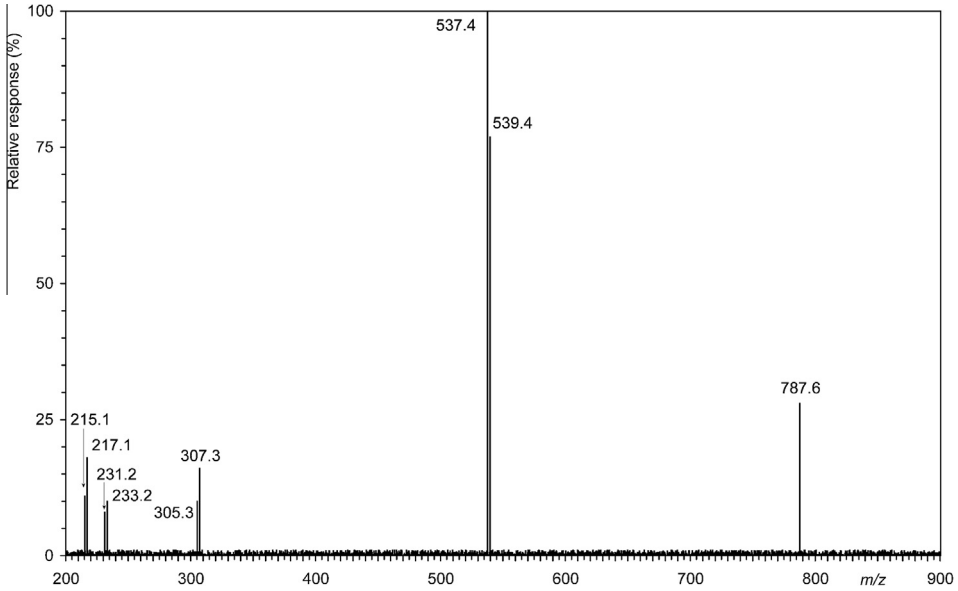

Fig. 6. Tandem mass spectrum of natural PnPnPm TAG.

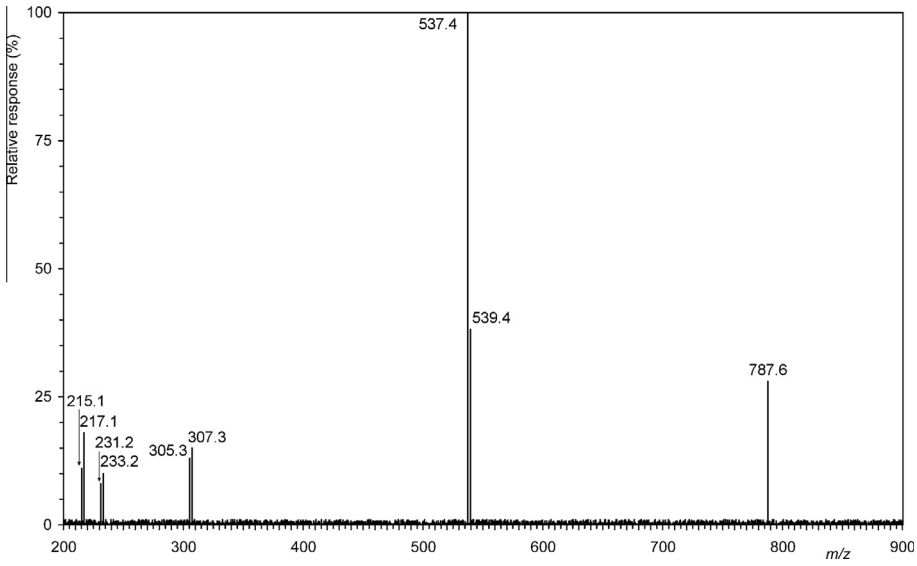

Fig. 7. Tandem mass spectrum of natural PnPmPn TAG.

