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Comprehensive lipid profiling of *Microchloropsis gaditana* by liquid chromatography - (tandem) mass spectrometry: Bead milling and extraction solvent effects

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ABSTRACT

Liquid chromatography - mass spectrometry (LC-MS) experiments were used to qualitatively and semi-quantitatively monitor the impact of bead milling and the influence of the nature of the extraction solvents, Bligh and Dyer (B&D) *versus* *n*-heptane, on the composition of the structural lipids extracted from the microalgae *Microchloropsis gaditana* (*M. gaditana*).

Our data reveal that lipid extraction with *n*-heptane as the solvent becomes as efficient as the standard Bligh and Dyer method when a milling step is included before the extraction. Indeed, lipid extraction yields amount to ~1% (*n*-heptane on intact cells), ~5% (B&D on intact cells), ~5% (*n*-heptane on milled cells) and ~6% (B&D on milled cells). In all the extracts, using liquid chromatography separation combined to accurate mass measurements and tandem mass spectrometry experiments, we identified a huge number of lipids belonging to different structural lipid families, including LPC (lyso-phosphatidylcholine), PC (phosphatidylcholine), DGTS (diacylglyceryl-*N*-trimethylhomoserine), MGTS (monoacylglyceryl-*N*-trimethylhomoserine), DGDG (digalactosyldiacylglycerol), DGMG (digalactosylmonoacylglycerol), MGDG (monogalactosyldiacylglycerol), PG (phosphatidylglycerol), PI (phosphatidylinositol), SQDG (sulfoquinovosyldiacylglycerol), SGMG (sulfoquinovosylmonoacylglycerol) and free fatty acids (FFA). We further demonstrated that the milling step induces strong modifications in the lipid composition due to extensive lipid degradation into free fatty acids - probably by enzymatic processes, even when the milled cells are conserved at −20 °C.

From a method development perspective, this work represents to the best of our knowledge the most complete structural analysis investigation of lipids extracted from intact and milled microalgae, including the use of a sector diagram representation that could be relevant for all lipidomic investigations. One of the main outcomes of

Abbreviations: B&D, Bligh and Dyer; BPI, Base Peak Intensity; CID, Collision-Induced Dissociation; DGDG, DiGalactosylDiacylGlycerol; DGMG, DiGalactosylMonoacylGlycerol; DGTS, DiacylGlyceryl-*N*-TrimethylhomoSerine; EPA, Eicosapentaenoic acid; ESI, Electrospray Ionization; FA, Fatty Acid; FAMEs, Fatty Acid Methyl Esters; FFA, Free Fatty Acids; FID, Flame Ionization Detector; GC, Gas Chromatography; GC-FID, Gas Chromatography - Flame Ionization Detector; HPLC, High Performance Liquid Chromatography; HRMS, High Resolution Mass Spectrometry; IC, Intact cells; IMS, Ion Mobility Spectrometry; K, ketene; LC-MS/MS, liquid chromatography - tandem mass spectrometry; LC-MS, liquid chromatography - mass spectrometry; LPC, LysoPhosphatidylCholine; *M. gaditana*, *Microchloropsis gaditana*; *m/z*, mass-to-charge ratio; MC, Milled cells; MeOH, methanol; *Mg*, *M. gaditana*; MGDG, MonoGalactosylDiacylGlycerol; MGTS, MonoacylGlyceryl-*N*-TrimethylhomoSerine; MTBE, Methyl *tert*-Butyl Ether; PC, PhosphatidylCholine; PE, PhosphatidylEthanolamine; PG, PhosphatidylGlycerol; PI, PhosphatidylInositol; PLs, PhosphoLipids; QToF, Quadrupole Time-of-Flight; S/L, Solid-Liquid extraction; SQDG, SulfoQuinovosylDiacylGlycerol; SQMG, SulfoQuinovosylMonoacylGlycerol; TAG, TriAcylGlycerol or TriAcylGlycerides; ToF, Time of Flight; UPLC, Ultra Performance Liquid Chromatography.

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the study is the confirmation that liquid chromatography - tandem mass spectrometry (LC-MS/MS) experiments are essential when lipid extraction procedures from any microalgae biomass have to be optimized. The analytical protocol developed in the present study certainly deserves to be extended to other microalgae.

1. Introduction

Due to actual environmental and energetical issues, efficient and green alternatives to replace fossil energy sources are mandatory in our energy-demanding society [1]. Biomass is now increasingly envisaged as a promising source to produce renewable electricity, thermal energy, or transportation fuels [2,3]. The 1st generation biofuels directly compete with the food industry since they are produced from raw materials that can be used in animal feed or human food [4]. The 2nd generation biofuels concern technologies that exploit cellulosic materials obtained from green waste such as wood, leaves and stems of plants. These materials, that are not directly used in food production, are referred to lignocellulosic biomass because they originate from woody or carbon-based components [2], with greater availability and non-competition for food compared to the 1st generation biofuels. Nevertheless, the production costs remain very high due to many technological barriers. Moreover, it is widely recognized that an ideal 2nd generation feedstock should grow on marginal land, *i.e.* land that cannot be used for "arable" crops [5,6]. Finally, the 3rd generation biofuels mostly rely on the use of microalgae for energy production [3] and these photosynthetic organisms are currently considered for the biofuel production because of their rapid growth rate on non-arable lands, their ability to fix CO_2 and their high lipid production capacity [7,8]. Microalgae are also increasingly studied since significant variations in their biochemical compositions may be induced and controlled by stress induction, *i.e.* nitrogen starvation, during cultivation [9]. Microalgae can accumulate fatty acids with yields 10 to 20 times higher than terrestrial oilseed species [10,11]. Some species of microalgae may also contain high level of polysaccharides that may be fermented into bioethanol. Ultimately, microalgae can be methanized to produce biogas [12]. Furthermore, the microalgal biomass can be used for applications other than energy production since, beside lipids, microalgae contain proteins, polysaccharides, pigments, and other specific metabolites. All those compounds may be valorized as pharmaceutical, nutraceutical, and cosmetic products, for instance [8,13–15].

Nannochloropsis gaditana (L.M. Lubián), renamed as *Microchloropsis gaditana* (*M. gaditana*) in 2015, is currently one of the most studied microalgae and its biomass is envisaged for numerous applications [16–18]. The genus *Microchloropsis* was reported for the first time by Hibberd in 1981 [19], and belongs to the *Eustigmatophyceae* class. Lubián *et al.* described *Nannochloropsis gaditana* in 1982 [20]. *M. gaditana* is a spherical unicellular alga with a diameter between 2 and 8 μm. Research efforts on *M. gaditana* are placed on the production of biodiesel and cosmetics since it can accumulate a high concentration of polyunsaturated fatty acids and has a rapid growth compared to other microalgae [17]. The chemical profile of *Microchloropsis* species (sp.) is composed by lipids (16–25%, *w/w*), proteins (26–32%), carbohydrates (32–37%) and ashes (5–10%). The lipid category includes neutral lipids (15%), polar lipids (25%), unsaponifiable lipids (15%) and chlorophyllides (6%). The 14:0; 16:0; 16:1; 18:1; 18:2; 20:5 fatty acids are mainly found amongst the *Microchloropsis* sp. lipids [21,22]. As for a reminder, the 16:1 fatty acid, for instance, is constituted by a 16-carbon chain length presenting 1 C=C bond at an unspecified position. This nomenclature is based on the LIPIDMAPS abbreviations. LIPIDMAPS is a consortium who develops online tools for predicting candidate structures from mass spectrometry data [23,24].

Analyzing the lipid content of *M. gaditana* is a crucial task since the lipid fraction represents a high proportion of the total biomass. Developing lipid profiling efficient methods is fundamental as soon as the microalgae are cultivated to produce larger amounts of lipids for diverse applications. Several sample preparation methods can be used for total lipid extraction, even if the reference method is the Bligh and Dyer (B&D) method [25]. This extraction procedure is based on the seminal work of Folch *et al.* [26], who used a mixture of chloroform ($CHCl_3$), methanol (MeOH) and water to extract lipids. Bligh and Dyer managed to reduce the organic solvent consumption by using a one-step extraction [26]. This method has been shown to be effective for the extraction of phospholipids (PLs), neutral lipids and sphingolipids [27,28]. Even if the use of chlorinated solvents remains problematic [29], the B&D method is to date widely considered as the standard method for lipid extraction and was demonstrated to be more efficient to extract lipids than $CHCl_3$, MeOH or *n*-heptane alone [30]. As for example, from the *Schizochytrium* sp. biomass, lipid extraction yields up to 22% (*w/w*) were obtained with the B&D procedure, whereas extractions with methanol (7%), heptane (11%) and chloroform (9.7%) were definitively less efficient [30]. Nevertheless, halogenated-solvent free methods are more and more explored nowadays since, for instance, chloroform is not compatible with food applications [29]. Matyash *et al.* recently reported that extraction using a mixture of methyl *tert*-butyl ether (MTBE) and methanol may reach similar extraction yields compared to the B&D extraction [31].

Cell wall rigidity also represents an issue for the quantitative extraction of (structural) lipids. There are several ways to disrupt cell walls to get access to intracellular compounds [32,33]. Mechanical as well as non-mechanical methods, *i.e.* enzymatic, microwave, ultrasound, acid treatment, sonication, autoclaving, and bead-beating, have previously been tested [34,35]. In biodiesel research, bead milling is efficiently used to increase the triacylglycerol (TAG) extraction yield, with limited decomposition of the TAG into the corresponding fatty acids [36]. Elst *et al.* [37] investigated using mass spectrometry the differences in the lipid profiles of intact and disrupted (freeze-drying steps) cells. *Nannochloropsis* sp. was used as the model microalgae and they also compared the supercritical CO_2 extraction to the standard B&D method [37]. They observed that the freeze-drying pretreatment increases the acylglyceride extraction yield, but also significantly induces the hydrolysis of structural lipids into free fatty acids. Moreover, the distributions of glyco- and phospholipids were shown to be affected, especially promoting digalactosyldiacylglycerols (DGDG) and phosphatidylcholines (PC) as compared to monogalactosyldiacylglycerols (MGDG) and phosphatidylglycerols (PG) [37].

