Lipidomic signature of the green macroalgae *Ulva rigida* farmed in a sustainable integrated multi-trophic aquaculture

Diana Lopes[1,2,3] , Ana S. P. Moreira[1,2], Felisa Rey[1,,32], Elisabete da Costa[1,2,3], Tânia Melo[1], Elisabete Maciel[1,2,3], Andreia Rego[4], Maria H. Abreu[4], Pedro Domingues[1], Ricardo Calado[3], Ana I. Lillebø[3], M. Rosário Domingues[1,2, *]

1 Centro de Espectrometria de Massa, Departamento de Química & QOPNA, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal. dianasalzedaslopes@ua.pt (DL) orcid.org/0000-0002-0072-0492; ana.moreira@ua.pt (AM); felisa.rey@ua.pt (FR); elisabetecosta@ua.pt (EC); taniamelo@ua.pt (TM); elisabete.maciel@ua.pt (EM); p.domingues@ua.pt (PD); mrd@ua.pt (MRD)

2 Departamento de Quimica & CESAM & ECOMARE, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal. E-mail:

3Departamento de Biologia & CESAM & ECOMARE, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal. E-mail: rjcalado@ua.pt (RC); lillebo@ua.pt (AIL)

4 ALGAplus - Produção e comercialização de algas e seus derivados, Lda., 3830-196 Ílhavo, Portugal. E-mail: amrego@algaplus.pt (AR); htabreu@algaplus.pt (MHA)

* Author to whom correspondence should be addressed: mrd@ua.pt; +351 234 401 505.

Abstract

Ulva species, green macroalgae, are widely distributed in the water across the globe, being one of the most heavily-traded edible seaweeds. Nonetheless, although this genus has been largely used in scientific studies, its lipidome remains rather unexplored. The present study sheds light over the lipid profile of *Ulva rigida* produced in a land-based integrated multi-trophic aquaculture (IMTA) system using liquid chromatography coupled to high resolution mass spectrometry for molecular lipid species identification. The lipidome of *U. rigida* revealed the presence of distinct beneficial *n*-3 fatty acids for human health, namely alpha-linoleic acid (ALA) and docosapentaenoic acid (DPA). A total of 87 molecular species of glycolipids, 58 molecular species of betaine lipids and 57 molecular species of phospholipids were identified in the lipidome of *U. rigida* including some species bearing PUFA and with described bioactive properties. Overall, the present study contributes to the valorization and quality validation of sustainably farmed *U. rigida*.

Keywords: Chlorophyta, Edible, Lipidome, Mass spectrometry, Seaweed, *Ulva rigida*

Introduction

Edible macroalgae are a good source of beneficial compounds for human health that display distinct functional properties that stimulate interest to number of high-value chains (e.g., medical, nutraceutical and cosmeceutical) (Holdt and Kraan 2011; Leal et al. 2013; Abreu et al. 2014; Rajauria 2015; Roohinejad et al. 2016). *Ulva* spp. have long been listed in FAO as one of the main macroalgae for commercial use (Naylor 1976). These popular green seaweeds can be used fresh, dried, or in liquid extracts, either for direct or processed consumption worldwide (McHugh 2003; Barriga et al. 2017). Popularly known in the human food market as sea lettuce, *Ulva* spp. belongs to class Ulvophyceae and can be found in marine and brackish waters, being widely distributed across the globe. *Ulva* species are well adapted to aquaculture production and can be successfully cultured by using an integrated multi-trophic aquaculture (IMTA) framework (Bolton et al. 2008; Msuya and Neori 2008; Marinho et al. 2013; Shpigel et al. 2017). This innovative and sustainable culture approach mimics the natural ecosystem of species from different trophic levels, associating the production of fed species (e.g. finfish) with other extractive organisms, namely marine invertebrates and/or algae, that incorporate organic and inorganic compounds resulting from the metabolism of fed species, as well as from uneaten feed. Overall, IMTA promotes a balanced production framework that is environmentally sustainable and viable from an economic point of view (Barrington et al. 2009; Chopin et al. 2012). The culture of seaweeds under an IMTA approach allows the removal of excess nutrients, namely phosphorus and nitrogen, from wastewater (Neori 2009; Lawton et al. 2013), while enhancing quality and stability of seaweeds biomass and their biochemical profile (Abreu et al. 2014).

Ulva species are consumed directly as “sea vegetables” and used as a food and feed ingredient. They are also recognized as an important source of valuable polysaccharides

77 (such as ulvans) and oligosaccharides rich in functional groups that bind important
78 microelements for human and animal nutrition (Lahaye and Robic 2007; Stengel et al.
79 2011; Berri et al. 2016; Wijesekara et al. 2017). However, to date, the lipid profile of *Ulva*
80 spp. is still poorly studied at molecular level and few articles have reported their lipid
81 characterization (Takahashi et al. 2002; Rozentsvet and Nesterov 2012; Ragonese et al.
82 2014), with most studies solely describing their fatty acid (FA) profile (van Ginneken et
83 al. 2011; Ragonese et al. 2014; Kendel et al. 2015). While lipids may solely represent
84 from 1 to 3% of the whole algal dry matter, they do display an important nutritional value,
85 with emphasis into polyunsaturated fatty acids (PUFAs) from the *n*-3 (e.g., alpha-
86 linolenic acid, eicosapentaenoic acid and docosahexaenoic acid) and *n*-6 (linoleic acid,
87 gamma-linolenic acid and arachidonic acid) (Kumari et al. 2010). As essential PUFAs are
88 not synthesized by humans, they need to be obtained through diet to provide energy and
89 others health benefits (e.g., reduce the risk of coronary disease and blood cholesterol)
90 (Ginzberg et al. 2000; Simopoulos 2008; Kendel et al. 2015). Furthermore, PUFAs are
91 also precursors of important mediators that play a key-role in inflammation and regulation
92 of immunity (Calder 2001). These biomolecules mostly occur in their esterified form in
93 polar lipids, namely phospholipids (PLs) and glycolipids. This feature enhances the
94 nutritional properties of these classes of polar lipids. Additionally, glycolipids isolated
95 from macroalgae have already been described as displaying bioactive proprieties, namely
96 antitumoral (Ohta et al. 1998; Eitsuka et al. 2004), anti-inflammatory (Banskota et al.
97 2013, 2014), antimicrobial (El Baz et al. 2013; Parveez et al. 2017) and antiviral activity
98 (Wang et al. 2007).
99 The potential added value of macroalgal polar lipids has received a new momentum with
100 the advent of mass spectrometry-based approaches, which have already been employed
101 to provide an in-depth characterization of lipidomic signatures of different macroalgae,

namely *Chondrus crispus* (Melo et al. 2015), *Codium tomentosum*, *Gracilaria* sp., and *Porphyra dioica* (da Costa et al. 2015, 2017, 2018). The aim of the present study is analyzed the lipidome of *Ulva rigida* (C.Agardh, 1823) from a land-based IMTA system using liquid chromatography high resolution mass spectrometry - based approach. The data presented will contribute to promote on-going efforts in the responsible, controlled and sustainable production of high-value macroalgae.

Material and methods

Reagents

HPLC grade chloroform ($CHCl_3$) and methanol (CH_3OH) were purchased from Fisher Scientific Ltd. (Loughborough, UK). All other reagents were purchased from major commercial sources. Milli-Q water was obtained from a water purification system (Synergy, Millipore Corporation, Billerica, MA, USA). Phospholipid internal standards 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(10-rac-glycerol) (dMPG), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (dMPS), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylinositol (dPPI), N-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (NPSM), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Biomass

The fresh biomass of *Ulva rigida* (C.Agardh, 1823) was produced by ALGAplus (production site located at Ria de Aveiro coastal lagoon, mainland Portugal, 40°36′43″N, 8°40′43″W) in an IMTA system, harvested in November 2016 (batch U1.4616.L). The

ALGAplus IMTA system is composed of a fish organic certified production units (seabass and seabream) and the seaweed land-based tank system. The water flows from the fish units, to the seaweed tanks and then to the exit channel that discharges clean water into the coastal lagoon. Seaweeds are cultivated using exclusively water input from the fish farm (nothing is added to the water). Stocking densities and water flows are manipulated in each season to achive optimal biomass yields and/or specific biomass quality traits (i.e. chemical composition, colour). After being harvest, all biological samples were cleaned to remove epiphytic foreign matters, washed with seawater that is sequentially filtered up to 25 micron and then sterilized by UV and Ozone treatment. The samples were then frozen at -80 °C, lyophilized, and stored at -80 °C until lipid extraction.