Analysis by Orbitrap is very fast; when using autosampler with LC pump and without LC column (direct injection) and low sample injection (low solvent flow, about 1 μL/s), the whole analysis takes about half a minute. To exclude contamination, especially in very similar samples such as those we analyzed, each even-numbered analysis was performed by filling the vial up with pure solvent as a sort of washing run. About 60 samples per hour can be analyzed in this way.

Identification of triacylglycerols by silver-LC/MS and NARP-LC/MS

Since TAGs of all three samples of snow algae represented the most abundant lipid class (determined gravimetrically), they were separated on a Sep-Pak Cartridge with aminopropyl-silica based polar bonded phase, and non-polar lipids were eluted by chloroform–methanol mixture. We focused on their analysis using silver-LC with a gradient of a hexane/2-propanol/acetonitrile mobile phase and identification by APCI. Optimization of silver-LC separation has been repeatedly discussed, in terms of selection of an appropriate column (laboratory-made silver-ion columns versus commercial cation exchange columns), the stability of the mobile phase (addition of low amounts of 2-propanol to a hexane–acetonitrile mixture), or the column temperature or length (Momchilova and Nikolova-Damyanova, 2012). The currently used combination of silver-LC/MS and NARP-LC/MS with tandem MS identification of structure was used not only for the analysis of a group of TAGs differing in the number of double bonds, but also for separation and identification of individual regioisomers of TAGs (Dugo et al., 2006).

Our TAGs were fractionated according to the number of double bonds; with increasing number of double bonds the interaction of TAGs with silver ions of the stationary phase is ever stronger, which leads to higher retention times. The total TAGs were separated into 13 fractions having from 0 to 12 double bonds, see Fig. 2, which were further analyzed by NARP-LC, see Fig 3. Saturated TAGs were eluted first while unsaturated TAGs were eluted

later according to the rising number of double bonds in the molecule. Retention times in silver-LC are affected above all by the number of double bonds, although, as evident from Fig. 2, TAGs having FAs of different chain length are partially separated; however, this was not sufficient to complete their identification, nor was it our aim. For example, Lisa et al. (2009) obtained a sufficient separation by three columns connected in series, but if the column length is extended 2- or 3-times, the retention time increases to reach more than two hours in TAGs having 10 double bonds. We therefore used the above-mentioned two-dimensional off-line separation. In the first dimension, the TAGs were separated only by the number of double bonds, while in the second dimension regioisomers of TAGs were analyzed by NARP-LC (Fig. 3) and identified by tandem MS, see Fig. 3. TAGs of snow algae are among the most complex mixtures so far analyzed in our laboratory (Rezanka and Sigler, 2007; Rezanka et al., 2008, 2011). Although they contain in principle only FAs with chain lengths C16 and C18 (the minor proportion of myristic acid, see Table 1, does not play any role in the fraction of TAGs), these TAGs contain 11 FAs, i.e. FAs with 0 to 4 double bonds, including positional isomers (oleic versus vaccenic acid). This is in contrast to the FA composition of another snow alga, *C. brevispina*, where 43 FAs were detected (Rezanka et al., 2008), which suggests a species-specific mechanism of maintaining membrane fluidity in cold environments.

If the TAGs contain 11 FAs, the number of all possible TAGs is very high, reaching 1000. When we neglect enantiomers, which can be separated only on a chiral column (Rezanka et al., 2013), the number of possible theoretical regioisomers is still 726 ($x = (n^3 + n^2)/2$).

Molecular species of TAGs are very often separated by using nonaqueous mobile phase with octadecylsilane columns, i.e. NARP-LC (Lisa et al., 2007; Rezanka and Sigler, 2007). In this separation mode the elution order depends on the length of the acyl chains and the number of double bonds. The method permits the identification of tens to hundreds of molecular species of TAGs from various plant and/or animal oils and fats.

