Development of new green processes for the recovery of bioactives from *Phaeodactylum tricornutum*

Bienvenida Gilbert-López, Almudena Barranco, Miguel Herrero, Alejandro Cifuentes, Elena Ibáñez*

Foodomics Laboratory, Bioactivity and Food Analysis Department, Institute of Food Science Research CIAL (UAM-CSIC), Campus de Cantoblanco, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

(*Food Research International, Special issue on "Microalgae and seaweeds as potential source of valuable nutrients, food additives and nutraceuticals for human and animal consumption"*)

*Corresponding author
Laboratory of Foodomics, Bioactivity and Food Analysis Department
Institute of Food Science Research, CIAL (UAM-CSIC)
C/ Nicolás Cabrera 9, Campus UAM Cantoblanco
28049 Madrid, Spain
e-mail: elena.ibanez@csic.es

Abstract

Two novel extraction techniques, pressurized liquid extraction (PLE) and microwave-assisted solvent extraction (MAE) have been evaluated for the recovery of bioactive compounds from *Phaeodactylum tricornutum*. Microalga *P. tricornutum* is rich in polyunsaturated fatty acids (PUFAs), such as EPA, and has a high content of the carotenoid fucoxanthin, which is a valuable pigment with several biological activities. Cells were disrupted at high pressure and then freeze-dried as a previous step to extraction. Additionally, only green solvents as water and ethanol (EtOH) -and mixtures of them- were used. For comparison purposes, the same response variables were considered in both processes: extraction yield (% w/w), total phenolic content (Folin-Ciocalteu assay), total carotenoids and chlorophylls, and antioxidant activity (ABTS assay, expressed as TEAC value). Factorial experimental designs were employed for both PLE and MAE optimization, being %EtOH in water and temperature the common experimental factors. Extraction time was also a factor considered for optimization in MAE. A detailed chemical characterization of pigments was performed by HPLC-DAD-MS/MS (high performance liquid chromatography-diode array detector-mass spectrometry), being fucoxanthin the main compound extracted. Optimum extraction conditions were 50 ºC, 100% EtOH, 20 min for PLE, while optimum conditions for MAE were 30 ºC, 100% EtOH and 2 min. Both technologies extract fucoxanthin as a main compound but higher recoveries were achieved using PLE due to a higher extraction yield. In addition, both MAE and PLE extracts contained different lipid classes potentially enriched in EPA, given an added-value to the extracts.

Keywords

Carotenoids, microalga, green extraction, response surface methodology, *Phaeodactylum tricornutum*, pressurized liquid extraction, microwave-assisted solvent extraction

Chemical compounds studied in this article

E-Fucoxanthin (PubChem CID: 5281239)

13Z-Fucoxanthin (PubChem CID: 102200897)

13'Z-Fucoxanthin (PubChem CID: 102200898)

1. Introduction

Microalgae are a rich source of bioactive compounds that have been underexploited, especially those from marine origin [Guedes et al., 2011]. *Phaeodactylum tricornutum* is a marine pennate diatom, unicellular brown microalgae. *P. tricornutum* cells can undergo morphological transitions stimulated by environmental conditions between three possible morphotypes (fusiform, triradiate, and oval) [De Martino et al., 2007]. Microalga *P. tricornutum* is typically rich in polyunsaturated fatty acids (PUFAs), such as EPA (eicosapentaenoic acid), and has a high content of the carotenoid fucoxanthin, which is a valuable pigment with several biological activities [Kim et al., 2011]. Considering its interesting biological activities, commercial production of fucoxanthin from *P. tricornutum* has been explored [Kim et al., 2012]. Nevertheless, its recovery entails the previous recovery of biomass and further downstream processing. Because most metabolites are located intracellularly, the disruption of cells is required as a previous step to improve the extraction with an appropriate solvent.

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), has an interesting potential for extracting bioactive compounds from macro- and microalgae [Herrero et al., 2015; Reyes et al., 2014]. The use of high pressure allows the solvents to maintain their liquid state at very high temperatures, above their boiling point. The use of these conditions accelerates the extraction by increasing the mass transfer rate of the analytes from the sample to the solvent. Moreover, the increased temperature reduces the viscosity and surface tension of solvents, promoting a better penetration into the matrix, thus, also improving the extraction rate [Mustafa & Turner, 2011]. This extraction technique allows obtaining higher yields than those achieved by conventional extraction