Moisture and ash determination

Moisture was determined by drying freeze-dried samples (250 mg x 5 replicates) in crucibles on an oven at 105 ºC for 15 h. For ash determination, the dried biomass in the crucibles was first pre-incinerated for 20 min using a heating plate and then placed in a muffle furnace at 575 °C for 6 h.

Nitrogen determination and protein estimation

Nitrogen content of freeze-dried samples (2 mg x 5 replicates) was obtained by elemental analysis on a Leco Truspec-Micro CHNS 630-200-200 elemental analyser at combustion furnace temperature 1075 ºC and afterburner temperature 850 °C. Nitrogen was detected using thermal conductivity. The protein content was estimated from the nitrogen determination using two nitrogen-protein conversion factors, 6.25 and 5 (Angell et al. 2016).

Total lipid extraction

Lyophilized samples were homogenized in a mortar and pestle until to obtain small-sized flakes. A biomass of 250 mg of macroalgae was mixed with 2.5 mL of CH_3OH and 1.25 mL of $CHCl_3$ in a glass PYREX tube and homogenized by vortexing for 2 min. After incubation in ice on rocking platform shaker (Stuart equipment, Bibby Scientific, Stone, UK) for 2.5 h, the mixture was centrifuged (Selecta JP Mixtasel, Abrera, Barcelona, Spain) for 10 min at 2000 rpm and the organic phase was collected in a new glass tube. The biomass residue was re-extracted twice with 2 mL of MeOH and 1 mL of $CHCl_3$. To wash the lipid extract and induce phase separation, 2.3 mL of Milli-Q water was added to the final organic phase, following by centrifugation for 10 min at 2000 rpm. The organic lower phase was collected in a new glass tube, dried under nitrogen stream. Lipid extracts were then transferred to amber vials, dried again, weighed, and stored at -20 ºC. Lipid content was estimated as dry weight percentage.

Fatty Acid analysis by Gas Chromatography-Mass Spectrometry (GC–MS)

Fatty acid methyl esters (FAMEs) were prepared using a methanolic solution of potassium hydroxide (2.0 M) (Melo et al. 2015). A volume of 2 µL of hexane solution containing FAMEs was analyzed by gas chromatography-mass spectrometry (GC–MS) on a GC system (Agilent Technologies 6890 N Network, Santa Clara, CA, USA) equipped with a DB-FFAP column with the following specifications: 30 m of length, 0.32 mm of internal diameter, and 0.25 µm of film thickness (J & W Scientific, Folsom, CA, USA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 50–550 in a 1s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C for 3 min, a linear increase to 160 °C

177 at 25 °C min^{-1}, followed by linear increase at 2 °C min^{-1} to 210 °C, then at 30 °C min^{-1}
178 to 250 °C, standing at 250 °C for 10 min. The injector and detector temperatures were
179 220 and 280 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.4 mL
180 min^{-1}. FA identification was performed considering the retention times and MS spectra
181 of FA standards (Supelco 37 Component Fame Mix, Sigma-Aldrich), and by MS
182 spectrum comparison with chemical databases (Wiley 275 library and AOCS lipid
183 library). The relative amounts of FAs were calculated by the percent area method with
184 proper normalization, considering the sum of all areas of identified FAs.
185
186 ***Lipid extract fractionation***
187 Isolation of polar lipids from pigments was performed using a modification of Pacetti's
188 method (da Costa et al. 2017). A sample of lipid extract (5 mg) was dissolved in 600 µL
189 of chloroform and transferred to a glass column with 500 mg of silica gel (40-60 µm, 60
190 A, Åcros Organics) followed by sequential elution with 5 mL of chloroform, 12 mL of
191 ether diethyl ether:acetic acid (98:2), 7 mL of acetone:methanol (9:1 v/v), and 10 mL of
192 methanol. Fractions 1 and 2, corresponding to neutral lipids and pigments, were
193 discarded. Fractions 3 and 4, rich in glycolipids and in phospholipids plus betaines,
194 respectively, were recovered, dried under nitrogen, and stored at −20 ◦C prior to analysis
195 by HILIC-ESI–MS.
196
197 ***Hydrophilic interaction liquid chromatography mass spectrometry (HILIC–ESI–MS)***
198 Lipid extracts and fraction were analyzed by hydrophilic interaction liquid chromatography
199 HILIC (Ascentis® Si column, 15 cm × 1 mm, 3 µm, Sigma-Aldrich) on a High-Performance LC
200 (HPLC) system (Thermo scientific AccelaTM) with a autosampler coupled online to a Q-
201 Exactive® mass spectrometer with Orbitrap® technology. Mobile phase A consisted of 25%
202 water, 50% acetonitrile and 25% methanol, with 1 mM ammonium acetate in relation to

the water volume, and mobile phase B consisted of 60% acetonitrile and 40% methanol, with the same amount of ammonium acetate in mobile phase A. The solvent gradient, flow rate through column and conditions used for acquisition of full scan LC-MS spectra and LC-MS/MS spectra in both positive and negative ion modes were the same as previously described (da Costa et al. 2015; Melo et al. 2015). Initially, 0% of mobile phase A was held isocratically for 8 min, followed by a linear increase to 60% of mobile phase A within 7 min and a maintenance period of 15 min, returning to the initial conditions in 10 min. A volume of 5 µL of each sample, containing 10 µg (10 µL) of lipid extract in $CHCl_3$, 4 µL of phospholipid standards mix (dMPC - 0.02 µg, dMPE - 0.02 µg, NPSM - 0.02 µg, LPC - 0.02 µg, dPPI - 0.08 µg, dMPG - 0.012 µg, dMPS - 0.04 µg) and 86 µL of eluent B, was introduced into the Ascentis Si column HPLC Pore column (15 cm × 1 mm, 3 µm, Sigma-Aldrich) with a flow rate of 40 µL min^{-1} at 30 ºC. The mass spectrometer with Orbitrap® technology was operated in simultaneous positive (electrospray voltage 3.0 kV) and negative (electrospray voltage -2.7 kV) modes with high resolution with 70,000 and AGC target of 1 x 10^6, the capillary temperature was 250 ºC, and the sheath gas flow was 15 U. In MS/MS experiments, a resolution of 17,500 and AGC target of 1 x 10^5 was used and the cycles consisted in one full scan mass spectrum and ten data-dependent MS/MS scans were repeated continuously throughout the experiments with the dynamic exclusion of 60 s and intensity threshold of 1 x 10^4. Normalized collision energy™ (CE) ranged between 25, 30 and 35 eV. Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). The identification of molecular species of polar lipids was based on the assignment of the molecular ions observed in LC–MS spectra, typical retention time, mass accuracy, and LC–MS/MS spectra interpretation that allows to confirm the identity of the polar head group and the fatty acyl chains for most of the molecular species.

Results

The total lipid content of the *U. rigida* was estimated by gravimetry of the lipid extracts. Also, samples were analyzed for the contents of moisture and ash, proteins, and carbohydrates and other compounds (estimated by difference). The mean moisture content (expressed as percentage of freeze-dried sample weight) of *U. rigida* was 6.41 ± 0.84, which was considered to express the content of ash and other components as percentage of dry weight (DW). The content (% DW) of ash and lipids was 26.47 ± 0.51 and 2.53 ± 0.22, respectively. Although the factor 6.25 is the most commonly used indirect nitrogen-to-protein conversion factor, studies have been shown that the protein content of seaweed is over-estimated by applying factor 6.25 (Hardouin et al. 2016). Angell et al. (2016) proposed the use of an universal nitrogen-to-protein conversion factor of 5 for determination of the protein content of seaweeds. Thus, both factors were used. Using factor 6.25 for protein estimation, the protein content (%DW) was 17.75 ± 0.492, and the content of carbohydrates and other compounds (% DW) was 53.25. Considering factor 5, the protein content decreased to 14.20 ± 0.393, while the content of carbohydrates and other compounds increased to 56.80.

