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Profiling of complex lipids in marine microalgae by UHPLC/tandem mass spectrometry

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ABSTRACT

Microalgal lipids are of great interest for their potential as food and feed ingredients, nutraceutical and cosmetic components, and as source of energy. Glycerol-based lipids constitute even >50% of the biomass of microalgae. How synthesis of these products takes place is still poorly explored, although remarkable differences with plants and among microalgal species have already emerged. These differences attain to both lipid class and fatty acyl composition, which could be modulated by specific growth conditions. Here we present a new method based on UHPLC coupled to high resolution tandem mass spectrometry for targeted analysis of the main lipid classes, i.e., glycolipids, phospholipids and triacylglycerols, applied to five marine microalgae exhibiting a great diversity in lipid composition. The LC/MS/MS approach is integrated with a recently proposed MTBE-based protocol for lipid extraction and the entire workflow is of general application to lipid profiling of cell extracts from different sources.

1. Introduction

Glycerol-based lipids are ubiquitous membrane and cytosolic constituents of living cells and recently have been the subject of many studies directed toward their qualitative and quantitative assessment [1–12]. The interest typically ranges from identification of individual species to biosynthesis and metabolic regulation of their production in order to unveil their complex biochemical network. In particular, marine microalgae are gaining increasing importance in the fields of biotechnology due to the high quality and large amount of lipids that are of interest as food and feed supplements, e.g., omega-3 EPA/DHA, and feasible biofuels [13–18]. However, except for very few species, lipid algal metabolism has been poorly investigated so far and we have a still limited knowledge of the biochemical processes underlying synthesis or plasticity of the major lipid classes including storage molecules as triacylglycerols (TG) and structural and functional lipids as phosphatidylglycerols (PG), phosphatidylcholines (PC), phosphatidylinositols (PI), phosphatidylethanolamines (PE), sulfoquinovosyldiacylglycerols (SQDG), monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG). On the other hand, aside the species-specific differences in levels and composition, it is well known that the relative amount of each lipid class as well as of each individual component greatly varies according to taxonomic group and physiological state. Furthermore, changes in culture conditions, including availability of elemental components (N, P and Si), temperature, salinity, light/dark cycle and growth phase affect lipid composition dramatically [19]. Although a picture of the lipid pathways in microalgae can be depicted by analogy with the well-known metabolism in plants [20], recent evidence from genetic and molecular studies have highlighted intriguing unique traits and have underlined the importance of further studies in these autotrophic microorganisms [21–22].

MS-based analytical techniques have become an elective tool to evaluate the entire set of cell metabolites and to detect the chemical diversity in biological samples [7,8,11,23,24]. Prompted by the interest for using algal triacylglycerols as biofuels [17,18], lipid profiling has been successfully employed for targeted analysis in microalgae grown under different physiological states and for untargeted analysis in high throughput applications, by both shotgun lipidomics on high resolution mass spectrometers such as FT-ICR [25] or on MALDI-MS [26], and by hyphenathed LC/ESIMS platforms with Q-Tof [27–30], triple quadrupole [31] or Orbitrap mass analyzer [32].

Abbreviations: DG, diacylglycerols; DGDG, digalactosyldiacylglycerols; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GL, glycolipids; HESI, Heated ElectroSpray Ionization; HILIC, hydrophilic interaction liquid chromatography; HRMS, high resolution mass spectrometry; MGDG, monogalactosyldiacylglycerols; MTBE, methyl *tert*-butyl ether; NP/LC, normal phase liquid chromatography; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PL, phospholipids; RP/LC, reversed phase liquid chromatography; SQDG, sulfoquinovosyldiacylglycerols; UHPLC, Ultra High Performance Liquid Chromatography.