77 techniques, in a shorter time and with less solvent consumption. PLE using ethanol has
78 been reported for the extraction of carotenoids from *Neochloris oleoabundans* [Castro-
79 Puyana et al., 2013], *Dunaliella salina* [Herrero et al., 2006] and *Chlorella ellipsoidea*
80 [Koo et al., 2011]. Additionally, 90% ethanol was used for the extraction of fucoxanthin
81 from *Eisenia bicyclis* [Shang et al., 2011] and the mixture of ethanol/limonene (1:1, v/v)
82 was proposed as a green approach for PLE extraction of lipids from different microalgae
83 [Golmakani et al., 2014]. Other microalgae from which bioactive compounds have been
84 extracted by PLE are *Haematococcus pluvialis* [Santoyo et al., 2009; Yuan & Chen, 2000],
85 *Chlorella vulgaris* [Wang et al., 2010] and *Spirulina platensis* [Herrero et al., 2005;
86 Herrero et al., 2007].
87 Microwave-assisted solvent extraction (MAE) is another novel technology that has been
88 used in a lesser extent than PLE to extract bioactive compounds from marine macro- and
89 microalgae, such as carotenoids, fucoidans (sulfated polysaccharides) and minerals [Kadam
90 et al., 2013; Xiao et al., 2012]. MAE transfers energy to the solution, which is heated by the
91 concurrence of dipole rotation and ionic conduction mechanisms. Dipole rotation means
92 realignment of dipoles with the applied electromagnetic field. Ionic conduction is the
93 electrophoretic migration of ions when an electromagnetic field is applied; the resistance of
94 the solution to this flow of ions results in friction and, thus, heats the solution. The solvent
95 can be heated above its boiling point, enhancing extraction efficiency and speed, applying
96 elevated pressures to maintain the solvent liquid. This high pressure forces the solvent to
97 penetrate into the matrix pores, which facilitates the extraction of target compounds. [Sparr
98 Eskilsson & Björklund, 2000]. Advantages of MAE include improved extraction rate,
99 lower use of solvents, and improved extraction yield. However, MAE requires an additional

100 separation process to remove solid residues after extraction, compared to PLE [Kadam et
101 al., 2013].
102 In order to optimize the extraction conditions of bioactive metabolites from microalgae,
103 response surface methodology (RSM) can be a useful tool. RSM is a compilation of
104 mathematical and statistical techniques based on the fit of a polynomial equation to the
105 experimental data, according to the selected experimental design. The fitted equation is
106 used to describe the behavior of a data set with the objective of making statistical
107 predictions. This methodology evaluates the interaction among the experimental factors, in
108 order to optimize the extraction conditions with a high level of confidence by performing
109 only a small batch of experiments. [Bezerra et al., 2008]. RSM has been extensively used
110 for the optimization of extraction conditions of bioactive compounds by pressurized
111 technologies as PLE [Koo et al., 2011; Shang et al., 2011], MAE [Tsiaka et al., 2015] or
112 supercritical fluid extraction (SFE) [Huang et al., 2008].
113 In this work, a comparison between MAE and PLE for the extraction of bioactive
114 compounds from *P. tricornutum* is proposed. The aim is the combination of novel
115 pressurized technologies with the use of green solvents for an environmentally-friendly
116 extraction of high added-value compounds from the marine microalgae. The effect of
117 temperature, solvent composition and extraction time on the extraction yield, total phenolic
118 content (TPC), and antioxidant activity (TEAC) are discussed in detail. Optimum extraction
119 conditions are selected according to an experimental design based on RSM.
120

121 # 2. Materials and methods

122 ## 2.1. Chemicals and samples

123 HPLC-grade methyl *tert*-butyl ether (MTBE), methanol, acetone, and ethanol were from
124 VWR (Leuven, Belgium). HPLC-grade isooctane was purchased to Carlo Erba (Val de
125 Reuil, France), while chloroform and tetrahydrofuran were from Avantor materials
126 (Gliwice, Poland). Sea sand (0.25–0.30 mm diameter) and potassium persulfate were from
127 Panreac. Butylated hydroxytoluene (BHT), trimethylamine, sodium chloride, formic acid
128 (LC-MS grade) and standards of fucoxanthin, chlorophyll a (from *Anacystis nidulans*
129 algae), ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt),
130 and Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) were obtained from
131 Sigma-Aldrich (St Louis, MO, USA). The water used was Milli-Q water (Millipore,
132 Billerica, MA, USA).
133 Freeze-dried samples of *P. tricornutum* were obtained from Fitoplancton Marino S.L.
134 (Cadiz, Spain), and stored under dry and dark conditions until use. *P. tricornutum* was
135 grown outdoors in horizontal tubular 2000L reactors. The reactors use pure CO_2 injection to
136 control pH in the culture by pH controller and flowmeters. pH was set at 7.5, while natural
137 light-dark cycles and ambient temperature were used (10-11 h of light, temperatures
138 ranging from 10-22 ºC). The reactors were inoculated with cultures grown in growth
139 chamber with the standard conditions of Fitoplancton Marino S.L. After harvesting, cells
140 were disrupted at high pressure (1200 bar) and then freeze-dried.
141