TAGs having the same ECN value can be separated on a high-efficiency column such as a combination of two 25 cm columns in series (Rezanka et al., 2011), which provides an efficiency of about 52,000 theoretical plates. The combination effectively separated most of the TAGs and, in addition, also regioisomers of TAGs as illustrated in Fig. 3.

Positive ion APCI mass spectra of TAGs typically exhibited $[M+H]^+$ ion and fragment ions of type $[M+H\text{-}RCOOH]^+$ ($[DAG]^+$), resulting from the loss of an acyl chain from TAG. Monoacyl glycerol ($[MAG]^+$) is equivalent to $[RCO+74]^+$ ion. The positive ion APCI mass spectrum (PmPmPm, for abbreviations of FAs see Table 1), i.e. the peak at tR 24.2 min represents $[M+H]^+$ ion at *m/z* 783.6 and the fragment ions $[M+H\text{-}RCOOH]^+$ at *m/z* 535.4 $[PmPm]^+$. Further ions at *m/z* 305.3 $[Pl+74]^+$ and $[RCO]^+$ at *m/z* 231.2 were also present. These ions were observed with sufficient intensity to analyze the mass spectrum.

TAGs are often present as regioisomers, i.e. positional isomers on the glycerol backbone (Mottram et al., 2001). Symmetrical TAGs (e.g. the PEP type) are eluted before the corresponding asymmetrical TAGs such as PPE (Momchilova et al., 2006). The regioisomers are identified based on the fact that the loss of FA in the secondary hydroxyl is likely to be energetically less favorable than the loss of FA from the primary hydroxyls.

Here we examined two pairs of regioisomers of the most abundant TAGs having C16 PUFAs, i.e. *sn*-PnPmPm (Fig. 4) and *sn*-PmPnPm (Fig. 5), *sn*-PnPnPm (Fig. 6) and *sn*-PnPmPn (Fig. 7), see also Table 2. The least abundant $[M\text{-}RCOO]^+$ ion ($[DAG]^+$ ion of *sn*-PmPnPm, i.e. $[PmPm]^+$ at *m/z* 535.4), arises by loss of palmitolinolenate (16:3) from the *sn*-2 position. The more abundant $[DAG]^+$ ion is due to loss of 16:4 from *sn*-1 or *sn*-3 position giving $[PmPn]^+$ ion at *m/z* 537.4. The $[PmPm]^+$:$[PmPn]^+$ ratio observed for PmPnPm is 37:100. Consequently, the two enantiomers show mass spectra differing only in intensities of $[DAG]^+$ ions.

Another pair of regioisomers, *sn*-PnPnPm (Fig. 6) and *sn*-PnPmPn (Fig. 7), were similarly separated with respective elution times 29.95 and 30.05 min. The mass spectra also demonstrate the structure of separated TAGs. $[PnPm]^+$ and $[PmPm]^+$ ions were

Table 2
NARP-LC/MS-APCI analysis of fraction having 8 to 12 double bonds of the TAGs mixture from snow alga *C. pichinchae*.