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Recently, we have reported a new NMR method for identification and broad quantitation of glycerolipids in oleaginous microalgae [33]. Following this work, here we present a comprehensive lipidomics workflow to study lipid changes in microalgae and other biological samples. The method relies on a halogenate-free methyl-*t*-butyl ether (MTBE) protocol for lipid extraction [34] and a quantitative UHPLC/ESI/MS/MS method based on a Kinetex Biphenyl column and a high resolution benchtop Q-Exactive spectrometer.

2. Materials and methods

2.1. General

Methanol and water, LC/MS grade, were from VWR International (Milan, Italy). All the other solvents were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Chemicals

Lipid standards 1,2,3-triheptadecanoyl-*sn*-glycerol (TG 17:0/17:0/17:0) 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine (PC 17:0/17:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (PG 17:0/17:0) and Bovine Liver Total Lipid Extract were purchased from Avanti Polar Lipids. Synthetic monogalactosylglycerides 1-(d_{31}-hexadecanoyl), 2-(d_{35}-octadecanoyl)-3-*O*-β-D-galactosyl-*sn*-glycerol (MGDG d_{31}-C16:0/d_{35}-C18:0) and 1,2-(dinonadecanoyl)-3-*O*-β-D-galactosyl-*sn*-glycerol (MGDG 19:0/19:0) were prepared as reported by Manzo *et al.* [35]. Synthetic sulfoquinovosydes 1,2-(dinonadecanoyl)-3-*O*-(6′-sulfo-α-D-quinovosyl)-*sn*-glycerol (SQDG 19:0/19:0) and 1-(d_{31}-hexadecanoyl), 2-(d_{35}-octadecanoyl)-3-*O*-(6′-sulfo-α-D-quinovosyl)-*sn*-glycerol (SQDG d_{31}-C16:0/d_{35}-C18:0) were prepared as reported by Manzo *et al.* [36].

2.3. Microalgal material

Cyclotella cryptica (CCMP331) and *Nannochloropsis salina* (CCMP369) were purchased from National Center for Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, USA), while *Thalassiosira weissflogii* (ICB-P09), *Alexandrium minutum* and *Alexandrium tamutum* were originally isolated in the Gulf of Naples from Laboratory of Ecophysiology of Stazione Zoologica Anton Dohrn (Naples, Italy). Diatoms *C. cryptica* and *T. weissflogii* were cultured in a 2 L polycarbonate carboy in f/2 medium [37] at room temperature (22.0 ÷ 1.0 °C) and gently bubbled with sterile air. Artificial light intensity (200 μmol m^{-2} s^{-1}) was provided by daylight fluorescent tube with a 14:10 h light: dark photoperiod. The diatoms were harvested at the end of the stationary phase by centrifugation in a swing-out Allegra X12R (Beckman Coulter Inc., Palo Alto, CA, USA) at 2300 *g*, 4 °C, for 10 min. The green alga *N. salina* was grown in a 2 L polycarbonate carboy in f/2-Si medium. Irradiance and photoperiod were the same as reported above for diatoms *T. weissflogii* and *C. cryptica*. The cultures were harvested at the stationary phase. Dinoflagellates *A. minutum* and *A. tamutum* were cultured in 10 L carboys filled with 0.22 μm FSW enriched with Keller (K) medium [38] at 20 °C under 12 h:12 h light: dark photoperiod (100 μmol $m^{-2}\cdot s^{-1}$). Cells were harvested at the stationary phase by centrifugation at 3750 rpm for 10 min at 4 °C in a swing-out rotor (DR 15P, Braun Biotechnology International, Allentown, PA, USA). Cell growth (cells mL^{-1}) was estimated daily using a Bürker counting chamber (Merck, Leuven, Belgium) (depth 0.100 mm) under an inverted microscope.