142 **2.2. Experimental design**

143 A factorial experimental design 3^2 was employed for PLE optimization, being percentage
144 of ethanol (0, 50, 100%) and temperature (50, 110, 170 ºC) the experimental factors. A total
145 of 12 experiments were conducted in a randomized order: nine points of the factorial design
146 and three additional center points to consider the experimental errors. The experimental

147 design for MAE considered three experimental factors: extraction time (2, 11, 20 min),
148 percentage of ethanol in the solvent mixture (0, 50, 100%) and temperature (30, 100, 170
149 ºC); therefore a 3^3 factorial design was developed for MAE. In this case, a total of 31
150 experiments were conducted: 27 points of the factorial design and four additional center
151 points to consider the experimental errors. **Table 1 and Table 2** show the experimental
152 matrix design for both extraction methods. For comparison, the same response variables
153 were studied in both processes: extraction yield (% dry weight of extract/ dry weight of
154 initial biomass), total phenols content (Folin-Ciocalteu assay), total carotenoids and
155 chlorophylls, and antioxidant activity (ABTS assay, expressed as TEAC). A quadratic
156 model was proposed for each response variable. The parameters of the models were
157 estimated by multiple linear regression using Statgraphics Centurion XVI software
158 (Statpoint Technologies, Warrenton, Virginia, USA). The model adequacy was evaluated
159 by the determination coefficient (R^2), the residual standard deviation (RSD) and the lack-
160 of-fit test for the model from the analysis of variance table, at a confidence level of 99% (p
161 = 0.01). From the fitted model, the optimum conditions were provided by the program.
162 Surface plots were developed using the fitted quadratic polynomial obtained.
163 **<Tables 1 and 2>**
164
165 **2.3. Extraction processes**
166 *2.3.1. Pressurized liquid extraction*
167 Pressurized liquid extractions of *P. tricornutum* were performed using an accelerated
168 solvent extraction system (ASE 200, Dionex, Sunnyvale, CA, USA) equipped with a
169 solvent controller. Extractions were performed at three different extraction temperatures
170 and solvent compositions (percentage of ethanol in the mixture), according to the

171 experimental design described in the previous section, and 20 min as the extraction time
172 (see **Table 1**). Prior to each extraction, an extraction cell heat-up step was performed for a
173 given time, which was fixed by the system (i.e., 5 min when the extraction temperature was
174 40 and 100 °C, and 8 min at 170 °C). All extractions were done using 11-mL extraction
175 cells at 100 bar containing 1 g of algae mixed homogeneously with 2.5 g of sea sand. The
176 extracts obtained were protected from light and frozen. Then ethanolic extracts were
177 evaporated under nitrogen stream, while water extracts were lyophilized in a freeze-drier
178 (Lyobeta, Telstar, Terrassa, Spain).
179
180 *2.3.2. Microwave-assisted solvent extraction*
181 Microwave-assisted solvent extractions of *P. tricornutum* were performed using a
182 Monowave EXTRA extraction system (Anton-Paar GmbH, Graz, Austria) composed by a
183 Monowave 300 and an autosampler MAS 24. Monowave 300 operates at a maximum
184 power of 850 W with a frequency of 2,455 MHz. The autosampler had 24 positions for 30-
185 mL vials, thus a sequence can be programmed for automatic analysis of up to 24 individual
186 experiments. The system can be operated at a maximum pressure of 30 bar over the sample
187 vial, which is dependent on the solvent composition and volume, and on the working
188 temperature. Fixed parameters were sample weight (0.5 g), solvent volume (10 mL) and
189 stirrer speed (1000 rpm). Experimental factors were extraction time, solvent composition
190 (%EtOH) and extraction temperature, as detailed in the experimental design described in
191 section 2.2 and **Table 2**.
192
193 *2.3.3. Conventional solid-liquid extraction*