The fatty acids (FAs) profile of *U. rigida* revealed the presence of saturated FAs (SFAs) such as 14:0, 16:0, 18:0 and 22:0, monounsaturated FAs (MUFAs) such as 16:1 and 18:1 and PUFAs such as 16:4, 18:3, 18:4, 20:4, 20:5 and 22:5, as detailed in Table 1. The FA profile showed 16:0 and 18:0 as the most abundant with relative abundance of 43.41% and 19.30%, respectively. It is also noteworthy the abundance of the PUFAs 16:4 (*n*-3) (3.76%), 18:3 (*n-3*) (4.45%), 18:4 (*n-3*) (8.82%) and 22:5 (*n-3*) (3.76%).

Polar lipid profile evaluated by HILIC–LC–MS and HILIC–LC–MS/MS allowed the identification at molecular level of glycolipids, betaine lipids and phospholipids in *U. rigida*. This lipidomic approach allowed the identification, in the case of glycolipids, the

acidic glycolipid sulfoquinovosyl diacylglycerol (SQDG) and it lyso form sulfoquinovosyl monoacylglycerol (SQMG), as well as the neutral glycolipid digalactosyldiacylglycerol (DGDG) and monogalactosyldiacylglycerol (MGDG). SQDGs and SQMGs were identified as negative $[M - H]^-$ ions in the LC–MS spectra. Overall, 20 molecular species of SQDG and 5 molecular species of SQMG (Table 2 and Fig. 1) were identified. The most abundant SQDG was assigned as SQDG (34:1) at *m/z* 819.5, identified as SQDG (18:1/16:0), while the most abundant SQMG was detected at *m/z* 555.3 and corresponded to SQMG (16:0) (Fig. 1). Typical fragmentation of SQMG and SQDG species observed in LC–MS/MS spectra as $[M - H]^-$ ions showed the product ion at *m/z* 225.0, corresponding to the anion of the sulfoquinovosyl polar head group that confirmed the presence of sulfoglycolipids, as seen in the LC–MS/MS spectra of SQMG at *m/z* 555.3 (Fig. 1-B) and SQDG at *m/z* 819.5 (Fig. 1-D). Furthermore, product ions corresponding to the neutral loss of fatty acyl chains as carboxylic acid (RCOOH) can be identified and confirm the composition of fatty acyl chains. SQMG species exhibit only one neutral loss of one fatty acid R_1COOH (El Baz et al. 2013; da Costa et al. 2015; Melo et al. 2015). LC–MS/MS spectrum of SQMG (16:0) at *m/z* 555.3 shows the neutral loss of palmitic acid (-16:0 R_1COOH, 256 Da) that lead to the formation of the product ion at *m/z* 299.0 (Fig. 1-B). LC-MS/MS spectrum at *m/z* 819.5, corresponding to SQDG (18:1/16:0), shows the loss of two fatty acyl chains R_1COOH and R_2COOH, that correspond to the neutral loss of 18:1 RC_1OOH (- 282 Da) and the neutral loss of palmitic acid 16:0 R_2COOH (- 256 Da) with formation of the product ions at *m/z* 537.3 and 563.3, respectively (Fig. 1-D).

The neutral molecular species monogalactosyldiacylglyceride (MGDG), digalactosyldiacylglyceride (DGDG) and their lyso forms, monogalactosylmonoacylglyceride (MGMG) and digalactosylmonoacylglyceride

278 (DGMG), were identified in the positive LC–MS spectra as $[M + NH_4]^+$ ions. Overall 27
279 molecular species of MGDG, 13 of MGMG, 13 of DGDG and 9 of DGMG were
280 identified (Table 3 and Fig. 2). The representative LC–MS spectra of MGDG and DGDG
281 classes are shown in Fig. 2, as well as the LC–MS/MS spectra of the most abundant
282 species of each class. The predominant MGDG were detected at *m/z* 760.5. The DGDG
283 were similarly predominate at *m/z* 932.6 and 936.7, representative spectrum in Fig. 2
284 concerns DGDG at *m/z* 932.6. The MGDG at *m/z* 760.5 corresponds to MGDG (34:8)
285 and was identified as MGDG (16:4/18:4), while the DGDG at *m/z* 932.6 refers to DGDG
286 (34:3) and was identified as DGDG (18:3/16:0). The typical fragmentation observed in
287 the LC–MS/MS spectra of MGDG and DGDG species as $[M + NH_4]^+$ ions allows to
288 confirm the presence of these neutral glycolipids. LC-MS/MS spectrum of MGDG (34:8)
289 at *m/z* 760.5 (Fig. 2-B) indicate the product ion at *m/z* 563.4, assigned as [M + NH4 –
290 197]$^+$, that results from combined loss of NH_3 (-17 Da) and loss of a hexose (-180 Da)
291 formed due to the cleavage of the sugar bond near the hemiacetal oxygen bond with
292 proton transfer to render a diacylglycerol structure. Similarly, in the LC-MS/MS spectrum
293 of DGDG (34:3) at *m/z* 932.6 (Fig. 2-D), we can observe the loss of the carbohydrate
294 moiety (loss of 180 + 162 Da) combined with loss of NH_3 (-17 Da), leading to the
295 formation of the product ion at *m/z* 573.5, indicated as $[M + NH_4 - 359]^+$. The fatty acyl
296 chains composition can be inferred by the presence of product ions corresponding to each
297 fatty acyl group as an acylium ion plus 74 (RCO + 74). These ions can be seen at *m/z*
298 305.2 and 333.2 in MGDG spectrum (Fig. 2-B) and correspond to 16:4 and 18:4,
299 respectively. In the case of DGDG spectrum (Fig. 2-D) the $[RCO + 74]^+$ ions can be seen
300 at *m/z* 313.3 and 335.3 and correspond to 16:0 and 18:3, respectively (Murphy 2015).
301 Betaine lipids identified in *U. rigida* included the diacylglyceroltrimethylhomoserine
302 (DGTS) and its lyso form monoacylglyceroltrimethylhomoserine (MGTS). The DGTS

303 and MGTS were identified in the LC–MS spectra as positive $[M + H]^+$ ions. Overall 40
304 molecular species of DGTS and 17 molecular species of MGTS were identified (Table 4
305 and Fig. 3). The structural features of betaine lipids were confirmed through the
306 identification of the typical product ions and fragmentation pathways observed in the LC–
307 MS/MS spectra. A representative LC–MS/MS spectrum of MGTS and DGTS is shown
308 in Fig. 3-B and Fig. 3-C, corresponding to the MGTS (18:4) at *m/z* 494.3 and DGTS
309 (34:4), identified as DGTS (18:4/16:0) at *m/z* 732.6. Both LC–MS/MS spectra of MGTS
310 (Fig. 3-B) and DGTS (Fig. 3-D) showed the typical reported ion of this class at *m/z* 236.1
311 corresponding to the combined loss of both fatty acids as keto derivatives (R_1CO+R_2CO)
312 (Melo et al. 2015; da Costa et al. 2018). The fatty acyl composition can be deducted by
313 the losses of fatty acyl chains as acid (-RCOOH) and ketene (-R=C=O) derivatives. The
314 ion at *m/z* 236.1 in LC–MS/MS spectrum of MGTS (18:4) (Fig. 3-B) also represents the
315 loss of 18:4 fatty acyl chain as keto derivative (-258 Da). In its turn, the LC–MS/MS
316 spectrum of DGTS (18:4/16:0) (Fig. 3-D) showed the ions at *m/z* 474.4 and 494.3
317 corresponding to the loss of fatty acyl chains as keto derivatives (-258 and -238 Da),
318 matching to 18:4 and 16:0 fatty acids. Moreover, the ion at *m/z* 456.4 confirmed the
319 presence of the fatty acid 18:4 since it corresponds to the loss of this fatty acyl chain as
320 an acid derivative (-276 Da).
321 PLs classes identified in *U. rigida* included phosphatidylglycerol (PG),
322 phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and
323 their lyso forms LPG, LPI, LPE and LPC. They were identified in negative mode as $[M
324 – H]^-$ ions. Overall 5 molecular species of LPG, 17 of PG, 6 of PI and 1 of LPI were
325 recognized (Table 5).
326 The LC-MS/MS spectra of PG (Fig. 4-A) and LPG species allowed to confirm their polar
327 head by the presence of the product ion at *m/z* 171.0, corresponding to $[C_3H_7O_2OPO_3H]$