In the present study, we will endeavor to monitor the influence of the nature of the extraction solvent and the impact of the bead milling process on the lipid content of the algae *M. gaditana* by focusing on the structural lipids whose structural diversity makes them relevant for high added-value applications. The main objective is thus to investigate, using mass spectrometry (Ultra Performance Liquid Chromatography - Mass Spectrometry, UPLC-MS), whether *n*-heptane as a halogen-free solvent and bead milling as a cell disruption method may be combined for the optimal extraction of intact structural lipids from *Microchloropsis gaditana*. To achieve these objectives, we will quantitatively and qualitatively compare different *M. gaditana* lipid extracts prepared using *n*-heptane or B&D as the extraction solvents on milled or intact microalgae.

2. Materials and methods

2.1. Solvents

For lipid extractions and mass spectrometry analysis, technical grade *n*-heptane, methanol and chloroform, as well as High Performance

Liquid Chromatography (HPLC) grade acetonitrile and methanol, were purchased from CHEM-LAB NV (Somme-Leuze, Belgium). Milli-Q water was prepared using a Veolia PureLab flex 2 generator (Veolia, Brussels, Belgium).

2.2. Chemicals

Lipid standards - digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) - were acquired from Sigma-Aldrich (Overijse, Belgium).

For the Gas Chromatography - Flame Ionization Detector (GC-FID) analysis, methanolic hydrogen chloride (HCl) 3 M and C_9 standards were acquired from Sigma-Aldrich (Overijse, Belgium). The Fatty Acid Methyl Esters (FAMEs) reference mixture - *Supelco Compenent Fatty Acid Methyl Ester mix* - was bought from Sigma-Aldrich.

2.3. Microchloropsis gaditana

Microchloropsis gaditana (L.M. Lubián) was bought as intact and lyophilized cells from Necton (Portugal) with the batch number: L3180317 1 070917. The algae samples (intact and milled cells) were conserved at −20 °C. The present study was conducted on a single batch of *M. gaditana* to avoid intercultural variations.

2.4. Lipid nomenclature

The lipid nomenclature used in the present study was developed by Liebisch *et al.* [38]. As for a typical example, DGDG (34:2) stands for: "DGDG" as the type of lipid, "34" as the total number of carbon atoms in the fatty acids and "2" as the number of C=C double bonds in the fatty acids. If the fatty acids are identified but not their position on the glycerol backbone, "_" is used, typically in DGDG (16:0_18:2). If the *sn*-positions (*sn*-1 or *sn*-2 positions; *sn*-3 being occupied by the head group substituent) are defined, the terminology becomes DGDG (16:0/18:2) where 16:0 is located on the *sn*-1 position and 18:2 on the *sn*-2 position.

2.5. Milling

For the bead milling process, a Dyno®-mill multi lab from Willy ABachofen AG (Muttenz, Switzerland) was used in the pendulum mode at 15 °C [39,40]. A solution of 90 g·mL^{-1} of *M. gaditana* in tap water was pumped at 150 mL·min^{-1} from an agitated feed tank to a 600 mL grinding chamber - rotation speed at 15 m.s^{-1} - containing the grinding medium - 0.35–0.45 mm Zirmil beads (ZrO_2) - at a 0.80 filling ratio. The basic procedure consisted in three consecutive milling steps [35]. All the samples were conserved at −20 °C immediately after the milling procedure.

2.6. n-Heptane extraction

The microalgae/*n*-heptane (ratio of 1/15, *w/v*) suspensions were stirred at room temperature (20 °C) for 18 h for both the milled and intact cells. The solutions were filtered on a Büchner flask using a Whatman™ filter (WHA100107) and the filtrates were evaporated under vacuum using an IKA Rotative Evaporator RV 10 (VWR International bv, Leuven, Belgium). The residues were extracted two more times with *n*-heptane. All the extractions were performed in triplicate.

2.7. Bligh and Dyer extraction [25]

1.34 g of intact microalgae was suspended in 11.25 mL of $CHCl_3$/MeOH (1/2, *v/v*) and 3 mL of demineralized water. The mixture was vortexed during 15 min at 2200 rpm on an IKA Top-Mix 20. Then 3.75 mL of $CHCl_3$ were added and vortexed for 2 min. 3.75 mL of demineralized water were then added and the suspension was vortexed for an additional 2 min. The mixture was further centrifuged using a Sigma 2-16P centrifuge (VWR International bv, Leuven, Belgium) during 10 min at 4500 rpm. The lower phase was evaporated under vacuum using an IKA Rotative Evaporator RV 10 (VWR International bv, Leuven, Belgium). All the extractions were performed in triplicate.

2.8. Total Lipid assay: Gas Chromatography – Flame Ionization Detector (GC-FID)

The different extracts were transmethylated by adding 1 mL of methanolic HCl 3 M to 1 mg of extract. The reaction was conducted at 50 °C for 6 h to allow the formation of fatty acid methyl esters (FAMEs), which were then extracted by adding 285 μL of *n*-heptane (*i.e.* 285 μL/mg extract) in order to obtain a FAME concentration in *n*-heptane of 3.5 mg·mL^{-1} (assuming that the lipid hydrolysis and the FAME extraction in *n*-heptane are quantitative). In a vial for gas chromatography (GC) analysis, 900 μL of sample solution were added to 100 μL of the internal standard (C9:0–1.045 mg·mL^{-1}).

FAMEs were analyzed using a Shimadzu GC-2010 Plus Gas Chromatograph (GC) (Shimadzu Benelux, Wemmel) coupled with a flame ionization detector (FID) equipped with a RT-2560 column (100 m length, 0.25 mm diameter, 0.20 μm film thickness, Restek). The injected volume of FAME solution was 1 μL. The carrier gas was helium (Westfalen analytical grade 99.99%) at a flow rate of 1.74 mL·min^{-1}. After injection, the temperature of the oven was initially held at 100 °C for 4 min and then increased to 240 °C with a rate of 3 °C.min^{-1} and maintained during 14.33 min for a global time of 65 min. The injector temperature was set to 225 °C and FAMEs were detected by flame ionization using a detector set at 285 °C. The gas flow in the detector was composed by a mix of three gases: 40 mL·min^{-1} of dihydrogen (H_2), 30 mL·min^{-1} of helium (He) and 400 mL·min^{-1} of air. Standard molecules of FAMEs (C4-C24) were injected for fatty acid identification [35].

2.9. Microscopic analysis

Microscopic investigations were realized on rehydrated cells of *M. gaditana* (*i.e.* in distilled water during 24 h). The algae were observed under a 100× immersion type objective with a Zeiss Axioscope A1 microscope coupled to a Zeiss AxioCam 305 color camera (Carl Zeiss NV, Zaventem, Belgium).

2.10. Ultra Performance Liquid Chromatography – Mass Spectrometry analysis (UPLC-MS)

UPLC-MS analysis was performed with a Waters Acquity H-class liquid chromatography device coupled to a Waters Synapt G2-Si mass spectrometer (Waters, Manchester, UK). The UPLC part consisted of a vacuum degasser, a quaternary pump and an autosampler. Sample volumes of 2 μL were injected. Chromatographic separation was performed on a non-polar column (Acquity UPLC BEH C18; 2.1 × 150 mm; 1.7 μm; Waters) at 35 °C. The mobile phase was programmed with a constant flow (0.2 mL·min^{-1}) and consisted of an elution gradient starting with 100% of eluent A (50:50 acetonitrile/water, *v/v* + 0.1% formic acid) and 0% of eluent B (90:10 isopropanol/acetonitrile, *v/v* + 0.1% of formic acid) and reaching 45% of eluent A and 55% eluent B at 1 min. This ratio was modified to reach 30% eluent A and 70% eluent B at 25 min, 100% of eluent B in 1 min, maintained for 4 min and, finally, brought back to 100% eluent A in 1 min for 10 min. The chromatographic run lasted 40 min based on Servaes *et al.* [41]. For the mass spectrometer parameters, the electrospray ionization (ESI) conditions were the same regardless of the ionization mode (positive or negative mode); capillary voltage: 3.1 kV (positive mode) and 2.5 kV (negative mode); cone voltage: 40 V; source offset: 80 V; source temperature: 150 °C; desolvation temperature: 300 °C. Dry nitrogen was used as the ESI gas with a flow rate of 500 L·h^{-1} for the desolvation gas. For the LC-MS analysis, the quadrupole was set to pass ions from *m/z* 50 to 2000 (*m/z*: mass-to-charge ratio) and

all ions were transmitted into the pusher region of the time-of-flight analyzer for mass-analysis with 1 s integration time. For the LC-MS/MS experiments, the so-called Tri-Wave® setup, which is composed of three successive T-Wave elements named the Trap cell, the Ion Mobility Spectrometry (IMS) cell, and the Transfer cell, in which the wave speed and amplitude are user-tunable, was used. LC-separated precursor ions were mass selected using the quadrupole mass filter and subjected to collisions against Argon in the Trap cell. The undissociated precursor ions as well as the product ions were further analyzed using the Time of Flight (ToF) analyzer. For the semi quantitative analysis, the ion current specific to a given ionized lipid was extracted from the LC-MS chromatogram and the relative abundances of all the detected ions were used to estimate the proportions of all the lipids in the extract. Positive and negative ions were analyzed separately for this relative quantification. For the PC, LPC, MGTS and DGTS lipids, the $[M+H]^+$, $[M+NH_4]^+$ and $[M+K]^+$ ions were considered for the semi quantitative analysis. For the MGDG, DGDG and DGMG lipids, the $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were considered, whereas, for the PI (phosphatidylinositol), PG (phosphatidylglycerol), FFA (Free Fatty Acids), SQMG (sulfoquinovosylmonoacylglycerol) and SQDG (sulfoquinovosyldiacylglycerol) lipids, only the $[M-H]^-$ ions were considered.