2.4. Sample preparation for LC/MS analysis and standard recovery

For each microalga, dry cell pellet (1.0–2.5 mg) was suspended with 180 μl of LC/MS grade MeOH in a glass vial. After sonication, 600 μl of MTBE were added and the extraction left at room temperature under shaking for 1 h. Upon addition of 150 μl of LC/MS grade water and shaking for 10 min, phase separation was induced. The sample was centrifuged at 1000 *g* for 10 min at 4 °C and the upper organic phase was recovered. The lower phase was re-extracted with MTBE, the upper phases combined and the organic solvent removed under nitrogen stream. Each extract was resuspended in CH_2Cl_2:MeOH (1:1) and diluted 1:50 with MeOH:IsoPrOH (1:1) for LC/MS analysis. A five-standard cocktail composed by synthetic α-SQDG (19:0/19:0) and MGDG (19:0/19:0), and commercial PG (17:0/17:0), PC (17:0/17:0) and TG (17:0/17:0/17:0) was used for quantitative analysis. For standard recovery, a first set of samples was spiked with 10 μg each of PC (17:0/17:0) and TG (17:0/17:0/17:0), 7.5 μg of α-SQDG (19:0/19:0) and 5 μg of MGDG (19:0/19:0). The extract was diluted 1:50 as indicated above and 10 μl injected for LC/MS/MS analyses. Area values for each standard were compared with a second set of samples spiked after extraction and % recovery was expressed as area ratio percent of the two set of samples. Experiments were performed in triplicate with five technical replicates and the results are reported as means ± standard deviation (SD).

2.5. Isolation of complex lipids as natural standards for LC/MS method assessment

Microalgal extracts were prepared as reported above from 750 mg of dry pellet of the marine diatom *T. weissflogii*. The raw extract was chromatographed on silica gel column by using a stepwise elution protocol: A, $CHCl_3$; B acetone/MeOH, 9:1; C, MeOH. TG were recovered from fraction A and identified by NMR and MS. Fraction B containing glycolipids was further purified by Sephadex LH-20 column with $CHCl_3$:MeOH (1:1) as eluent, affording monogalactosyl, digalactosyl and sulfoquinovosyl pools, identified by NMR and MS. These natural lipids were used to set the LC/MS conditions.

2.6. Ultra high performance liquid chromatography/high resolution mass spectrometry (UHPLC/HRMS)

Chromatographic separations were achieved on Infinity 1290 UHPLC System (Agilent Technologies, Santa Clara, CA, USA), equipped with a Kinetex Biphenyl 2.6 μm, 150 × 2.1 mm column, (Phenomenex, Castel Maggiore, Bologna, Italy) at 28 °C. Eluent A: water and eluent B: MeOH. The elution program consisted of a gradient from 40 to 80% B in 2 min, then to 100% B in 13 min, holding at 100% B for 7 min. A post run equilibration step of 5 min was included prior to each analysis. Flow rate was 0.3 ml/min. The injection volume was 10 μl and the autosampler was maintained at 10 °C.

MS analyses were carried out on Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a HESI source. Source parameters were as follows: Spray voltage positive polarity 3.2 kV, negative polarity 3.0 kV, Capillary temperature 320 °C, S-lens RF level 55, Auxiliary gas temperature 350 °C, Sheath gas flow rate 60, Auxiliary gas flow Rate 35.

Full MS scans were acquired over the range 200–1800 with a mass resolution of 70,000. The target value (AGC) was $1e^6$ and the maximum allowed accumulation time (IT) was 100 ms. For the data dependent MS/MS ($ddMS^2$) analyses a Top10 method was used. The ten most intense peaks were selected for fragmentation with a stepped normalized energy of 25–28–35 and 20–40% in positive and negative ionization mode, respectively. AGC was $2e^5$ with IT 75 ms and 17,500 mass resolution. Analyses were performed with five replicates and injections were of 10 μl.

2.6.1. Lipid identification and statistical analysis

Each experiment was run in triplicate. For lipid identification and quantification LC/MS/MS data from five measurements for each sample were processed by Xcalibur 3.0.63 and LipidSearch 4.1.16 package (Thermo Scientific). Absolute quantitation was carried out by a single point calibration using the five-standards that represent all the lipid