RT	TAG	Double bond	BUD_FIT	MEANDRY	UDOLI
24.2	PmPmPm	12	6.2	5.0	5.5
25.9	PmPmSt	12	3.3	3.5	3.4
26.4	PnPmPm	11	0.8	0.8	1.0
29.1	StStPm	12	1.6	1.7	1.8
29.5	PnPmSt	11	0.4	0.3	0.4
29.8	PmPmLn	11	6.2	6.4	6.3
30.0	PnPnPm	10	0.6	0.6	0.6
30.4	PlPmPm	10	0.7	0.7	0.6
33.0	StStSt	12	0.7	0.7	0.8
33.4	StStPn	11	0.2	0.2	0.4
33.8	LnStPm	11	3.1	3.0	4.3
34.0	PnPnSt	10	0.1	0.1	0.1
34.2	PlPmSt	10	0.2	0.2	0.2
34.5	PlStPm	10	0.2	0.2	0.2
34.8	LPmPm	10	0.9	0.9	0.9
35.1	PnPnPn	9	0.1	0.1	0.1
35.2	PlPnPm	9	0.2	0.2	0.2
35.6	PoPmPm	9	2.7	2.9	2.2
36.5	LnStSt	11	1.6	1.7	1.7
36.8	PlStSt	10	0.1	0.1	0.1
37.1	LPmSt	10	0.4	0.4	0.4
37.2	LnPmSt	10	1.8	1.9	1.9
37.4	LnLnPm	10	6.2	5.1	5.5
37.7	PlPnSt	9	0.1	0.1	0.1
37.9	PlLnPm	9	0.4	0.4	0.4
38.1	LPnPm	9	0.6	0.6	0.6
38.2	PoPmSt	9	0.8	0.8	0.8
38.3	OPmPm	9	6.2	6.4	6.4
38.3	PlPlPm	8	0.2	0.2	0.2
38.4	PlPnPn.	8	1.2	1.2	1.2
38.5	PoPnPm	8	0.1	0.1	0.1
38.6	PPmPm	8	0.3	0.3	0.3
39.4	PnPnLn	9	0.5	0.5	0.5
40.2	PoStSt	9	0.4	0.4	0.4
40.4	PlLnSt	9	0.9	0.9	0.9
40.5	OStPm	9	3.3	2.3	2.9
40.6	LStSt	10	0.2	0.2	0.2
40.7	LnLnSt	10	3.1	3.2	3.2
40.8	LnLnPn	9	3.9	4.1	3.9
40.9	LLnPm	9	4.2	5.3	4.8
40.9	PStPm	8	2.1	2.3	2.2
41.0	StPlPl	8	0.1	0.1	0.1
41.1	PoLnPm	8	2.6	2.9	3.2
41.2	LPlPm	8	0.4	0.4	0.4
41.4	PlLnPn	8	0.4	0.4	0.4
41.5	PoPmSt	8	0.7	0.7	0.7
41.6	SPmPm	8	1.2	1.3	1.2
41.8	LPnPn	8	0.1	0.1	0.1
44.1	OStSt	9	1.6	1.7	1.7
44.4	LnLnLn	9	3.6	4.6	3.4
45.2	LLnSt	9	2.1	2.2	2.2
46.0	PlStL	8	0.2	0.2	0.2
46.3	OPnSt	8	0.6	0.7	0.6
47.3	PlLnLn	8	2.8	2.7	2.5
47.4	StPSt	8	0.7	0.7	0.7
47.5	PmLL	8	1.2	1.2	1.2
47.6	PoLnSt	8	0.8	0.9	0.8
47.7	SStPm	8	0.9	0.9	0.9
47.7	PStSt	8	1.1	1.1	1.1
47.8	LLnPn	8	0.6	0.6	0.6
48.7	LLnLn	8	6.2	7.3	7.7
48.9	SStSt	8	0.3	0.3	0.3
49.6	LLSt	8	0.4	0.4	0.4
50.3	OLnSt	8	4.6	2.6	1.9

again differently abundant, their ratio for PnPmPm being 75:100 and for PmPnPm 37:100.

Triacylglycerols of all three samples of snow algae consisted only of molecules with an even number of acyl carbons, i.e. from 48 to 54. TAGs with 46 acyl carbons or less were not determined. Since the fatty acids identified here (see Table 1) and by Lang et al. (2011) include myristic acid, this acid is obviously not present in TAGs. The most frequent were TAGs having 6–8 double bonds, and the most abundant TAGs were 50:7 (PPmLn), 54:8 (LLnLn) and 54:9 (LnLnLn), see Fig. 3. Only small differences in the abundance of TAGs were detected between the samples, which is understandable given their characteristics (the same species and life cycle stage) and similar environmental conditions of the three sampling sites.