3. Results and discussion

3.1. Preparation of the lipid extracts: bead milling and extraction

Dried intact cells (IC) and milled cells (MC) were extracted with *n*-heptane or the B&D mixture according to the procedures described in the experimental section and schematized in Fig. 1. Four extracts were thus prepared. After filtration and solvent evaporation, the dried solid-liquid extracts were weighed to determine the gravimetric extraction yields, *i.e.* weight of dried extract relative to weight of the dried cells (in %).

As presented in Fig. 1 and Table 1, using the B&D protocol compared to the *n*-heptane extractions, higher extraction yields were obtained, both on the intact (13.50 ± 0.87% *vs* 3.15 ± 0.91%) and milled (15.45 ± 0.64% *vs* 11.81 ± 0.13%) cells. Whereas the increase was weak for the milled cell extractions, an important increase from 3.15 ± 0.91% to 13.50 ± 0.87% was measured in the case of the intact cells, nicely correlating the results obtained by Elst *et al.* on *M. gaditana* [37]. They demonstrated that the supercritical CO_2 extraction became comparable to the standard B&D procedure for lipid extraction only when the microalgae membranes were first destroyed by applying multiple freeze drying steps [37]. This could indicate (i) that the B&D protocol is less selective than the *n*-heptane extraction inducing a more efficient extraction of other metabolites, (ii) that the *n*-heptane extraction is more selective toward apolar lipids than the B&D protocol that also efficiently extract polar lipids and/or (iii) that the B&D solvents modify to some extent the properties of the microalgae cell membrane. This second hypothesis correlates with the similar extraction yields (15.45 ± 0.64% and 11.81 ± 0.13%) determined for both the different solvents when starting from the milled cells.

Table 1
Quantification of the lipid extraction yields: (a) Gravimetric extraction yields (see also Fig. 1) and (b) total lipid content determined by Gas Chromatography - Flame Ionization Detector (GC-FID). (c) The extracted lipid data (%) were obtained by multiplying the extraction yield by the total lipid content to roughly estimate the percentage of lipids - expressed in fatty acid methyl ester (FAME) equivalents - in the extract. *Mg* is for *Microchloropsis gaditana*, hept and B&D correspond to *n*-heptane (hept) and Bligh and Dyer (B&D) extractions, respectively and IC and MC for intact and milled cells.

	Mg-hept-IC	*Mg*-hept-MC	*Mg*-B&D-IC	*Mg*-B&D-MC
(a) Extraction yield (%)	3.15% ± 0.91	11.81% ± 0.13	13.50% ± 0.87	15.45% ± 0.64
(b) GC-FID: total lipid content (%)	36.3% ± 1.0	42.0% ± 5.2	39.0% ± 2.1	37.9% ± 1.7
(c) Extracted lipids (%)	1.1%	4.9%	5.3%	5.9%

To further test these hypotheses, we monitored the cell integrity upon milling and/or extraction using optical microscopy. As shown in Fig. 2, the milling procedure appeared to be very efficient in destroying the cell membranes as no intact algal cells were observed (Fig. 2B, D and F), contrarily to the spherical cells observed in Fig. 2A [35]. However, from Fig. 2C and E, we observed that neither the *n*-heptane nor the B&D extractions quantitatively disrupt the membrane integrity since numerous spheroid cells were still detected in absence of the milling step. Therefore, only a permeabilization effect can be suspected when intact cells are submitted to the B&D protocol. Choi *et al.* demonstrated that, when cells are treated with organic solvents, the outer cell membrane permeability is significantly decreased due to phospholipid extraction. Chloroform, a B&D solvent, was determined to be the most effective permeabilizing agent, nicely corroborating our data [42].

Before questioning the nature of the lipids extracted by all these protocols using mass spectrometry experiments, we determined the total lipid content of the four prepared extracts by gas chromatography - flame ionization detector (GC-FID) experiments, see the experimental section [35]. These data indicate the lipid proportion, in fatty acid equivalents, within the extracts. Interestingly, whatever the used extraction procedure (B&D or *n*-heptane, milling or not), the total lipid content, as determined by GC-FID, was measured around 35–40%, see Table 1. In other words, in all the extracts, the percentages of total fatty acids were measured similar and thus the use of *n*-heptane did not seem to increase the selectivity of (less polar) lipids *versus* other metabolites.

By associating the extraction yields and the total lipid contents in

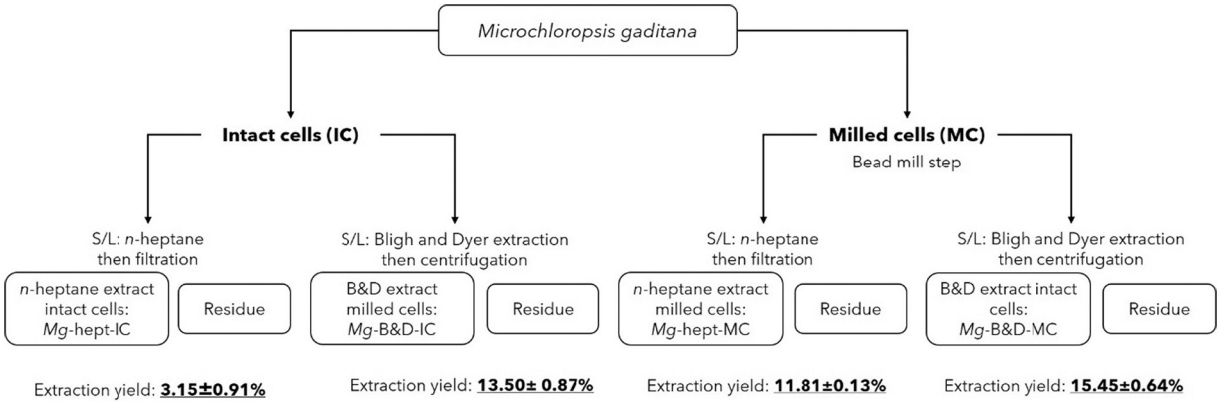

Fig. 1. Solid-liquid (S/L) extractions of the *M. gaditana* (*Mg*) biomass (milled *versus* intact cells) with *n*-heptane (hept) and Bligh and Dyer (B&D) mixture. The extraction yields correspond to the gravimetric ratios between the dried extracts and the dried cells (in %).

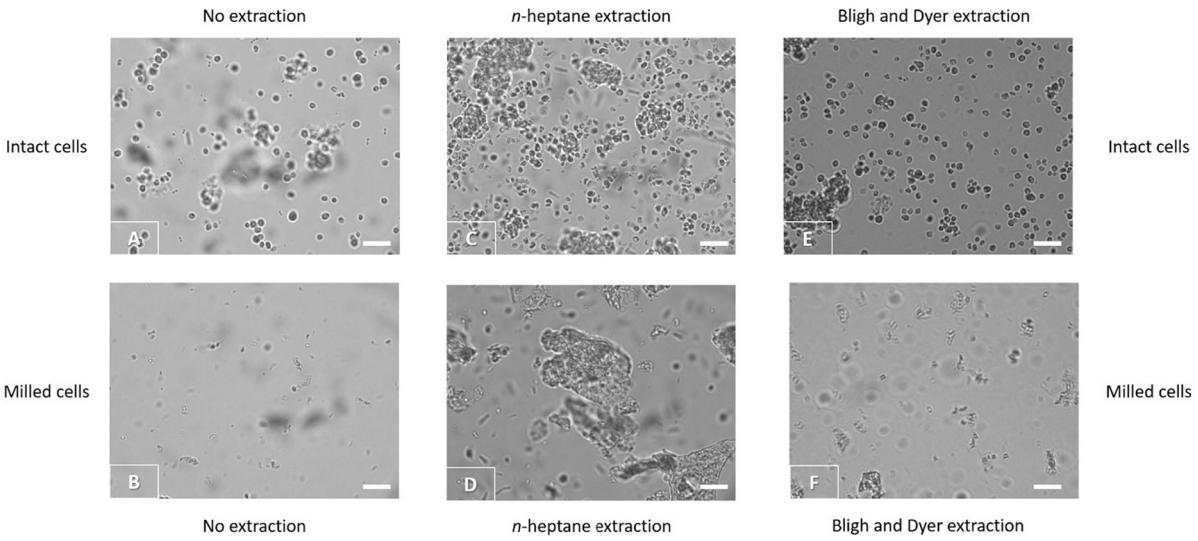

Fig. 2. Optical microscopy analysis of the cell suspension: A: intact cells, before extraction; B: milled cells, before extraction; C: intact cells, after *n*-heptane extraction; D: milled cells, after *n*-heptane extraction; E: intact cells, after Bligh and Dyer extraction; F: milled cells, after Bligh and Dyer extraction. The scale bars represent 20 μm.

Table 1, we determined the extracted lipid proportions from the biomass (in fatty acid equivalents) and we obtained 1.1%, 4.9%, 5.3%, and 5.9% for the *Mg*-hept-IC, *Mg*-hept-MC, *Mg*-B&D-IC and *Mg*-B&D-MC, respectively. At first glance, *n*-heptane extraction became thus as efficient as the standard B&D protocol to selectively extract lipids provided a milling step is included in the lipid extract procedure.

At this point of the procedure comparison, lipid integrity must also be questioned since the milling procedure is likely to induce lipid degradation by, for instance, releasing enzymes such as lipases [43]. To eliminate or slow down these possible reactions, all the extracts obtained, including the lyophilized milled cells, were stored at −20 °C. The lipid profiles of the different extracts (intact cells extract and milled cells extract) must also be compared to evaluate the potential selectivity (*n*-heptane *versus* B&D method) for the extraction of different lipid families.