194 Conventional acetone extraction was performed (in triplicate) to determine the total
195 extractable compounds in *P. tricornutum* [Reyes et al., 2014]. Briefly, 200 mg of
196 lyophilized algae were mixed with 20 mL acetone containing 0.1% (w/v) BHT in a 50-mL
197 Falcon tube and the mixture was shaken for 24 h in an orbital shaker (DOS-20L, Elmi Ltd.,
198 Riga, Latvia) at 250 rpm in the dark. Following extraction, the exhausted substrate was
199 precipitated out in a refrigerated centrifuge (Rotina 380R, Hettich, Tuttlingen, Germany)
200 operating at 4863 x *g* (5000 rpm) at 4 ºC during 10 min. The supernatant was collected, and
201 the solvent was removed using a stream of N_2. Dry acetone extracts were weighted and
202 stored at −20 ºC.
203
204 **2.4. Total phenols content (TPC)**
205 TPC was estimated according to the Folin–Ciocalteu assay [Koşar et al., 2005] with some
206 modifications [Sánchez-Camargo et al., 2016]. Briefly, 10 µL aliquot of extract solution
207 (typically, concentration ranging from 5 to 10 mg mL^{-1}, although depending on sample)
208 and 600 µL ultrapure water are mixed, to which 50 µL undiluted Folin–Ciocalteu reagent
209 (Merck, Darmstadt, Germany) is subsequently added. After 1 min, 150 µL of 20% (w/v)
210 Na_2CO_3 are added and the volume is made up to 1 mL with water. The mixture is vortexed
211 and incubated for 2 h at room temperature in the darkness. After the incubation time, 300
212 µL of each reaction mixture were transferred to a 96-well microplate. The absorbance is
213 measured at 760 nm in a microplate spectrophotometer reader Synergy HT (Bio Tek
214 Instruments, Winooski, VT). A standard curve with serial gallic acid solutions (0.031–2 mg
215 mL^{-1}) is used for calibration. Data are presented as the average of triplicate analyses
216 expressed as mg gallic acid equivalents (GAE) g^{-1} extract.

2.5. Antioxidant capacity assay

The TEAC (Trolox equivalents antioxidant capacity) value was determined using the method described by Re et al., 1999, with some modifications [Sánchez-Camargo et al., 2016]. $ABTS^{\bullet+}$ (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature for 16 hr. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM sodium phosphate buffer pH 7.4 to an absorbance of 0.7 (±0.02) at 734 nm. Ten microliters of sample (5 different concentrations) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in an eppendorf vial and 300 μL of the mixture was transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a microplate spectrophotometer reader (Synergy HT, BioTek). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as reference standard and results are expressed as TEAC values (mmol trolox equivalentsg^{-1} extract). These values are obtained from five different concentrations of each sample tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were done in triplicate.

2.6. Carotenoids and chlorophylls determination.

2.6.1. Total carotenoids and chlorophylls determination.

A spectrophotometric method was used to determine the total carotenoid and total chlorophylls concentration, based on their characteristic absorbance. Extracts were dissolved in methanol at concentrations ranging 0.05 - 1 mg mL^{-1}. Absorbance of these solutions was recorded at two specific wavelengths, 470 and 665 nm, for carotenoids and

240 chlorophylls, respectively. External standard calibration curves of fucoxanthin (0.5 – 10 µg
241 mL^{-1}) and chlorophyll a (0.5 – 7.5 µg mL^{-1}) were used to calculate the total carotenoid and
242 chlorophyll content. Total carotenoids were expressed as mg carotenoids g^{-1} extract, by
243 interpolating the absorbance of the extract at 470 nm in the calibration curve of
244 fucoxanthin. Total chlorophylls were expressed as mg chlorophyll g^{-1} extract, by
245 interpolating the absorbance of the extract at 665 nm in the calibration curve of chlorophyll
246 a.
247
248 *2.6.2. Chemical characterization of carotenoids and chlorophylls by HPLC-DAD-APCI-*
249 *MS/MS*
250 Carotenoids and chlorophylls profile of *P. tricornutum* extracts was determined by HPLC-
251 DAD (diode-array detector) according to a method previously described [Castro-Puyana et
252 al., 2013; Gilbert-López et al., 2015]. HPLC analyses of the extracts were conducted using
253 an Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA) equipped with a
254 diode-array detector, and using a YMC-C_{30} reversed-phase column (250 mm × 4.6 mm
255 inner diameter, 5 µm particle size; YMC Europe, Schermbeck, Germany) and a pre-column
256 YMC-C_{30} (10 mm x 4 mm i.d., 5 µm). The mobile phase was a mixture of methanol–
257 MTBE–water (90:7:3 v/v/v) (solvent A) and methanol–MTBE (10:90 v/v) (solvent B)
258 eluted according to the following gradient: 0 min, 0 % B; 20 min, 30 % B; 35 min, 50 %
259 B; 45 min, 80 % B; 50 min, 100% B; 60 min, 100% B; 62 min, 0% B. The flow rate was
260 0.8 mL min^{-1} while the injection volume was 10 µL. The detection was performed at 280,
261 450 and 660 nm, although spectra from 240 to 770 nm were collected using the DAD (peak
262 width > 0.1 min (2 s), slit 4 nm). The instrument was controlled by LC ChemStation 3D
263 Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA). Extracts were