Conclusion

HRMS in combination with ESI makes it possible to analyze a broad range of lipid classes in the crude algal extract (in our case snow algae) without preceding purification or separation by SPE or HPLC. The method allowed us to identify several hundreds of molecular species from many classes of both polar and nonpolar lipids including phospholipids and glycolipids, at the rate of up to 60 samples per hour. Apart from its merits the method has also some shortcomings; especially important is its inability to identify certain isomers, e.g. the TAGs triolein (OOO) versus trivaccenin (VVV), etc.

Detailed characterization of the three samples of snow alga by lipidomic analysis with HR MS, silver-LC/APCI-MS and NARP-LC/APCI-MS enabled us to identify more than 300 intact lipids and, after chromatography, also intact TAGs. Although the combination of all three methods is very time-consuming, it provides a maximum number of results that cannot be obtained by any of the methods alone.

The use of off-line two-dimensional silver-LC in the first dimension and NARP-LC in the second dimension, after a previous lipidomic analysis by HR MS with APCI-MS as detection, has made it possible to analyze complex samples of snow algae collected in nature.

We succeeded in separating TAGs having from 0 to 12 double bonds; these TAGs were found to contain only C16 and C18 fatty acids with 0 to 4 double bonds. This shows this alga as a unique biotechnological source of essential PUFAs.

Despite their obvious importance, metabolic adaptations of photoautotrophic extremophiles involving lipids are poorly known. The complex approach used in this study may represent a powerful tool enabling comprehensive studies of lipid content in microorganisms that are exposed to various environmental stresses both in the field and in laboratory experiments. This method can thus provide new insights into the adjustments of metabolic pathways in microorganisms living in cold environments.

Experimental

Plant material

Snow algae were aseptically collected in May 2012 from snow fields in the Krkonoše and Jizera Mountains (Czech Republic). All the three sampling sites were situated in a spruce forest, and the algae formed patchy green coloration of the surface layers of snow. Both sites in the Krkonoše Mountains were located in the Labský důl Valley, but they differed in altitude: The site Bud' Fit was located at an altitude of 810 m a.s.l. (50°44′35″N, 15°36′13″E), the site Meandry Labe at an altitude of 1060 m a.s.l (50°45′42″N, 15°33′08″E). The sampling locality in the Jizera Mountains (Údolí Jizery) was situated near the Jizera River at an altitude of 780 m a.s.l. (50°48′09″N, 15°21′53″E). The snow cover at all sites is seasonal. Snow samples were transported to the laboratory in a thermos bottle, identified under a light microscope, and the algal biomass was kept frozen until further analysis. The green blooms were formed entirely by vegetative stages of *C. pichinchae* Wille, and no zygospores were observed in the samples.

Lipid extraction and isolation of TAGs

Acetonitrile, 2-propanol, hexane, chloroform and methanol were purchased from Sigma–Aldrich (Prague, CR). The extraction procedure was based on the method of Bligh and Dyer (1959), except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases (Kates, 1986). The alcohol–water mixture of the frozen cells was cooled, one part chloroform was added and the lipids 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.

Total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 grams of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted neutral lipids with 40 mL of chloroform–methanol (7:3), and acidic lipids with 30 mL of chloroform–methanol–concentrated aqueous ammonia (70:30:2) containing 0.4% (w/v) ammonium acetate. The eluate of neutral lipids was reduced in volume, mixed with 1 mL of hexane and the mixture was stirred for 15 min. The solution was filtered, hexane was evaporated and the oil samples were dissolved in an acetonitrile–2-propanol–hexane mixture (1:1:1, v/v/v), which was injected on the HPLC column.

FAMEs analysis

The total TAGs (~5 mg) were saponified overnight in 10% KOH–MeOH at room temperature. A fatty acid fraction obtained from the saponification was partitioned between alkali solution (pH 9) and diethyl-ether 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 BF_3/MeOH (14% solution of BF_3 from Sigma–Aldrich).