3.2. Tandem mass spectrometry procedure for the qualitative lipid profiling by mass spectrometry

As stated before, lipid profiling was performed in the present study using LC-MS and LC-MS/MS experiments by exclusively considering the structural lipids. The liquid chromatography protocol was selected to afford an efficient separation between the different structural lipids, see experimental section.

Depending on their chemical structures, lipids can be analyzed either in the positive and/or negative ionization modes as shown in Table 2 [28]. Structural characterization of lipids is best achieved using liquid chromatography coupled to mass spectrometry by associating retention time and molecular mass determination (exact mass measurements, HRMS for High Resolution Mass Spectrometry) to tandem mass spectrometry (MS/MS) data. In particular, numerous studies report on the structural analysis of lipids based on MS/MS experiments, *i.e.* Collision-induced Dissociations (CID), with a special attention paid at the identification of ion decompositions characteristic to the different lipid families [47–50]. Lipid profiling is best achieved by monitoring from the LC-MS(MS) data the occurrence of fragment ions that are specific to lipid families. Table 2 presents tell-tale fragment ions and neutral losses that are used in the present study to ascribe the corresponding precursor ions, *i.e.* $[M+H]^+$ or $[M+Na]^+$ in the positive ionization mode and the $[M-H]^-$ in the negative ionization mode, to a specific lipid family. Table 2 was built based on literature data or on LC-MS/MS analysis performed on commercially available standard lipids (see experimental). All the MS/MS spectra are presented in the Supporting Information (SI), together with fragment ion structure assignments.

As far as the DGMG (digalactosylmonoacylglycerol) family is

Table 2
Mass spectrometry analysis of lipids (see experimental section for the acronyms): characteristic fragment ions or neutral losses for the different families of lipids; [a] based on the fragmentation of the DGDG standard minus one fatty acid linked to the glycerol backbone. All the tandem mass spectra (MS/MS reference) are presented in the supporting information. In the ion mode column, (+) and (−) stand for positive and negative ionization modes. The detected lipids are: MGTS (monoacylglyceryl-*N*-trimethylhomoserine); DGTS (diacylglyceryl-*N*-trimethylhomoserine); LPC (lysophosphatidylcholine); PC (phosphatidylcholine); DGMG (digalactosylmonoacylglycerol); DGDG (digalactosyldiacylglycerol); MGDG (monogalactosyldiacylglycerol); PI (phosphatidylinositol); PG (phosphatidylglycerol); FFA (free fatty acid); SQMG (sulfoquinovosylmonoacylglycerol); and SQDG (sulfoquinovosyldiacylglycerol).

Lipids	Ion mode	Precursor ions	Fragment ions	Neutral losses (Da)	MS/MS reference	*sn*-Position reference
MGTS	+	$[M+H]^+$	*m/z* 236		Fig. SI1	
DGTS	+	$[M+H]^+$	*m/z* 236		Fig. SI2	
LPC	+	$[M+H]^+$	*m/z* 184		Fig. SI3	Okudaira *et al.* [44]
PC	+	$[M+H]^+$	*m/z* 184		Fig. SI4	Houjou *et al.* [45]
DGMG	+	$[M+Na]^+$		−162	Figs. 3 and SI5[a]	
DGDG	+	$[M+Na]^+$		−162 (2 times)	Fig. SI6	Guella *et al.* [46]
MGDG	+	$[M+Na]^+$		−162	Fig. SI7	Guella *et al.* [46]
PI	−	$[M-H]^-$	*m/z* 241		Fig. SI8	Pi *et al.* [47]
PG	−	$[M-H]^-$	*m/z* 171		Fig. SI9	Hsu *et al.* [48]
FFA	−	$[M-H]^-$		−44	Fig. SI10	Aslan *et al.* [49]
SQMG	−	$[M-H]^-$	*m/z* 225		Fig. SI11	Cutignano *et al.* [50]
SQDG	−	$[M-H]^-$	*m/z* 225		Fig. SI12	Cutignano *et al.* [50]

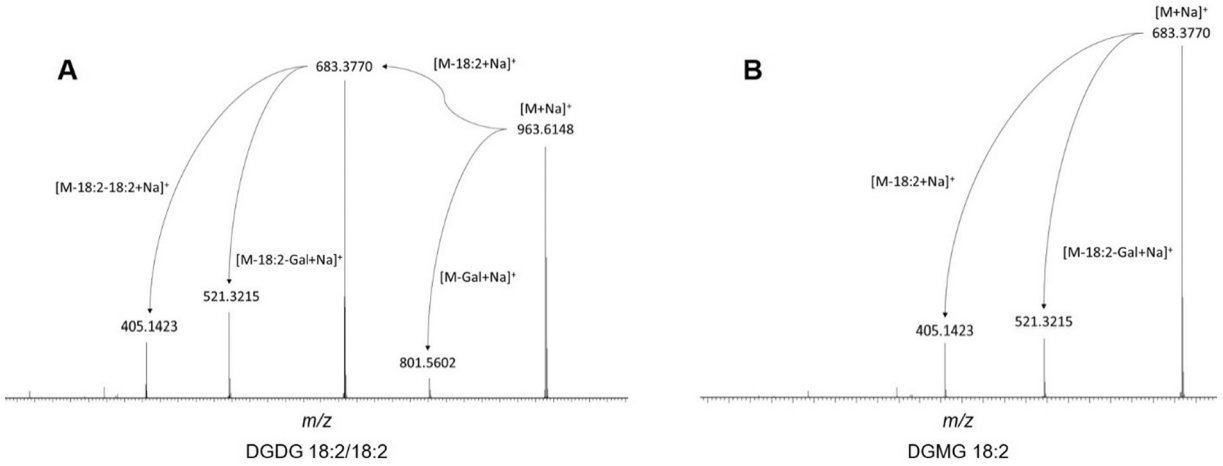

Fig. 3. Collision-induced Dissociation (CID)-Ion Mobility Spectrometry (IMS)-CID analysis of digalactosyldiacylglycerol (DGDG) standard molecule in direct infusion: (A) Collision-induced dissociation (CID) mass spectrum of the $[DGDG+Na]^+$ precursor ions performed in the Trap cell (Argon, Collision energy = 50 V) and (B) CID mass spectrum in the Transfer cell (Argon, 50 V) of the $[DGMG+Na]^+$ fragment ions. This "apparent" MS^3 analysis was setup to create a CID reference spectrum for the DGMG (digalactosylmonoacylglycerol) molecules (see Table 2).

concerned, no standard molecules nor literature data were commercially available. We used a DGDG 18:2/18:2 (digalactosyldiacylglycerol) molecule available as a standard to create a CID reference mass spectrum for the DGMG molecules. Indeed, as shown in Fig. 3A, when subjected to MS/MS experiments, the $[DGDG+Na]^+$ ions readily expel a neutral fatty acid residue to generate $[DGMG+Na]^+$ fragment ions that could be used as reference ions for the DGMG family. As presented in Fig. 3A, upon collisional activation, the $[DGDG+Na]^+$ precursor ions (*m/z* 963.6) suffer a 162 u neutral loss that corresponds to the loss of the galactosyl head group [51]. The precursor *m/z* 963.6 ions competitively expel a fatty acid residue to generate $[DGMG+Na]^+$ ions detected at *m/z* 683.4 in Fig. 3A [46]. A unique signal is detected for the fragment ions since the *sn*-1 and *sn*-2 positions are occupied by a 18:2 fatty acid residue. Using the TriWave device of the Waters Synapt G2-Si that consists of three consecutive cells, *i.e.* Trap cell, ion mobility spectrometry (IMS) cell and Transfer cell, a CID-IMS-CID sequence of ion manipulation can be envisaged. In such an experiment, the *m/z* 963.6 ions are subjected to collisions against argon in the Trap cell and all the produced ions are separated by ion mobility experiments before being subjected to CID in the Transfer cell. This allows recording the CID spectrum of the *m/z* 683.4 ions corresponding to $[DGMG+Na]^+$ ions. The generated spectrum is presented in Fig. 3B and confirms that the 162 u loss of a galactosyl residue can be considered as a key neutral loss characterizing the DGMG family members, see Table 2.

3.2.1. *LC-MS lipid profiling in the positive ionization mode*

The lipid extracts from the *M. gaditana* were analyzed using MS-based methods that combined LC-(HR)MS and LC-MS/MS [41]. Lipid identification was achieved by comparing the generated MS(/MS) data to the data gathered in Table 2 obtained from standards (PC, LPC, MGDG, DGDG) and literature data. We also took advantage of the free online database LIPIDMAPS to ascertain the lipid identification. Fig. 4 compares the LC-MS chromatograms obtained upon analysis of the *n*-heptane extracts of intact and milled cells, *i.e. Mg*-hept-IC and *Mg*-hept-MC extracts, in the positive ionization mode. These analyses are referenced as *Mg*-hept-IC-ESI(+) and *Mg*-hept-MC-ESI(+), respectively. Using