264 dissolved at a concentration of 5 mg mL^{-1} in ethanol/solvent A (1:1) prior to HPLC
265 analysis.
266 For the calibration curve, six different concentrations of fucoxanthin in ethanol, ranging
267 from 0.0125 to 0.16 mg mL^{-1}, were analyzed at least by triplicate using the LC-DAD-MS
268 instrument.
269 The liquid chromatograph equipped with DAD detector was directly coupled to an ion trap
270 mass spectrometer (Agilent ion trap 6320) via an atmospheric pressure chemical ionization
271 (APCI) interface. operating under positive ionization mode using the following parameters:
272 capillary voltage, −3.5 kV; drying temperature, 350 °C; vaporizer temperature, 400 °C;
273 drying gas flow rate, 5 L min^{-1}; corona current (which sets the discharge amperage for the
274 APCI source), 4,000 nA; nebulizer gas pressure, 60 psi. Full scan was acquired in the range
275 from *m*/*z* 150-1,300. Automatic MS/MS analyses were also performed, fragmenting the two
276 highest precursor ions (10,000 counts threshold; 1V Fragmentor amplitude).
277

278 **2.7. Analysis of the lipid fraction**

279 *2.7.1. Total lipids analysis.*

280 Total lipid extraction was performed as proposed by Axelsson & Gentili, 2014. Briefly, 25
281 mg of PLE extract, MAE extract or freeze-dried algae were weighted in a 15-mL centrifuge
282 tube. Then, 8 mL of chloroform/methanol 2:1 (v/v) were added and mixed with the sample
283 in a vortex. Subsequently, 2 mL of NaCl 0.73% (w/v) in water were added and the tube was
284 shaken again in a vortex. Following extraction, two phases were separated in a centrifuge
285 (Rotina 380R, Hettich, Tuttlingen, Germany) operating at 350 x *g* at room temperature
286 during 5 min. The lower layer was collected with a glass pipette and transferred to a pre-
287 weighed glass vial. The solvent was removed using a stream of N_2 and the vial was

288 weighted again. Therefore, total lipids were calculated by a gravimetrical determination and
289 the results were expressed as percentage (w/w).
290
291 *2.7.2. Chemical characterization of lipid class compositions by HPLC-Evaporative Light*
292 *Scattering Detection (ELSD).*
293 Separation of lipid classes was done using the method described by Castro-Gómez et al.,
294 2014, with minor modifications. The analysis was performed using an HPLC system
295 (model 1290 Infinity II, Agilent Technologies Inc.) coupled with an evaporative light
296 scattering detector (ELSD, 1260, Agilent Technologies Inc.) using nitrogen as the
297 nebulizing gas at a flow rate of 2 SLM (Standard liter per minute). Nebulizer and
298 evaporator temperatures were set at 50°C and 90°C, respectively; led intensity was set at
299 50% and the gain was set at 1. Two columns were used in series (250 mm × 4.5 mm i.d.
300 Zorbax Rx-SIL column with 5-µm particle diameter; Agilent Technologies Inc.) and a
301 precolumn (12.5 mm × 4.5 mm i.d.) with the same packing was used. Before analysis,
302 samples were dissolved in CH_2Cl_2 (5 mg mL^{-1}) and 40 µL were injected. Autosampler
303 temperature was kept at 8ºC, while column temperature was set at 40°C. Solvent mixtures
304 and gradient is detailed in reference Castro-Gómez et al., 2014. In order to identify the
305 chromatographic peaks corresponding to pigments co-extracted with lipids, a diode-array
306 detector (1290 Infinity II model, Agilent Technologies Inc.) was connected to the HPLC,
307 previous to ELSD detector. Absorbance signals were collected at 450 and 640 nm to
308 identify carotenoids and chlorophylls.
309