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC (Varian BV, Middelburg, The Netherlands), Varian 240-MS ion trap detector with electron ionization (EI), and CombiPal autosampler (CTC, USA) equipped with split/splitless injector. A DB-5MS column was used for separation (60 m, 0.25 mm ID, 0.25 μm film thickness). The temperature program started at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 160 °C at a rate of 25 °C min^{-1}. The second temperature ramp was up to 280 °C at a rate of 2.5 °C min^{-1}, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 3 scans s^{-1} under electron ionization at 70 eV, mass range *m/z* 50–450. FAMEs (fatty acid methyl esters) were identified according to their mass spectra [29, 30] and using a mixture of chemical standards obtained from Sigma–Aldrich.

Lipidomic analysis by HR ESI

The high-resolution hybrid mass spectrometer LTQ Orbitrap Velos (Thermo Scientific, FL, USA) was used. ESI–MS analysis was performed in the positive ion mode. MS spectra were obtained by the FT mode. MS spectra were acquired with target mass resolution of

R = 30,000 at *m/z* 400 (lock mass 413.6662 Da). The ion spray voltage was set at -2500 V and the scan range of the instruments was set at *m/z* 200–1500. Nitrogen was used as a nebulizer gas set at 18 arbitrary units (sheath gas) and 7 arbitrary units (aux gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected by the low-resolution FT mode. Flow Injection Analysis (FIA) was used for sample introduction into the heated ESI (H-ESI) ion source. Acetonitrile/water (50/50 v/v) was used at the flow rate of 150 μL/min. The H-ESI temperature was set to 250 °C.

NARP-LC/APCI-MS

LC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Agilent, USA) and two Hichrom columns HIRPB-250AM 250 × 2.1 mm ID, 5 μm particle size, in series. This setup represented a high-efficiency column – approximately 26,000 plates/250 mm, a flow rate of 1 mL/min, an injection volume of 10 μL, and column temperature of 25 °C. The TAGs were separated using a gradient solvent program with acetonitrile (ACN) and 2-propanol (iPrOH) as follows: initial ACN/iPrOH (99:1, vol/vol); linear from 5 min to 110 min ACN/iPrOH 30:70 (vol/vol); held until 10 min; the composition was returned to the initial conditions over 10 min. The performance was measured by triheptadecenoin as an internal standard under the conditions given above.

The detector was an AB Sciex API 4000 mass spectrometer (AB Sciex, Ontario, Canada) using APCI mass spectra. The ionization mode was positive, using the following settings: source temperature 420 °C, nebulizer gas (GS1) 5, nebulizer current 3 μA, curtain gas 10, collision gas high and declustering potential 100 V. The optimal collision energy was dependent on the type of experiment and was set to +10 V (full scan mode) or +30 V (product ion mode). In all full scan runs spectra were obtained from *m/z* 200–1100. To identify the exact composition of high *m/z* value TAG species, an enhanced product ion spectrum (optimal CE +35 V) was made of all ions with an *m/z* value over 800.

CID ion mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas as a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 200–1100 with a step size of *m/z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.2 software.

Silver-ion HPLC

The LC system used for separation in the silver-ion mode was the same as that used in the reversed-phase mode. TAGs (~1 μg/μL in methanol) were chromatographed on two silver-ion columns Chrom-Spher Lipids (250 × 4.6 mm, 5 μm, Varian, Palo Alto, CA, USA) connected in series. Mobile phase was a gradient from 100% of A and 0% of B at 0 min to 60% of A and 40% of during 120 min, where A is hexane-2-propanol-acetonitrile (99.8:0.1:0.1, v/v/v) and B is mixture of hexane-2-propanol-acetonitrile (96:2:2, v/v/v). The flow rate, column temperature, and injection volume were 0.5 mL/min, 25 °C, and 10 μL, respectively. Columns were conditioned by mobile phase A at flow rate of 0.5 mL/min for one hour before the every analysis. Other separation conditions used were as in the RP-LC, see above.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.01.017.

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