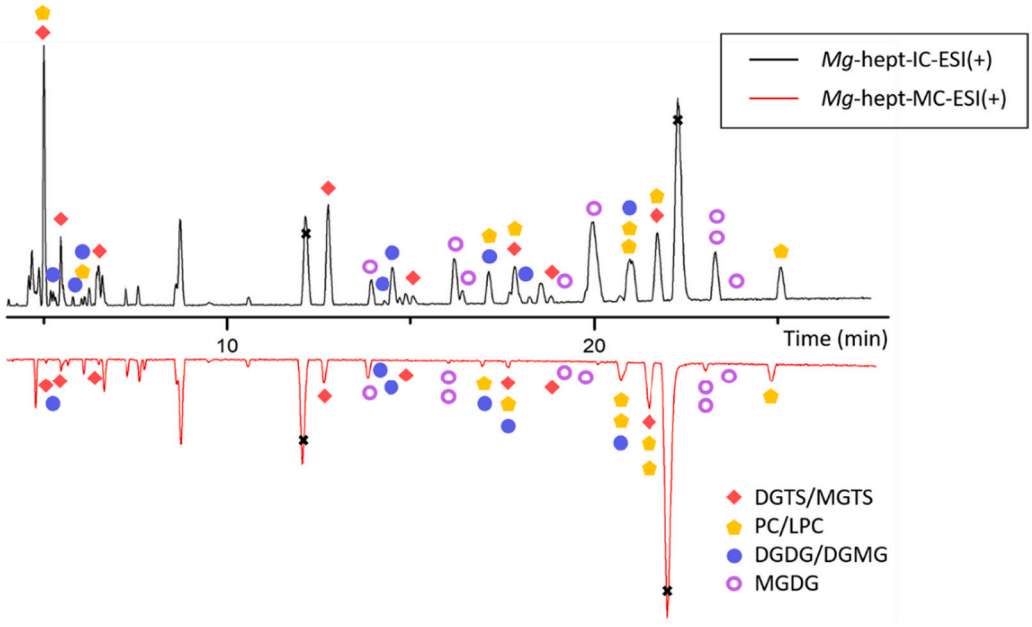

Fig. 4. Liquid chromatography - mass spectrometry (LC-MS) analysis in the positive ionization mode (ESI(+)) of the *n*-heptane (hept) extracts of intact (IC) and milled cells (MC) of *Microchloropsis gaditana* (*Mg*): BPI (Base Peak Intensity) chromatograms recorded for the *Mg*-hept-IC (top chromatogram - black line) and for the *Mg*-hept-MC (bottom chromatogram - red line) extracts. The retention times highlighted with black crosses were attributed to chemical noise, since they were already detected in the blank. The detected lipids were: MGTS (monoacylglyceryl-*N*-trimethylhomoserine); DGTS (diacylglyceryl-*N*-trimethylhomoserine); LPC (lysophosphatidylcholine); PC (phosphatidylcholine); DGMG (digalactosylmonoacylglycerol); DGDG (digalactosyldiacylglycerol); and MGDG (monogalactosyldiacylglycerol). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HRMS and MS/MS data, the lipid analysis of *Mg*-hept-IC(MC) in the positive ionization mode allowed identifying seven lipid families, namely LPC, PC, DGTS, MGTS, DGDG, DGMG and MGDG. All these lipids are the common constituents of the membrane lipid bilayers. They are structural lipids and they hence support the normal cell functions [52]. For instance, DGDG are well-known bilayer lipids making them essential for structural and functional stabilization of the chloroplast and so the cell viability [53]. DGTS and MGDG are proposed to represent the major sources of eicosapentaenoic acid (EPA) (C20:5) in the extraplastidic and plastidic membranes [54].

As for a typical example of our identification strategy, the phosphatidylcholine family (PC) is presented here with more details. The *m/z* 184.1 fragment ions are used as the PC and LPC signatures (see Table 2) allowing a cleaning-up of the BPI chromatograms from lipid ions of other families, as shown in Fig. 5. To increase the sensitivity of such an analysis, the abundances of the *m/z* 184.1 fragment ions must be significantly increased by subjecting all the source-generated ions (no mass selection in the quadrupole analyzer) to collisional activation in the Trap cell. In Fig. 5, the BPI chromatogram recorded with applying 30 V collision energy in the Trap cell reveals the presence of numerous signals, corresponding to the lipids detected in the positive ion mode as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ (fragment) ions. By specifically extracting from the ion chromatogram the *m/z* 184.1 LPC and PC signature ions, the ion chromatogram is significantly simplified by only revealing LPC and PC ions that are further identified based on HRMS and MS/MS experiments, see Table SI1.

As far as the PC lipids are concerned, the identification of the fatty acid residues, as well as their *sn*-1/*sn*-2 positions, can be achieved based on CID experiments. For instance, both the chromatogram signals at 22.2 and 22.9 min correspond to *m/z* 758.6 $[M+H]^+$ ions and their compositions are clearly confirmed using HRMS experiments as presented in Table SI1. Using LC-MS/MS experiments, these isomeric ions were subjected to CID experiments in the Trap cell and the corresponding CID spectra are presented in Fig. 6. The CID decomposition reactions undergone by $[PC+H]^+$ ions are described in the literature

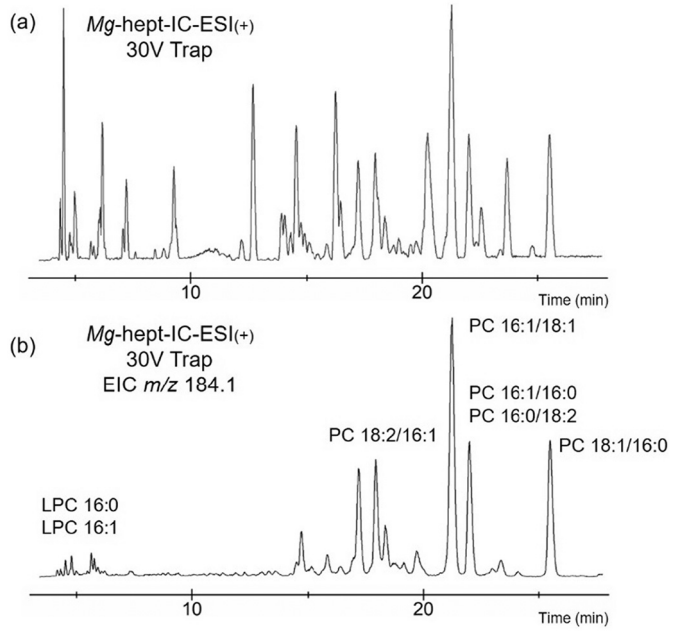

Fig. 5. Liquid chromatography - mass spectrometry (LC-MS) analysis of the *n*-heptane (hept) extract of intact cells (IC) for the identification of the phosphatidylcholine lipids (PC): (a) BPI (Base Peak Intensity) chromatogram with 30 V collision energy in the Trap cell and (b) EIC (Extracted Ion Chromatogram) of the *m/z* 184.1 as the MS signature of the PC and LPC (lysophosphatidylcholine) lipid ions.

[45] and, for each of the fatty acid residues, two competitive exit channels are observed, *i.e.* the loss of the acyl chain as a fatty acid (FA in Fig. 6) or as a neutral ketene (K in Fig. 6). If the PC molecule is decorated by two different fatty acid residues, four fragment ions are then detected in the CID spectrum, as observed in Fig. 6. This allows identifying the nature of the acyl chains (without permitting to localize the double bonds). Doing so, from the CID spectra in Fig. 6, the PC molecules eluting at 22.2 and 22.9 min were shown to respectively contain 16:1/18:1 and 16:2/18:0 as fatty acid residues. As far as the regioisomer distinction is concerned, it is proposed in literature reports that the fragment ions coming from the ketene loss are more abundant than the fragment ions for the fatty acid loss, when the fatty acid residue is on the *sn*-2 position and *vice versa* [45]. The data for PC and LPC presented in Table SI1 have been composed sequentially based on such LC-MS and LC-MS/MS experiments.

By combining the $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ion abundances, including all the isotopic signals, for all the identified PC and LPC, and assuming that the nature of the fatty acid residues did not significantly affect the ionization efficiencies, we determined the molar fractions of the LPC and PC in the extract and visualized them within a sector diagram, as recently proposed for natural product analysis [55]. In these diagrams (Fig. 7), the inner circle presents the mass-to-charge ratios (*m/z*) of the ions, whereas the outer circle highlights the presence of isomers, if applicable. From such a representation, we immediately determined that, for the PC family, four compositions were present and that unique retention times were recorded for these PC compositions, except for the *m/z* 758.6 ions. This indicated the presence of unique fatty acid compositions for PC 16:0/18:1, PC 16:0/16:1 and PC 16:1/18:2, whereas two isomers were detected for the *m/z* 758.6 ions, namely PC 16:0/18:2 and PC 16:1/18:1. We strongly believe that such a representation is ideal for presenting lipidomic results. The sector diagrams for the other lipid families are presented in the Supporting information (Figs. SI13 to SI19).

We then repeated the same procedure for all the lipid families presented in Table 1. The global sequence was (i) identification of the different lipid families by extracting key fragment ions or monitoring neutral losses from the LC-MS chromatograms, (ii) exact mass measurements to determine the elemental composition of the lipid molecules, (iii) CID experiments to identify the fatty acid residues and (iv) assignment of the fatty acid relative positions (*sn*-1 or *sn*-2), if applicable. We already noted that, for the PC ions, the relative intensities of ketene or fatty acid fragment ions can be used for the regioisomer distinction [45]. Guella *et al.* demonstrated that MS/MS experiments provide definitive information about the position of the acyl groups on the glycerol backbone of the DGDG and MGDG by indicating a preferential loss of the acyl chain as neutral carboxylic acid from the *sn*-1 rather than from the *sn*-2 position from the protonated lipids [46].