328 $^{-}$. On the other hand, the polar head of PI (Fig. 4-B) and LPI is observed at *m/z* 241.0,
329 corresponding to an inositol-1,2-cyclic phosphate anion $(C_6H_{10}O_5PO_3]^{-}$. The carboxylate
330 anions R_1COO^{-} and R_2COO^{-} allowed the identification of fatty acyl chains (Murphy
331 2015).
332 LPE, PE, LPC and PC molecular species were identified in positive mode as $[M + H]^{+}$
333 ions. Overall, 7 molecular species of LPE, 3 of PE, 3 of LPC and 15 of PC were identified
334 (Table 6). Typical loss of 141 Da was noted in LC–MS/MS spectra of $[M+H]^{+}$ ions of
335 LPE and PE , while the acyl chains were identified in negative mode by the presence of
336 carboxylate $RCOO^{-}$ anions observed in the LC–MS/MS spectra of the respective $[M–H]^{-}$
337 ions. The LC–MS/MS spectra of $[M+H]^{+}$ ions of LPC and PC showed the typical product
338 ion of the polar head at *m/z* 184.0, while the carboxylate $RCOO^{-}$ anions that allowed the
339 identification of fatty acyl composition were observed in the LC–MS/MS spectra of the
340 respective $[M–CH_3COO]^{-}$ ions (Murphy 2015).
341
342 **Discussion**
343 To the best knowledge of the authors, the present study represents the first in depth
344 characterization of lipidomic signature of the green macroalgae *U. rigida*. *U. rigida*
345 screened in the present work was produced in a land-based IMTA system, with this culture
346 approach being considered as a sustainable and environmentally friendly approach to produce
347 seaweeds and provide high grade safe biomass. When compared to the harvesting of seaweeds
348 from the wild, this production system has as main the advantages the production of high biomass
349 loads under controlled and replicable conditions, a less variable biochemical profile that allows
350 product standardization, as well as the implementation of mandatory traceability protocols for
351 seaweeds and seaweed-based-products targeting premium markets (Ridler et al. 2007; Chopin et
352 al. 2012). Fatty acids profile identified was similar with that reported for the same species
353 (Ak et al. 2014) and for other species belonging to the genus *Ulva*, namely *Ulva lactuca*,

354 *Ulva rotundata*, *Ulva clathrata* and *Ulva intestinalis* (Fleurence et al. 1994; Peña-
355 rodríguez et al. 2011; van Ginneken et al. 2011; Rozentsvet and Nesterov 2012). As the
356 PUFAs reported in the present study are essential FAs for humans, the macroalgae *U.*
357 *rigida* can be an affordable dietary source of these FAs (Li et al. 2009; Cottin et al. 2011).
358 There are several studies that defend an ideal *n*-6/*n*-3 ratio. While *n*-3 PUFAs exhibit anti-
359 inflammatory and antioxidant activity, improve the cardiac system and prevent breast
360 cancer (Mozaffarian et al. 2005; Siriwardhana et al. 2012; Fabian et al. 2015), *n*-6 PUFAs
361 tend to promote tumor growth and inflammatory processes (Patterson et al. 2011). One
362 of the important dietary factor in the obesity prevention is a balanced *n*-6/*n*-3 ratio of 1-
363 2/1 (Simopoulos 2016). Therefore, the consumption of *n*-6 FAs should be lower than *n*-
364 3, in order to avoid several diseases including depressive disorder (Okuyama et al. 1997;
365 Husted and Bouzinova 2016). In addition, lower *n*-6/*n*-3 ratio was associated with
366 decreased risk of breast cancer in women (Simopoulos 2008). In this context, *U. rigida*
367 presented a relative abundance of *n*-6 and *n*-3 PUFAs of 1.51% and 21.77%, respectively.
368 Therefore, its *n*-6/*n*-3 ratio is lower than 1, highlighting the potential health promoting
369 properties of this macroalgae for human consumption. Although *n*-6/*n*-3 ratios are known
370 to vary between species and growth condition, to the authors best knowledge *U. rigida*
371 farmed using a sustainable land based IMTA approach described in the present study
372 displayed the lowest *n*-6/*n*-3 ratio report so far for *Ulva* spp. (van Ginneken et al. 2011;
373 Kendel et al. 2015). This finding confirms the added value of algal biomass originating
374 from land-based IMTA, as a higher contents in *n*-3 fatty acids are commonly associated
375 with health promoting benefits for consumers (Simopoulos 2002).

376 Identified FAs are esterified into lipid molecules such as glycolipids, betaine lipids and
377 phospholipids (PLs). The glycolipids detected include sulfolipids and galactolipids which

together represented the most abundant structural compounds of chloroplast membranes (Hölzl and Dörmann 2007) with up to 87 molecular species being identified in *U. rigida*.

There are several studies that demonstrated glycolipids bioactivity from different algae species, such as antiviral, antibacterial and antitumoral activity (Plouguerné et al. 2014; Blunt et al. 2016). Wang et al. (2007) described the antiviral activity attributed to SQDG (32:0) from the green macroalgae *Caulerpa racemosa* (Forsskål) J.Agardh, (1873). Furthermore, Baz et al. (2013) analyzed the SQMG (16:0) as antitumoral and antimicrobial activity Other authors demonstrated the inhibitory effect of SQDG and DGDG from the brown macroalgae *Sargassum horneri* (Turner) C.Agardh (1820) suggesting the use of these compounds like chemotherapy agents (Hossain et al. 2005). It is also reported that seaweeds with an abundant presence of PUFAs in their composition proved to display anti-inflammatory activity by inhibiting nitric oxide release by macrophages (Banskota et al. 2013; Lopes et al. 2014). Betaine lipids (DGTS and MGTS) represent a group of polar lipids low studied to date and few studies have characterized their profile in seaweeds (da Costa et al. 2015, 2017; Melo et al. 2015). Some species of DGTS identified in *U. rigida* have already been reported in green microalgae like *Chlamydomonas reinhardtii* P.A.Dangeard (1888) and Chlorarachniophytes (Vieler et al. 2007; Roche and Leblond 2010). It has been suggested that DGTS has the same function as PC due to their similar zwitterionic structure. Moreover, they are interchangeable with each other in their roles within the cell (Riekhof et al. 2005). Organisms that contain a high level of DGTS display either an absence of PC or its presence is very low (Dembitsky and Rezanka 1995; Kunzler and Eichenberger 1997). Furthermore, Ginneken et al. (2017) revealed that *Ulva* sp. uses a mechanism rarely reported in euckaryotes, as it applies the biochemical pathway to produce DGTS that can replace PC in seaweed cell

wall (Klug and Benning 2001). It was suggested that the high DGTS/PC ratio occur communlyin in species of the genus *Ulva*.

Regarding PLs, their beneficial effects have been studied since the early 1900s (Küllenberg de Gaudry et al. 2012). The positive effect of PLs is supported by several studies that showed an improvement of the pharmacokinetics of some drugs when associated with PLs compounds, and a reduction of side effects of some drugs when administered together, namely indomethacin (NSAID) (Dial et al. 2006; Lichtenberger et al. 2009). Their cytoprotectively effects and anti-fibrogenic potential have already been highlighted (Gundermann et al. 2011). Moreover, PLs from marine organisms have shown a remarkable effect in the regulation of the blood lipid profile in patients suffering from hyperlipidaemia (Bunea et al. 2004). PLs beneficial dietary effect is the result of their interaction with cellular membranes influencing a vast number of signaling processes and also the effect of their fatty acid composition. The great advantage of these molecules is related with the ability of their esterified *n*-3 FAs to compensate *n*-3 FA deficiency in a more efficient way than other *n*-3 FA supplements (e.g. as triacylglycerides or as free FAs). Thus, PLs from foodstuff are major supplies of *n*-3 PUFAs for living systems (Jannace et al. 1992). Furthermore, the antioxidant potential of PG found in *U. rigida* could be explored (Banskota et al. 2014).