310 ## 3. Results and discussion

3.1. Pressurized liquid extraction of *P. tricornutum*.

As indicated in section 2.2, a factorial experimental design 3^2 was employed for PLE optimization, being %EtOH (0, 50, 100%) and temperature (50, 110, 170 ºC) the experimental factors. The quadratic model proposed for each response variable (*Yi*) was:

$$Y_i = \beta_0 + \beta_1 A + \beta_2 S + \beta_{1,1} A^2 + \beta_{1,2} A \cdot B + \beta_{2,2} B^2 + \varepsilon,$$

where A is the temperature, B is the solvent composition (percentage of EtOH in the mixture), β_0 is the intercept, β_1 and β_2 are the linear coefficients, $\beta_{1,1}$ and $\beta_{2,2}$ are the quadratic coefficients, $\beta_{1,2}$ is the interaction coefficient, and ε is the error variable.

Experimental results of all studied variables are detailed in **Table 1**. Results obtained for ANOVA test of each response variable are detailed in **Table S1** (Electronic Supplementary Material). Standardized Pareto charts and response surface for each variable are depicted in **Figure S1** (Electronic Supplementary Material). Extraction yield was positively influenced by the temperature: an increase in temperature has different favorable effects such as an improved mass transfer from the sample to the extraction solvent, an increase of the solubility of compounds and a reduction in solvent viscosity which favors the penetration of the solvent into the matrix [Mustafa & Turner, 2011]. The composition of the solvent also affected the extraction yield, as the quadratic term of solvent composition showed a significant ($p = 0.01$) negative effect on the yield (**Table S1** and **Figure S1**), obtaining higher yields with the mixture of water:ethanol (50:50) than with the pure solvents (see **Table 1**). Therefore, the model proposed 170ºC and 40% EtOH as the optimum conditions to maximize the extraction yield.

Total phenols content of the extracts showed a different behavior. This response was mainly influenced by the composition of the solvent (**Table S1** and **Figure S1**), obtaining higher phenols content in pure ethanol extracts. Antioxidant activity (TEAC) showed a

335 similar trend to TPC, being higher in pure ethanol extracts (see **Table 1**). Solvent linear and
336 quadratic terms of the model showed a positive significant effect on the antioxidant
337 activity, as well as the quadratic term of temperature (**Table S1** and **Figure S1**). The
338 optimum conditions obtained to maximize TPC and TEAC included the use of 100% EtOH
339 at 50ºC.
340 The content of carotenoids was mainly affected by the composition of the solvent, followed
341 by the quadratic term of temperature (see **Table S1** and **Figure S1**). Up to 110 ºC, the
342 amount of carotenoids increased when the percentage of ethanol was higher in the solvent,
343 and decreased with the temperature. However, the highest amount of carotenoids was found
344 at 170ºC with 50% EtOH (see **Table 1**). Nevertheless, it has to be considered that the
345 determination coefficient (R^2), which indicates the variability of the response variable
346 explained by the model, was 0.7983 for carotenoids, distinctly lower than the determination
347 coefficients obtained for yield (R^2 = 0.9181), TPC (R^2 = 0.9250) or TEAC (R^2 = 0.9727),
348 therefore some deviations in the predictions can occur for carotenoids content. The model
349 proposed 170ºC and 100% EtOH as the optimum conditions to maximize the carotenoids
350 content in the extract.
351 Multiple response optimization was performed to maximize the studied variables, namely,
352 extraction yield, total phenols content, total carotenoids and antioxidant activity. To do that,
353 all response variables were considered equally important (the weight factor and the impact
354 were set at 1.0 and 3.0, respectively). The optimum conditions proposed by the model were
355 170 ºC and 97% EtOH, with an overall desirability value of 0.699. This result was expected
356 considering the different behavior of the extraction yield compared to the rest of responses
357 in terms of the extraction solvent and temperature. Clearly, *P. tricornutum* contains
358 important amounts of polar compounds such as proteins and sugars that are better extracted

using more polar solvents, while phenolic compounds and carotenoids (which are related to the antioxidant activity) are preferentially extracted using 100% ethanol. Moreover, in general, solubility increases with the temperature. Therefore, to reach a compromise is quite difficult in this particular case, so, in order to improve the desirability (and therefore the prediction of the mathematical model), the extraction yield was excluded and the rest of response variables were maximized. As a result, the adjusted model proposed as optimum extraction conditions 50ºC and 100% EtOH, with an overall desirability value of 0.905. Surface response plots are depicted in **Figure 1 a)** and **b)**. Predicted values of yield, TPC, TEAC and total carotenoids by both models are detailed in **Table 3**.