Finally, as a semi-quantitative approach, we compared using sector diagrams the relative abundances of all the lipid families. Of course, such a comparison is largely biased by the fact that lipids from different families are not likely to present similar ionization efficiencies. Nevertheless, since the main objective of our study is to define whether the lipid extraction procedure quantitatively and qualitatively impacts on the lipid profiles, such a comparison will remain valuable and useful. In Fig. 8, we compared the lipid profiles of the extracts prepared from the intact and milled cell using *n*-heptane as the extraction solvent. The direct comparison of both sector diagrams reveals that the different lipid families were detected in both cases and that their relative proportions did not seem to be drastically affected upon milling, although the PC lipids seemed to be more abundant and the MGTS family less represented after the milling step. One of the expected issues upon milling is the release of enzymes, such as lipases, that could hydrolyze the lipid molecules producing free fatty acid (FFA) molecules [56]. Using LC-MS analysis in the positive ionization mode, FFA molecules are not sampled, and we must thus analyze the same extracts in the negative ionization mode. This will also allow analyzing other families of lipids such as PI,

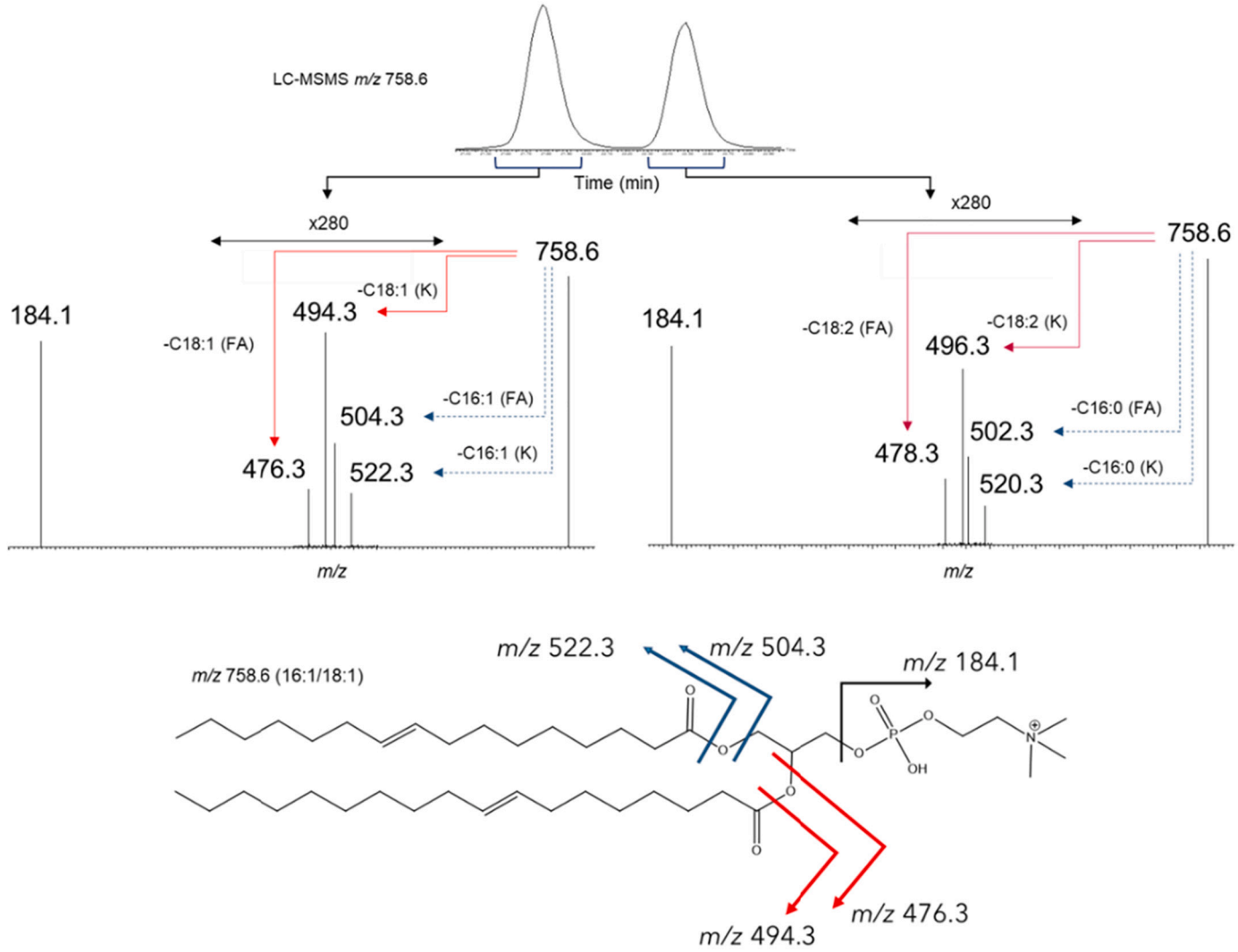

Fig. 6. Liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of the phosphatidylcholine (PC) lipids eluting after 22.2 and 22.9 min. The K losses (red) correspond to the elimination of the acyl group as a neutral ketene residue, whereas the acyl chain can be expelled as a neutral fatty acid - FA losses - (blue) from the mass-to-charge ratio (*m/z*) 758.6 $[PC+H]^+$ precursor ions [48]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

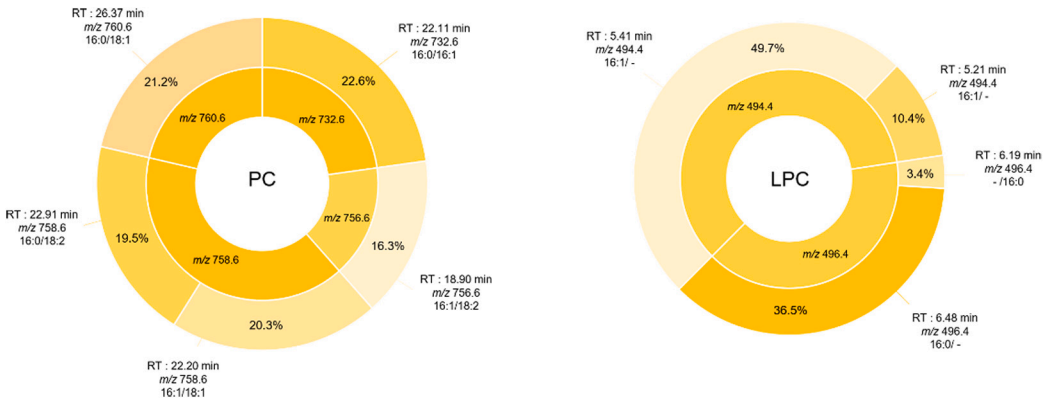

Fig. 7. Relative quantification of the phosphatidylcholine (PC) and lyso-phosphatidylcholine (LPC) lipids detected in *n*-heptane (hept) extract of the intact cells (IC) of *Microchloropsis gaditana* (*Mg*). For each lipid, the retention time (RT), the mass-to-charge ratio (*m/z*) of the $[M+H]^+$ ions, the fatty acid composition and their positions on the glycerol backbone are presented. The relative proportions were determined using extracted liquid chromatography - mass spectrometry (LC-MS) signal integration (see experimental).

PG, SQMG and SQDG (Table 1).

We also analyzed the B&D extracts of the intact and milled cells using exactly the same protocol and the semi-quantitative data are gathered in the sector diagrams presented in Fig. 8. When compared to the *n*-heptane extracts, the same lipid families were detected in the B&D extracts, even if the relative proportions between the different families were found different, as expected when considering the different polarities of the extraction solvents. A key difference was that MGTS lipids were significantly more present in the B&D extracts. These charged lipids presenting only one acyl chain are probably the most polar lipids