Traditionally the study of algal lipids has targeted fatty acids analysis through GC-MS or GC-FID (Marshall et al. 2002). However, the overall information acquired through these techniques is limited and solely refers to fatty acids, which in living systems are mostly linked to polar lipids. In the last decade, with the advent of mass spectrometry, the commercialization of new devices with higher sensitivity, resolution and sample screening speed, such as Orbitrap ant Q-TOF instruments, allowed to gain a more in depth knowledge of lipids. The used of liquid chromatography (LC) online with mass

spectrometry is nowadays an advanced and promising approach to study lipids in living systems. The LC-MS platforms allows to identify and quantify molecular structural details in one single run over very short periods of time (Maciel et al. 2016). In one LC-MS run, more than two hundred lipid species from different lipid classes are routinely identified and quantified. Lipid species identification is based on the ions in MS and, in the case of high-resolution MS, through confirmation of mass accuracy. The structural details are confirmed by MS/MS data of each molecular species, namely through the analysis of typical ion fragments. In recent years, this lipidomic approach has been successfully used to unravel the lipidome of seaweeds (da Costa et al. 2015, 2017, 2018; Melo et al. 2015) and has become a powerful tool to screen for high value lipid species with potential biotechnological applications.

Conclusion

The mass spectrometry–based approach employed in the present study allowed the identification of 202 molecular species of polar lipids shared between glycolipids, betaine lipids and phospholipids, most of them confirmed by their fatty acids composition. The knowledge of lipid composition of *U. rigida* from a sustainable land-based IMTA system, comes to inspire future studies of valorization of this seaweed, as its aquaculture production under controlled conditions will continue to increase as it offers consumers a safer and more standardized product, from an organoleptically (industry communication) and biochemical point of view. Moreover, the present study may also serve to stimulate the consumption of *U. rigida* produced under controlled conditions, as its lipidome displays a number of molecular species with beneficial bioactive properties that may also foster new biotechnological applications.

Acknowledgements

The authors are grateful to ALGAplus- Produção e Comércio de algas e seus derivados, Lda. for supplying the seaweed samples. Thanks are due to Fundação para a Ciência e a Tecnologia (FCT, Portugal), European Union, QREN, POPH, FEDER and COMPETE for funding the QOPNA research unit (FCT UID/QUI/00062/2013), to RNEM (LISBOA-01-0145-FEDER-402-022125) for the Portuguese Mass Spectrometry Network, to CESAM (UID/AMB/50017/2013) financed by Portuguese funds through the FCT/MEC and when applicable co-financed by FEDER under the PT2020 Partnership Agreement. Thanks are also due to FCT for the grants of Diana Lopes (SFRH/BD/119027/2016) and Felisa Rey (SFRH/BPD/115347/2016). Ana S.P. Moreira (BPD/UI51/5041/2017) and Elisabete da Costa (BPD/UI51/5042/2018) are grateful for the grants within framework of the project GENIALG – Genetic diversity exploitation for innovative macro-alga biorefinery (ANR-15-MRSE-0015) funded by European Union's Horizon 2020 Framework Programme. Tânia Melo is grateful for her Post-Doc grant (BPD/UI 51/5388/2017) funded by RNEM. This work is a contribution of the Marine Lipidomics Laboratory and was also supported by the Integrated Programme of SR&TD "Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate" (Centro-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund.

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Captions

Figure 1. LC–MS spectra in negative ion mode of SQMG (A) and SQDG (C) classes identified as $[M - H]^-$ ions. LC–MS/MS spectra of the $[M - H]^-$ ions of the most abundant species of SQMG at *m/z* 555.3 (B) and SQDG at *m/z* 819.5 (D).

Figure 2. LC–MS spectra in positive ion mode of MGDG (A) and DGDG (C) classes identified as $[M + NH_4]^+$. LC-MS/MS spectra of the $[M + NH_4]^+$ ions of the most abundant specie of MGDG at *m/z* 760.6 (B) and DGDG at *m/z* 932.5 (D). The ions group assigned with symbol (*) are a background.

Figure 3. LC–MS spectra in positive mode of MGTS (A) and DGTS (C) classes identified as $[M + H]^+$ ions. LC–MS/MS spectra of the $[M + H]^+$ ions of the most abundant specie of MGTS at *m/z* 494.3 (B) and DGTS at *m/z* 732.6 (C).

Figure 4. LC–MS/MS spectrum in negative mode of PG (34:4) specie at *m/z* 741.5 (A) and PI (34:3) specie at *m/z* 831.5 (B) identified as $[M–H]^-$ ions.

Table 1. Fatty acid profile of *U. rigida* sustainably produced under IMTA conditions, expressed as relative abundance (%). Values are means of seven samples ± standard deviation (SD).

Table 2. Molecular species of SQDGs and SQMGs identified by HILIC–ESI–MS as negative $[M – H]^-$ ions. Identification as sulfoglycolipids and fatty acyl composition was confirmed by the analysis of the LC–MS/MS spectra of each $[M – H]^-$ ion. C represents

727 the total number of carbon atoms and N the total number of double bonds on the fatty
728 acyl chains. The most abundant species are marked in bold.
729
730 **Table 3.** Molecular species of MGDG, MGMG, DGDG and DGMG identified by
731 HILIC–ESI–MS as positive $[M + NH_4]^+$ ions. Identification as galactoglycerolipids and
732 fatty acyl composition was confirmed by the analysis of the LC– MS/MS spectra of each
733 $[M + NH_4]^-$ ion. C represents the total number of carbon atoms and N the total number of
734 double bonds on the fatty acyl chains. The most abundant species are marked in bold.
735
736 **Table 4**. Molecular species of DGTS and MGTS identified by HILIC–ESI–MS as
737 positive $[M + H]^+$ ions. Identification as betaines and fatty acyl composition was
738 confirmed by the analysis of the LC–MS/MS spectra of each $[M + H]^+$ ion. C represents
739 the total number of carbon atoms and N the total number of double bonds on the fatty
740 acyl chains. The most abundant species are marked in bold.
741
742 **Table 5.** Molecular species of LPG, PG, LPI, PI identified by HILIC–ESI–MS as negative
743 $[M – H]^–$ ions. Identification of different PL classes and fatty acyl composition was
744 confirmed by the analysis of the LC–MS/MS spectra of each $[M – H]^–$ ion. C represents
745 the total number of carbon atoms and N the total number of double bonds on the fatty
746 acyl chains. The most abundant species are marked in bold.
747
748 **Table 6.** Molecular species of LPE, PE, LPC and PC identified by HILIC–ESI–MS as
749 positive $[M + H]^+$ ions. Identification of PL class was confirmed by the analysis of the
750 LC–MS/MS spectra of each $[M + H]^+$ ion. Identification of fatty acyl composition was
751 performed by the analysis of the LC–MS/MS spectra of respective $[M–H]^–$ ions for LPE

752 and PE and $[M–CH_3COO]^-$ ions for LPC and PC, if observed. C represents the total
753 number of carbon atoms and N the total number of double bonds on the fatty acyl chains.
754