Three replicate extractions were performed in each of the proposed optimum conditions. Extracts at 170ºC and 97% EtOH showed a concentration of carotenoids lower than the predicted. The deviation in the prediction is not surprising as the model for carotenoids was not able to explain 20% the variability of the response. For the rest of response variables (yield, TPC, TEAC), experimental values obtained were close to the predicted ones (RSD between experimental and predicted values below 10%), as can be seen in **Table 3**. On the other hand, extracts obtained at 50ºC and 100% EtOH showed lower yield, but higher amounts of carotenoids and phenols, and a higher antioxidant activity (TEAC value); all of the experimental values were close to those predicted, as detailed in **Table 3**.

<Figure 1 and **Table 3>**

3.2. Microwave-assisted solvent extraction of *P. tricornutum*.

As indicated in section 2.2, a 3^3 factorial experimental design was developed for MAE. The experimental design for MAE considered three experimental factors: extraction time (2, 11,

382 20 min), %EtOH (0, 50, 100%) and temperature (30, 100, 170 ºC). The quadratic model
383 proposed for each response variable (*Yi*) was:
384 $Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 t + \beta_{1,1} A^2 + \beta_{1,2} A \cdot B + \beta_{1,3} A \cdot C + \beta_{2,2} B^2 + \beta_{2,3} B \cdot C + \beta_{3,3} C^2 + \varepsilon$,
385 where A is the temperature, B is the solvent composition (percentage of EtOH in the
386 mixture), C is the extraction time, β_0 is the intercept, β_1, β_2 and β_3 are the linear
387 coefficients, $\beta_{1,1}$, $\beta_{2,2}$ and $\beta_{3,3}$ are the quadratic coefficients, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$ are the
388 interaction coefficients, and ε is the error variable.
389 Experimental results of all studied variables are detailed in **Table 2**. Results obtained for
390 ANOVA test of each response variable are detailed in **Table S2** (Electronic Supplementary
391 Material). Standardized Pareto charts and response surface for each variable are depicted in
392 **Figure S2** (Electronic Supplementary Material). As it was previously observed for PLE
393 experimental design, the temperature and the presence of water in the solvent mixture
394 positively influenced extraction yield using MAE, obtaining the lowest yields when pure
395 ethanol was used as extraction solvent (see **Table 2**). The extraction time did not show a
396 strong influence in the extraction yield. Statistically, it can be observed linear and quadratic
397 terms of temperature showed significant positive effect, while the increase of EtOH in the
398 mixture has a significant negative effect and the extraction time didn't exert a significant
399 effect. The model ($R^2 = 0.7745$) proposed 170ºC, pure water and 20 min of extraction time
400 as the optimum conditions to maximize extraction yield.
401 Total phenols content of the extracts showed a different behavior. As in the case of PLE,
402 TPC was mainly influenced by the composition of the solvent (linear and quadratic terms of
403 ethanol percentage exhibited a positive significant effect) and by the quadratic term of the
404 temperature (**Figure S2**), obtaining higher phenolic content in pure ethanol and working at
405 lower and higher temperatures (see **Table 2**). The extraction time did not show a significant

406 influence in the phenolic content. The model (R^2 = 0.9130) proposed 30ºC, 100% EtOH
407 and 2 min of extraction time as the optimum conditions to maximize the total phenols
408 content.
409 Antioxidant activity (TEAC) was mainly affected by the solvent, obtaining high TEAC
410 values for all extracts obtained in pure ethanol (see **Table 2**). The lowest TEAC values
411 were obtained for water extracts. Temperature showed significant a quadratic behavior,
412 contributing to maximum TEAC values at the highest and the lowest temperatures tested;
413 on the other hand, the extraction time did not significantly affect the results (**Table S2** and
414 **Figure S2**). Therefore the model (R^2 = 0.8043) proposed 170ºC, 100% EtOH and 2 min of
415 extraction time as the optimum conditions to maximize the antioxidant activity.
416 Total carotenoid content was mostly influenced by the solvent composition (**Table S2** and
417 **Figure S2**); carotenoids were mainly extracted by pure ethanol (see **Table 2**). On the other
418 hand, the amount of carotenoids decreased with the temperature, which can be indicative of
419 compound degradation. Statistical model (R^2 = 0.9780) proposed 30ºC, 100% EtOH and 2
420 min of extraction time, as the optimum conditions to maximize the total content of
421 carotenoids.
422 Multiple response optimization was performed to maximize the variables: extraction yield,
423 total phenolic content, total carotenoids and antioxidant activity. To do that, all response
424 variables were considered equally important (the weight factor and the impact were set at
425 1.0 and 3.0, respectively), as in the case of PLE design. Under these conditions, the
426 optimum conditions proposed by the model of MAE were 170ºC, 100% EtOH and 5.8 min
427 of extraction time, with an overall desirability value of 0.623. In order to improve the
428 desirability, the extraction yield was excluded and the rest of response variables were
429 maximized. As happened for PLE design, the proposed optimum temperature decreased