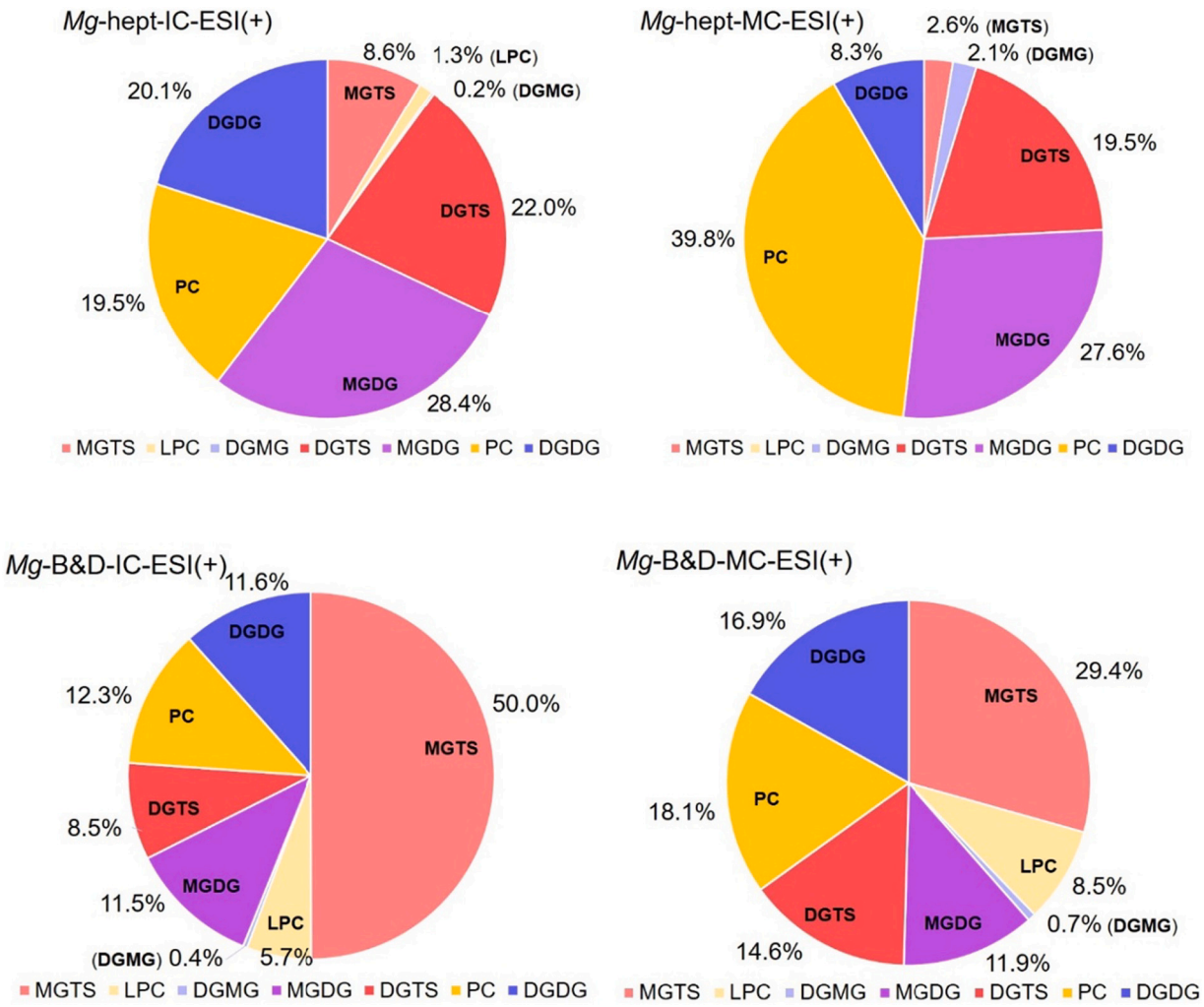

Fig. 8. Lipid profiling by liquid chromatography - mass spectrometry (LC-MS) analysis (ESI(+): positive ionization mode) of the *n*-heptane (hept) extracts of intact (IC) and milled cells (MC), respectively *Mg*-hept-IC-ESI(+) and *Mg*-hept-MC-ESI(+) and of the Bligh and Dyer (B&D) extracts of intact and milled cells, respectively *Mg*-B&D-IC-ESI(+) and *Mg*-B&D-MC-ESI(+). The relative proportions were determined using extracted LC-MS signal integration (see experimental). The detected lipids are: MGTS (monoacylglyceryl-*N*-trimethylhomoserine); DGTS (diacylglyceryl-*N*-trimethylhomoserine); LPC (lysophosphatidylcholine); PC (phosphatidylcholine); DGMG (digalactosylmonoacylglycerol); DGDG (digalactosyldiacylglycerol); and MGDG (monogalactosyldiacylglycerol).

extracted from the cells making their higher proportion in the B&D extract comprehensive. Similarly, the LPC family was also more represented in the B&D extract when compared to the *n*-heptane one. On the other hand, the relative proportions between the 4 other lipid families, *i.e.* DGDG, PC, DGTS and MGDG, appeared similar whatever the used solvents. After milling, the MGTS proportion drastically decreased from 50.0% to around 29.4% in the B&D extract. This is probably due to the fact that the hydrolysis of MGTS removes the unique acyl chain rendering the hydrolysis product no longer detected amongst the lipid families. One can believe that a similar behavior could apply for the LPC molecules, except that they are competitively produced by PC hydrolysis.

3.2.2. *LC-MS lipid profiling in the negative ionization mode*

Fig. 9 presents the LC-MS chromatograms of the *Mg*-hept-IC and *Mg*-hept-MC extracts analyzed using LC-MS in the negative ionization mode. In the intact cell *n*-heptane extract LC-MS chromatogram, *i.e. Mg*-hept-IC-ESI(−), 21 lipid signals were detected and were ascribed to $[M\text{-}H]^-$ ions of PG, PI, SQDG, SGMG and FFA. Their elemental compositions were confirmed based on accurate mass measurement (Table SI2) and CID mass spectrometry. Note that, upon CID, the $[M\text{-}H]^-$ ions from lipids generate deprotonated free fatty acid ions whose observation indicates the nature of the acyl chain appended on the glycerol backbone [47].

The LC-MS analysis of the intact cell extract was further compared to the LC-MS experiment conducted on the milled cell extract in Fig. 9. Compared to the positive ionization mode data, the differences between the intact and milled cells analysis were striking and clearly pointed to an overproduction of FFA upon milling. Also, after milling, the signals corresponding to SQDG and SQMG have almost totally disappeared. This is clearly observed when comparing the sector diagrams for both analysis in Fig. 10 and observing that most of the ion intensity is ascribed to FFA. It is however envisaged that the relative proportion of the FFA is overestimated due to their high ionization efficiency. Nevertheless, the PI, PG, SQMG and SQDG lipids were still detected in our LC-MS analysis, even for the milled cell extract. It is likely that the free fatty acid (FFA) proportion significantly increases due to the release of enzymes, for example lipases, upon milling as already proposed in the literature [43]. A previous study conducted on *Microchloropsis* sp. demonstrated that the FFA concentration is largely increased within disrupted cell extracts compared to intact cells, since FFA are generated by lipolysis of different types of lipids when the cell wall is disrupted [56]. The degradation of polar lipids is presumably associated with the action of phospholipases and/or glycolipases after cell membrane disruption [56–58].

Fig. 11 presents the relative proportions of the different lipids by discarding the FFA contribution. It is worthy of note that the intact structural lipid relative proportions were conserved upon milling, as

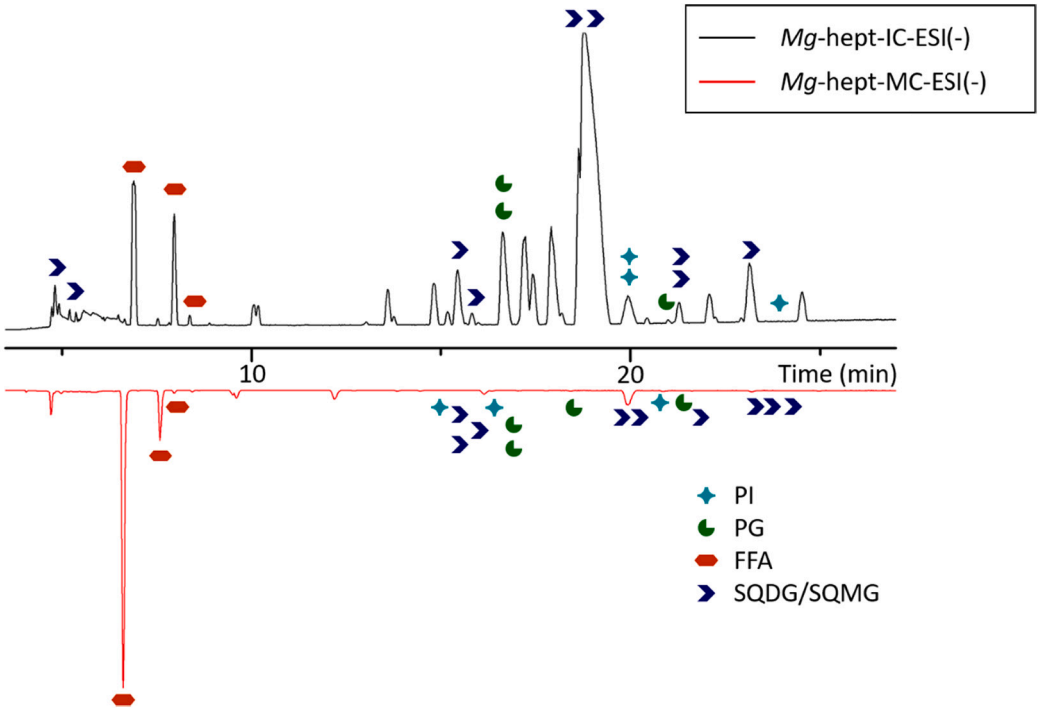

Fig. 9. Liquid chromatography – mass spectrometry (LC-MS) analysis in the negative ionization mode (ESI(−)) of the *n*-heptane (hept) extracts of intact (IC) and milled cells (MC) of *Microchloropsis gaditana* (*Mg*): BPI (Base Peak Intensity) chromatograms recorded for the *Mg*-hept-IC (top chromatogram - black line) and for the *Mg*-hept-MC (bottom chromatogram - red line) extracts. The retention times highlighted with black crosses were attributed to chemical noise, since they were already detected in the blank. The detected lipids were: PI (phosphatidylinositol); PG (phosphatidylglycerol); FFA (free fatty acid); SQMG (sulfoquinovosylmonoacylglycerol); and SQDG (sulfoquinovosyl-diacylglycerol). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

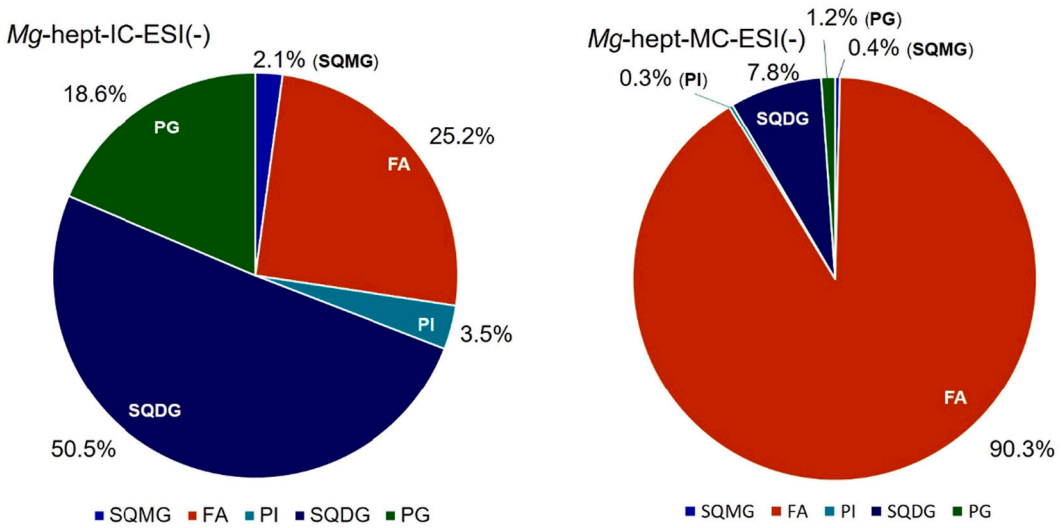

Fig. 10. Lipid profiling by liquid chromatography – mass spectrometry (LC-MS) analysis (ESI(−): negative ionization mode) of the *n*-heptane (hept) extract of intact (IC) and milled cells (MC) of *Microchloropsis gaditana* (*Mg*), respectively *Mg*-hept-IC-ESI(−) and *Mg*-hept-MC-ESI(−): the relative proportions were determined using extracted LC-MS signal integration (see experimental). The detected lipids were: PI (phosphatidylinositol); PG (phosphatidylglycerol); FFA (free fatty acid); SQMG (sulfoquinovosylmonoacylglycerol); and SQDG (sulfoquinovosyldiacylglycerol).