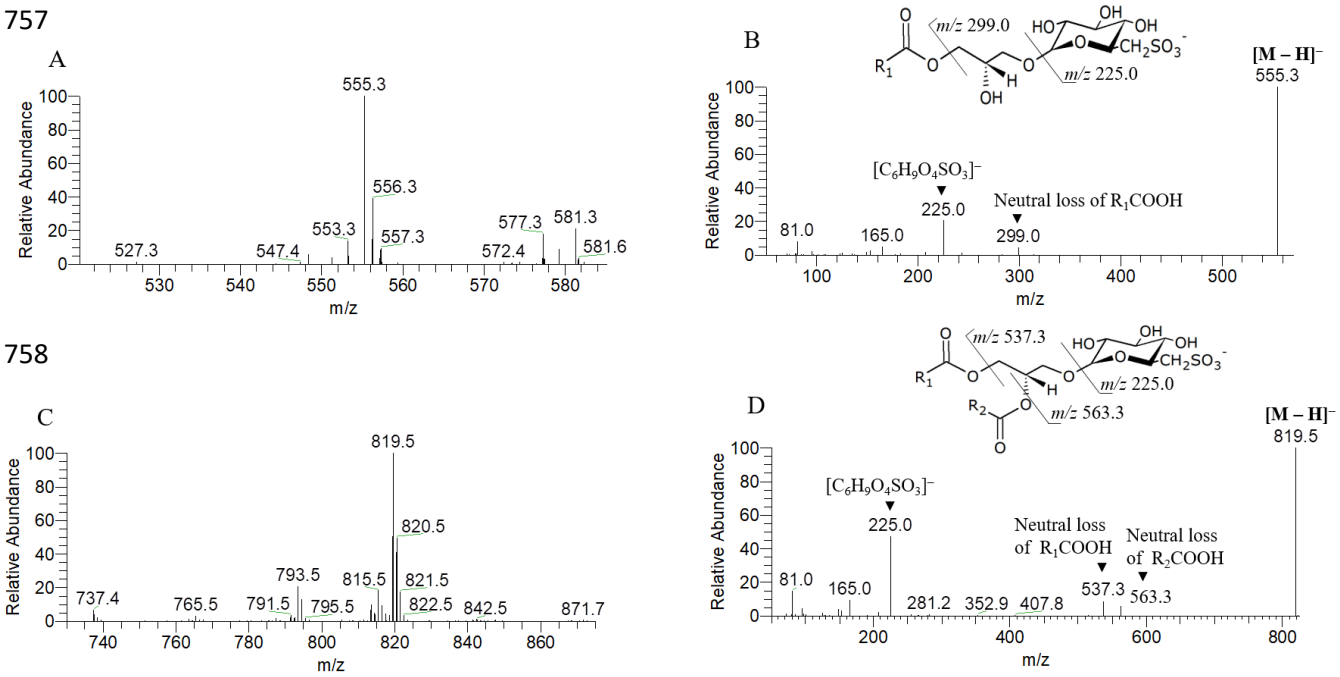
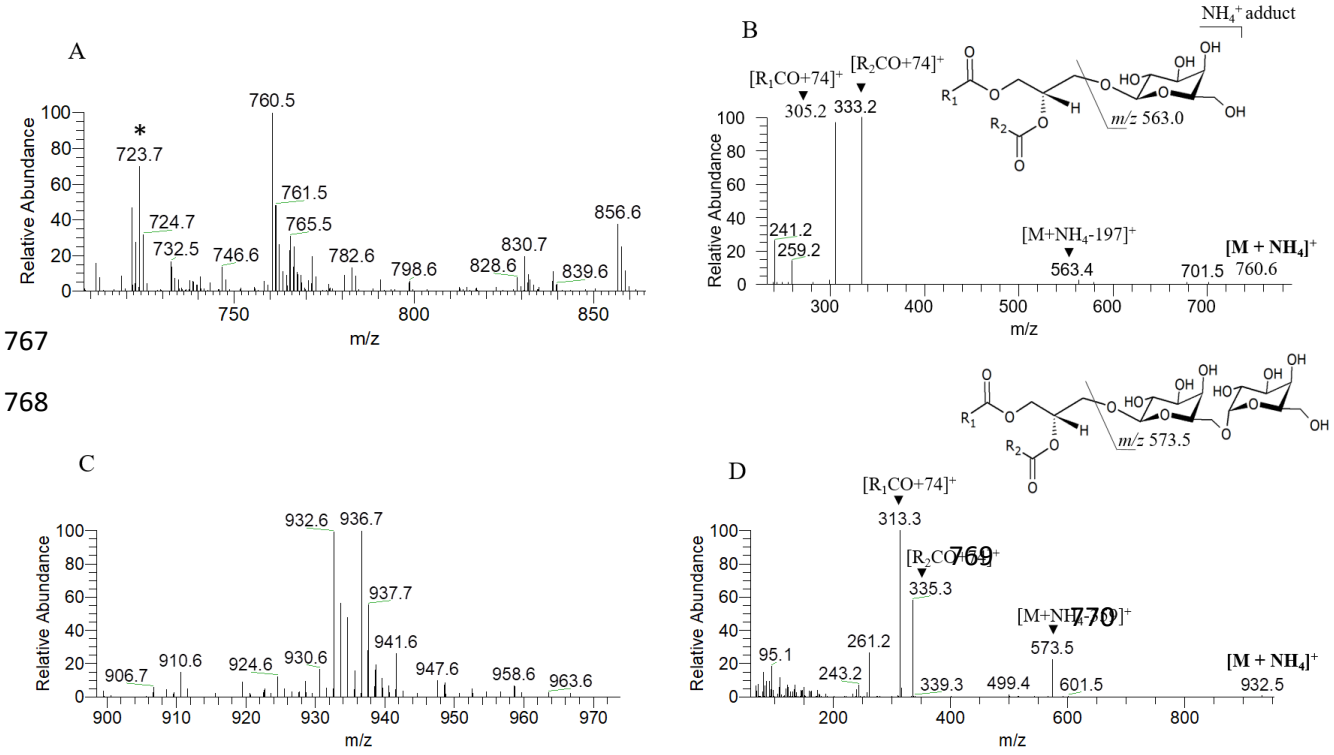

A
Relative Abundance
555.3
556.3
553.3
557.3
577.3
581.3
581.6
527.3
547.4
572.4
m/z
B
m/z 299.0
m/z 225.0
[M – H]⁻
555.3
[C6H9O4SO3]⁻
225.0
Neutral loss of R1COOH
299.0
81.0
165.0
Relative Abundance
m/z
C
819.5
820.5
793.5
815.5
821.5
737.4
765.5
791.5
795.5
822.5
842.5
871.7
Relative Abundance
m/z
D
m/z 537.3
m/z 225.0
m/z 563.3
[M – H]⁻
819.5
[C6H9O4SO3]⁻
225.0
Neutral loss of R1COOH
Neutral loss of R2COOH
537.3
563.3
81.0
165.0
281.2
352.9
407.8
Relative Abundance
m/z

Figure 1.

766



767

768

771

772

773 **Figure 2.**

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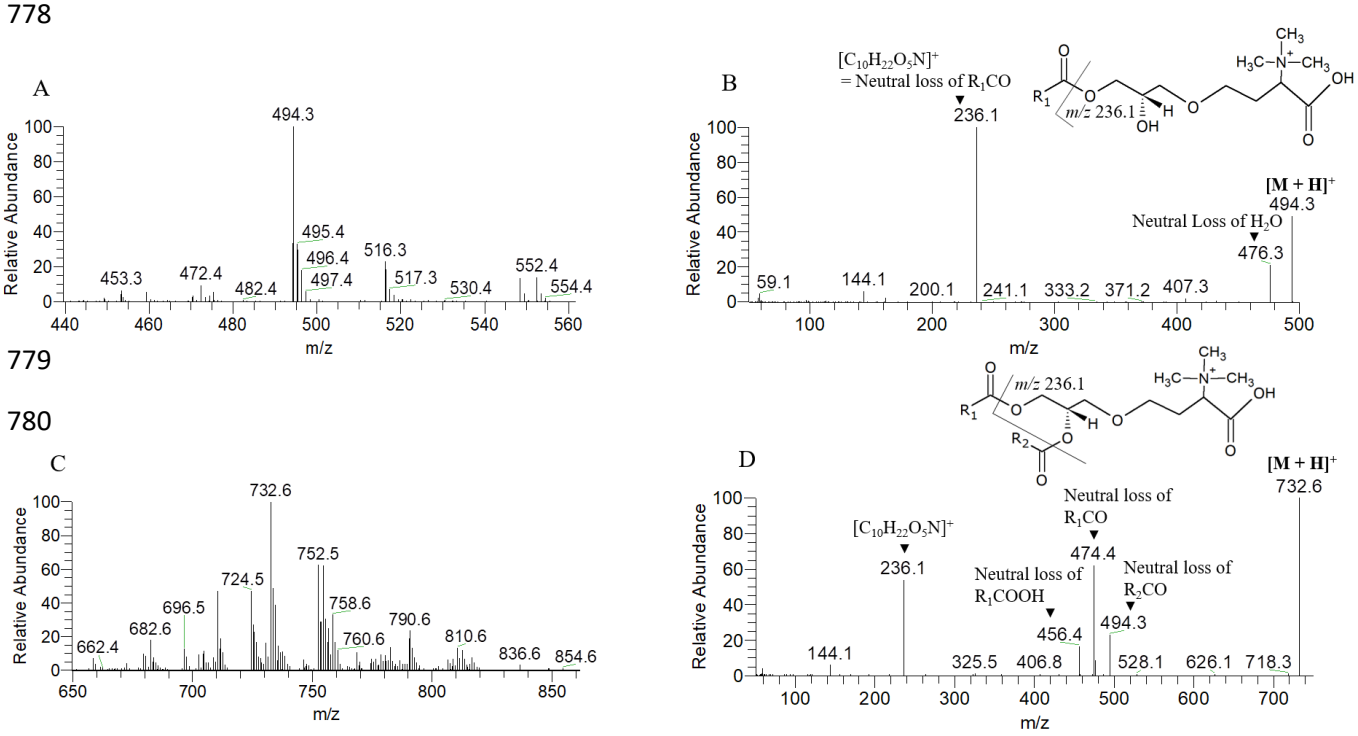
A
494.3
495.4
496.4
497.4
516.3
517.3
453.3
472.4
482.4
530.4
552.4
554.4
Relative Abundance
m/z
B
[C10H22O5N]+
= Neutral loss of R1CO
236.1
m/z 236.1
[M + H]+
494.3
Neutral Loss of H2O
476.3
59.1
144.1
200.1
241.1
333.2
371.2
407.3
C
732.6
752.5
724.5
696.5
682.6
758.6
790.6
760.6
810.6
662.4
836.6
854.6
D
Neutral loss of
R1CO
474.4
Neutral loss of
R1COOH
456.4
Neutral loss of
R2CO
494.3
144.1
325.5
406.8
528.1
626.1
718.3

Figure 3.

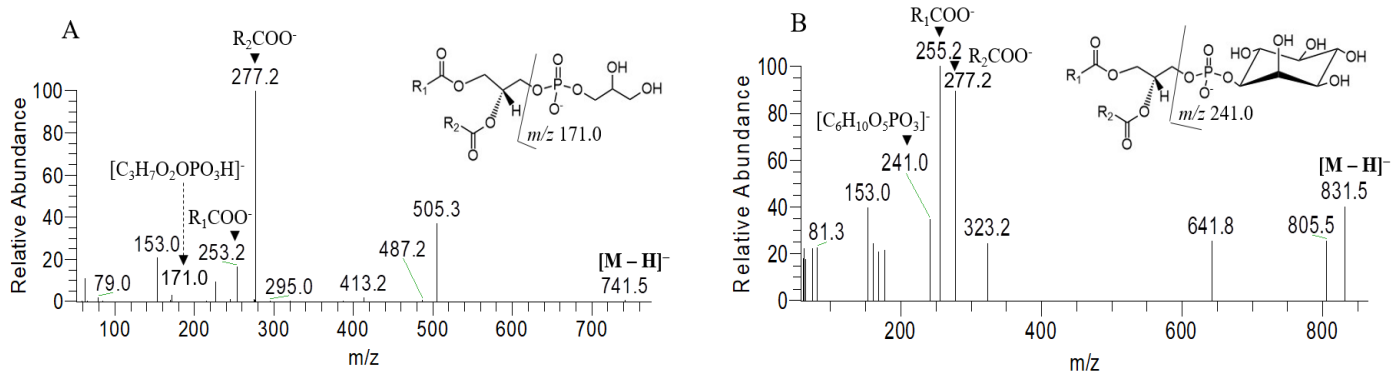

Figure 4.

Table 1.

Fatty acids	Relative abundance (%) ± SD
14:0	1.33 ± 0.21
16:0	43.41 ± 0.75
16:1 (*n*-7)	1.39 ± 0.12
16:1 (*n*-9)	1.76 ± 0.16
16:4 (*n*-3)	3.76 ± 0.17
18:0	19.30 ± 1.64
18:1	8.56 ± 1.21
18:2 (*n*-6)	1.21 ± 0.10
18:3 (*n*-6)	0.29 ± 0.04
18:3 (*n*-3)	4.45 ± 0.22
18:4 (*n*-3)	8.82 ± 0.40
20:4 (*n*-3)	0.65 ± 0.06
20:5 (*n*-3)	0.84 ± 0.10
22:0	0.46 ± 0.08
22:5 (*n*-3)	3.76 ± 0.54
Σ SFAs	64.50 ± 2.10
Σ MUFAs	11.71 ± 0.78
Σ PUFAs	23.78 ± 1.33
Σ (*n*-3)	22.28 ± 1.22
Σ (*n*-6)	1.50± 0.13

Table 2.

[M – H]⁻ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains	[M – H]⁻ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains
527.3	SQMG (14:0) [a]		805.5	SQDG (33:1)	17:1/16:0
553.3	SQMG (16:1)	16:1	807.5 [a]	SQDG (34:7)	
555.3	**SQMG (16:0)**	16:0	811.4	SQDG (34:5)	20:5/14:0
577.3	SQMG (18:3)	18:3	813.5	SQDG (34:4)	18:4/16:0
581.3	SQMG (18:1)	18:1	815.5	SQDG (34:3)	18:3/16:0
737.5	SQDG (28:0)	14:0/14:0 and 12:0/16:0	**819.5**	**SQDG (34:1)**	18:1/16:0
763.5	SQDG (30:1)	14:0/16:1	839.5	SQDG (36:5)	20:5/16:0
765.5	SQDG (30:0)	14:0/16:0	841.5	SQDG (36:4)	20:4/16:0 and 18:1/18:3
785.5	SQDG (32:4)	16:4/16:0 and 14:0/18:4	843.5	SQDG (36:3)	20:3/16:0
787.5	SQDG (32:3)	14:0/18:3 and 16:3/16:0	845.5	SQDG (36:2) [b]	
789.5	SQDG (32:2)	18:2/14:0	847.5	SQDG (36:1)	20:1/16:0 and 18:0/18:1
791.5	SQDG (32:1)	16:1/16:0 and 18:1/14:0	867.5	SQDG (38:5)	22:5/16:0
793.5	SQDG (32:0)	16:0/16:0			

[a] Molecular specie identified only by retention time and mass accuracy calculation.

[b] Molecular species identified only by retention time, mass accuracy calculation and typical product ion at *m/z* 225.0.

Table 3.

$[M+NH_4]^+$ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains	$[M+NH_4]^+$ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains
502.3	MGMG (16:4)	16:4	800.6	MGDG (36:2) [a]	
504.3	MGMG (16:3)	16:3	792.5	MGDG (36:6)	18:3/18:3 and 18:4/18:2
506.3	MGMG (16:2)	16:2	796.6	MGDG (36:4) [a]	
508.3	MGMG (16:1)	16:1	826.6	MGDG (38:3) [a]	
510.4	MGMG (16:0) [a]		828.7	MGDG (38:2) [a]	
530.3	MGMG (18:4) [a]		830.7	MGDG (38:1) [a]	
532.4	MGMG (18:3) [a]		854.7	MGDG (40:3) [a]	
534.4	MGMG (18:2) [a]		856.7	MGDG (40:2) [a]	
536.4	MGMG (18:1) [a]		858.7	MGDG (40:1) [a]	
556.4	MGMG (20:5) [a]		644.4	DGMG (14:0) [a]	
558.4	MGMG (20:4) [a]		664.4	DGMG (16:4) [a]	
584.4	MGMG (22:5) [a]		666.4	DGMG (16:3) [a]	
592.4	MGMG (22:1) [a]		668.4	DGMG (16:2) [a]	
712.5	MGDG (30:4) [a]		670.4	DGMG (16:1)	16:1
714.4	MGDG (30:3) [a]		672.4	DGMG (16:0)	16:0
732.5	MGDG (32:8)	16:4/16:4	692.4	DGMG (18:4) [a]	
734.5	MGDG (32:7)	16:3/16:4	694.4	DGMG (18:3) [a]	
736.5	MGDG (32:6)	16:2/16:4 and 16:3/16:3	746.4	DGMG (22:5) [a]	
738.5	MGDG (32:5) [a]		894.5	DGDG (32:8) [a]	
740.5	MGDG (32:4)	16:4/16:0 and 16:1/16:3	908.6	DGDG (32:1)	16:1/16:0 and 18:1/14:0
742.5	MGDG (32:3)	16:3/16:0 and 18:3/14:0	910.6	DGDG (32:0)	16:0/16:0
748.6	MGDG (32:0)	16:0/16:0	922.6	DGDG (34:8) [a]	
760.5	**MGDG (34:8)**	18:4/16:4	924.6	DGDG (34:7) [a]	
764.5	MGDG (34:6) [a]		926.6	DGDG (34:6) [a]	
766.6	MGDG (34:5)	18:1/16:4	928.6	DGDG (34:5) [a]	
768.6	MGDG (34:4)	18:4/16:0 and 18:3/16:1	930.6	DGDG (34:4) [a]	
770.6	MGDG (34:3)	18:3/16:0 and 18:2/16:1	**932.6**	**DGDG (34:3)**	18:3/16:0
774.6	MGDG (34:1)	18:1/16:0	934.6	DGDG (34:2)	18:2/16:0
786.5	MGDG (36:9) [a]		**936.7**	**DGDG (34:1)**	18:1/16:0
788.5	MGDG (36:8)	18:4/18:4 and 20:5/16:3	956.6	DGDG (36:5) [a]	
790.5	MGDG (36:7)	18:4/18:3 and 20:3/16:4	958.6	DGDG (36:4)	18:3/18:1

[a] Molecular species identified only by retention time and mass accuracy calculation.