already shown for the structural lipids analyzed under LC-MS in positive ion mode (Fig. 8). This could reveal that, although, upon milling, the lipid hydrolysis is occurring certainly due to the release of lipids and enzymes, the specificity of the hydrolysis reactions is not high enough to be monitor in our investigations. From these data, we observed that PG lipids are more sensitive to the hydrolysis than the SQDG ones. Finally, in Fig. 11, the *n*-heptane and B&D extracts are compared (neglecting the FFA contribution) and we observed that all the extracts were qualitatively and quantitatively similar. In Fig. 8 that concerns the positive ionization mode, the relative proportions between the lipid families extracted by *n*-heptane or B&D were observed to be different. One of the major differences between the lipid families analyzed in the positive and negative ionization modes is that the ESI(+) detected lipids are either neutral (DGMG, MGDG and DGDG) or charged (PC, DGTS, MGTS and LPC), whereas the ESI(−) detected lipids are only charged lipids (SQMG, PI, SQDG and PG). The coexistence of neutral and charged polar lipids amongst the ESI(+) detected lipids is at the origin of the differences between the *n*-heptane and the B&D extracts only when analyzed in ESI (+).

4. Conclusions

Lipid profiling of microalgae biomass remains challenging (i) due to the huge diversity of lipid families, (ii) due to the different localizations of lipids within the cells (related to their biological roles) and (iii) due to the presence of specific enzymes that may be released during the

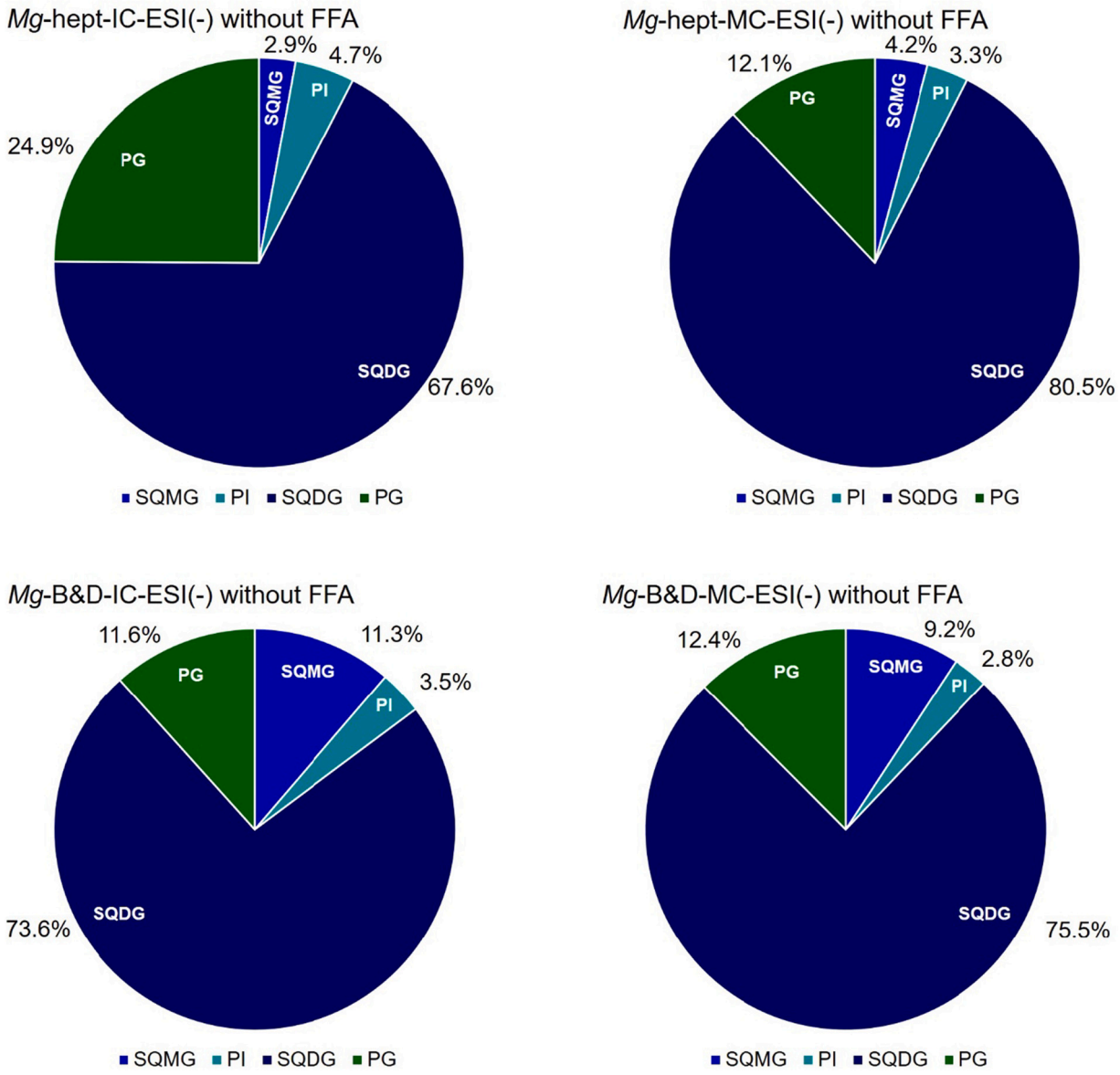

Fig. 11. Lipid profiling by liquid chromatography – mass spectrometry (LC-MS) analysis (ESI(−): negative ion mode) of the *n*-heptane (hept) extract of intact (IC) and milled cells (MC) of *Microchloropsis gaditana* (*Mg*), respectively *Mg*-hept-IC-ESI(−) and *Mg*-hept-MC-ESI(−), and of the Bligh and Dyer (B&D) extraction of intact and milled cells, respectively *Mg*-B&D-IC-ESI(−) and *Mg*-B&D-MC-ESI(−) when discarding the free fatty acids (FFA) contribution: the relative proportions were determined using extracted LC-MS signal integration (see experimental). The detected lipids were: PI (phosphatidylinositol); PG (phosphatidylglycerol); FFA (free fatty acid); SQMG (sulfoquinovosylmonoacylglycerol); and SQDG (sulfoquinovosyldiacylglycerol).

biomass processing. A typical example concerns the so-called structural lipids, whose presence in the different membranes renders their extraction challenging. Often cell membrane disruption protocols are applied prior to the extraction steps for increasing the lipid extraction yields [33]. Recent studies demonstrated that such step may be accompanied by enzyme release that further degrades the lipids generating free fatty acids [56]. When the lipid extraction aims to be exploited for biofuel preparation these enzymatic processes may be considered as anecdotic. However, when the objectives of the extraction are to conserve the lipid integrity, this is definitively a drawback. Lipid profiling therefore appears mandatory and to date lipid extraction optimizations are scarcely accompanied by a full structural analysis investigation.

In the present study, we extensively used LC-MS and LC-MS/MS experiments to monitor the impact of bead milling and the influence of the extraction solvents, Bligh and Dyer *versus* *n*-heptane, on the nature of the structural lipids extracted from *Microchloropsis gaditana* (*M. gaditana*), that is an important microalga whose biomass, including the lipid fraction, is being considered for many applications.

We first set up a LC-MS strategy, including accurate mass measurement and collision-induced dissociation experiments, in both the positive and negative ionization modes, to highlight the huge diversity of lipids. We also clearly demonstrated that LC-MS experiments must be performed in both the positive and negative ionization modes for a full lipid profiling since some lipid molecules are detected in a specific ionization mode.

We demonstrated that the Bligh and Dyer method remains more efficient for lipid extraction when considering the gravimetric extraction yield, including the total lipid content (GC-FID), making it to date the method of choice for lipid extraction from microalgae. However, when qualitatively analyzing the extracted lipid families, some differences are detected. In particular, *n*-heptane was shown to afford extracts richer in strongly apolar lipids, such as glycerolipids. Also, single acyl chain lipids are less present in the *n*-heptane extracts. Really important, we shown

that the differences between the *n*-heptane and B&D extracts are mostly observed in the positive ion mode due to the detection of neutral and charged polar lipids. In the negative ionization mode, only charged polar lipids are detected as deprotonated molecules.

We further shown that the milling step is accompanied by extensive lipid degradation into free fatty acid upon enzyme release, even when the biomass is conserved at −20 °C. However, the lipid degradation seems to affect all the lipid families in similar extent. Again, the differences between *n*-heptane/B&D extracts of intact/milled cells are mostly detected in the positive ionization mode, whereas the FFA molecules are only detected in the negative ionization mode. Globally, LC-MS experiments again clearly appear essential when lipid extraction procedures from the microalgal biomass are optimized.

From a method development perspective, this work presents one of the most complete structural analysis studies of lipids extracted from intact and milled microalgae, including the use of a sector diagram representation that could be relevant for all lipidomic investigations. One of the main outcomes of the study is the confirmation that LC-MS/MS experiments are essential when lipid extraction procedures from any microalgae biomass are optimized and that LC-MS experiments must be conducted in both the positive and negative ionization modes. The analytical protocol developed in the present study certainly deserves to be extended to other microalgae.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no potential financial or other interests that could be perceived to influence the outcomes of the research. No conflicts, informed consent, human or animal rights applicable.

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Appendix A. Supplementary data

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