Table 4.

[M+H]$^+$ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains	[M+H]$^+$ *m/z*	Lipid Species (C:N)	Fatty Acyl Chains
446.3	MGTS (14:0)	14:0	724.6	DGTS (34:8)	16:4/18:4
464.3	MGTS (16:3)	16:3	726.6	DGTS (34:7)	16:4/18:3
466.3	MGTS (16:4)	16:4	728.5	DGTS (34:6)	16:2/18:4
470.3	MGTS (16:2)	16:2	730.6	DGTS (34:5)	16:1/18:4 and 16:2/18:3
472.4	MGTS (16:1)	16:1	**732.6**	**DGTS (34:4)**	16:0/18:4
474.4	MGTS (16:0)	16:0	734.6*	DGTS (34:3)	16:0/18:3
492.3*	MGTS (18:5)	18:5	736.6	DGTS (34:2)	16:0/18:2 and 16:1/18:1
494.3	**MGTS (18:4)**	18:4	738.6	DGTS (34:1)	16:0/18:1
496.4	MGTS (18:3)	18:3	746.6	DGTS (35:4)	17:0/18:4
498.4	MGTS (18:2)	18:2	750.6	DGTS (36:9)	18:4/18:5
500.4	MGTS (18:1)	18:1	752.5	DGTS (36:8)	18:4/18:4
502.4	MGTS (18:0)	18:0	754.6*	DGTS (36:7)	18:3/18:4
520.4	MGTS (20:5)	20:5	756.6*	DGTS (36:6)	18:3/18:3 and 18:2/18:4
522.4	MGTS (20:4)	20:4	758.6*	DGTS (36:5)	18:1/18:4
524.4	MGTS (20:3)	20:3	760.6	DGTS (36:4) [a]	
530.4	MGTS (20:0)	20:0	762.6	DGTS (36:3)	18:1/18:2
548.4	MGTS (22:5)	22:5	764.6	DGTS (36:2)	18:1/18:1
558.5	MGTS (22:0)	22:0	776.6	DGTS (38:10) [a]	
656.5	DGTS (28:0)	14:0/14:0	778.6	DGTS (38:9)	16:4/22:5 and 20:5/18:4
676.5	DGTS (30:4) [a]		780.6*	DGTS (38:8)	20:4/18:4
682.6	DGTS (30:1)	14:0/16:1	782.6*	DGTS (38:7)	20:4/18:3 and 20:3/18:4
684.6	DGTS (30:0)	16:0/14:0	784.6	DGTS (38:6)	20:2/18:4 and 16:1/22:5
700.6	DGTS (32:6)	16:2/16:4	786.6	DGTS (38:5)	16:0/22:5
702.6	DGTS (32:5) [a]		808.6*	DGTS (40:8)	22:5/18:3
704.5	DGTS (32:4)	14:0/18:4	812.6	DGTS (40:6)	22:5/18:1
706.6	DGTS (32:3)	16:1/16:2	816.7	DGTS (40:4)	22:0/18:4
708.6	DGTS (32:2)	16:0 /16:2 and 16:1/16:1	830.6	DGTS (42:11) [a]	
710.6	DGTS (32:1)	16:0/16:1 and 14:0/18:1	832.6	DGTS (42:10)	22:5/20:5
712.6	DGTS (32:0)	16:0/16:0	860.6	DGTS (44:10)	22:5/22:5

[a] Molecular species identified only by retention time and mass accuracy calculation.

* Ion with contribution of sodium adduct $[M + Na]^+$ of DGTS observed as $[M + H]^+$ with mass difference of 22 Da.

Table 5.

[M – H]⁻ *m/z*	**Lipid Species (C:N)**	**Fatty Acyl Chains**	**[M – H]⁻ *m/z***	**Lipid Species (C:N)**	**Fatty Acyl Chains**
481.3	LPG (16:1)	16:1	747.5	PG (34:1)	18:1/16:0 and 16:1/18:0
483.3	LPG (16:0)	16:0	749.5	PG (34:0)	18:0/16:0
505.3	LPG (18:3) [a]		765.5	PG (36:6)	16:1/20:5
507.3	LPG (18:2) [a]		767.5	PG (36:5)	20:5/16:0 and 18:1/18:4
509.3	LPG (18:1)	18:1	769.5	PG (36:4)	18:1/18:3 and 18:2/18:2
691.5	PG (30:1)	14:0/ 16:1	771.5	PG (36:3)	18:1/18:2
693.5	PG (30:0)	14:0/16:0	773.5	PG (36:2)	18:1/18:1
711.5	PG (32:5)	16:1/16:4	571.3	LPI (16:0)	16:0
713.5	PG (32:4)	16:0/16:4 and 16:1/16:3	781.5	PI (30:0)	14:0/16:0
717.5	PG (32:2)	16:1/16:1	829.5	PI (34:4)	16:0/18:4
719.5	PG (32:1)	16:1/16:0 and 14:0/18:1	**831.5**	**PI (34:3)**	16:0/18:3
739.5	PG (34:5)	16:1/18:4	833.5	PI (34:2)	16:0/18:2
741.5	**PG (34:4)**	16:1/18:3	835.5	PI (34:1)	16:0/18:1
743.5	PG (34:3)	18:3/16:0 and 16:1/18:2	873.5	PI (38:10) [a]	
745.4	PG (34:2)	16:1/18:1 and 18:2/16:0			

[a] Molecular species identified only by retention time and mass accuracy calculation.

Table 6.

926

$[M + H]^+$ ***m/z***	**Lipid Species (C:N)**	**Fatty Acyl Chains**	**$[M + H]^+$** ***m/z***	**Lipid Species (C:N)**	**Fatty Acyl Chains**
496.3	LPC (16:0) [a]		806.6	PC (38:6) [b]	
542.3	LPC (20:5) [a]		808.6	PC (38:5) [a]	
568.3	LPC (22:6) [a]		828.6	PC (40:9) [a]	
706.5	PC (30:0) [a]		830.6	PC (40:8) [b]	
728.5	PC (32:3) [a]		452.3	LPE (16:1) [a]	
730.5	PC (32:2)	16:1/16:1	454.3	LPE (16:0)	16:0
754.6	PC (34:4) [a]		478.3	LPE (18:2)	18:2
756.6	PC (34:3) [b]		480.3	LPE (18:1) [a]	
758.6	PC (34:2)	16:1/18:1	500.3	LPE (20:5)	20:5
760.6	PC (34:1)	16:0/18:1	502.3	LPE (20:4)	20:4
780.6	PC (36:5) [b]		528.3	LPE (22:5)	22:5
784.6	PC (36:3)	18:1/18:2	688.5	PE (32:2) [c]	
786.6	**PC (36:2)**		690.5	PE (32:1) [c]	
804.6	PC (38:7) [b]		**716.5**	**PE (34:2)**	16:1/18:1 and 16:0/18:2

927 [a] Molecular species identified only by retention time and mass accuracy calculation.

928 [b] Molecular species of PC identified by retention time, mass accuracy calculation and typical product ion

929 observed at *m/z* 184 in the LC-MS/MS spectrum of $[M + H]^+$ ion.

930 [c] Molecular species of PE identified by retention time, mass accuracy calculation and typical neutral loss

931 of 141 in the LC-MS/MS spectrum of $[M + H]^+$ ion.

932