430 from the maximum assayed to the minimum. Therefore, the new model proposed as
431 optimum extraction conditions 30ºC, 100% EtOH and 2 min of extraction time, with an
432 overall desirability value of 0.925. Surface response plots are depicted in **Figure 1 c)** and
433 **d)**. Predicted values of yield, TPC, TEAC and total carotenoids by both models are detailed
434 in **Table 3**.
435 Three replicate extractions were performed in each of the proposed optimum conditions.
436 Extracts at 170ºC, 100% EtOH and 5.8 min, showed an extraction yield lower than the
437 predicted, with a relative standard deviation (RSD) of 17.75%. For the rest of response
438 variables (TPC, TEAC, carotenoids), experimental values obtained were close to those
439 predicted, as can be seen in **Table 3**. On the other hand, extracts obtained for 2 min at 30 ºC
440 and 100% EtOH showed lower yield, but higher amounts of carotenoids and phenols, and a
441 higher antioxidant activity (TEAC value); all the experimental values were close to those
442 predicted, as detailed in **Table 3**.
443
444 **3.3. Analysis of carotenoids and chlorophylls present in the extracts**
445 Total carotenoids and chlorophylls calculated by the native absorbance of the extracts (see
446 section 2.6) showed a similar trend: they were extracted mainly with ethanol at low
447 temperatures. Extracts obtained at optimum conditions were analyzed by HPLC-APCI-
448 MS/MS to obtain more information about the particular carotenoids and chlorophylls
449 composition in the extracts. Chromatographic separation was performed in a C30 stationary
450 phase, which provides better selectivity for long-chain analytes than conventional C18
451 reverse-phase columns and enhanced resolution of geometric isomers (E- and Z- isomers)
452 of xanthophylls and carotenes [Řezanka et al., 2009; Amorim-Carrilho et al., 2014]. A
453 tentative identification of different carotenoids was attained combining the information

454 provided by the two detectors (DAD and MS), the use of commercial standards, and data
455 found in the literature. Information about characteristic UV–vis spectra, $[M+H]^+$, and the
456 main fragments obtained by MS/MS for the different pigments detected in PLE and MAE
457 optimum extracts is given in **Table 4. Figure 2** shows their corresponding chromatographic
458 profiles. The main compounds determined correspond to carotenoids, most-notably
459 fucoxanthin isomers, being E-fucoxanthin the most abundant compound by far.
460 Fucoxanthin isomers (peaks 10-12) could be tentatively assigned due to their UV-Vis and
461 MS/MS spectra [Gilbert-López et al., 2015]. Protonated molecule $[M+H]^+$ was only
462 observed for fucoxanthin 13Z and 13'Z-isomers; the other main ion detected for these
463 isomers was the dehydrated molecule ($[M+H-H_2O]^+$, *m/z* 641.5). E-fucoxanthin presented
464 also the dehydrated molecule and a fragment ion corresponding to a loss of 78 Da (*m/z*
465 581.5), consistent with the sequential losses of the C-3 carbomethoxy group (acetic acid)
466 and a water molecule. MS/MS analyses from these ions exhibited a loss of 92 Da (*m/z*
467 549.7 and *m/z* 489.4, respectively) that could be attributed to the loss of toluene from the
468 polyene chain.
469 The second most intense peak in the chromatogram (peak 23) could be tentatively
470 identified as diatoxanthin by its sodium adduct (*m/z* 589.6) and its protonated molecule
471 (*m/z* 567.4), which presented the loss of water as main fragment (*m/z* 549.5). Its UV-Vis
472 spectrum was consistent with data found in the literature [Britton et al., 2004]. Other two
473 carotenoids (peaks 22 and 28) and one carotenoid ester (peak 26) were detected in the
474 extracts, but they could not be completely identified.
475 The rest of the minor peaks in the chromatogram presented the characteristic absorbance
476 spectrum of chlorophylls, and therefore those that have not been identified have been
477 designed as chlorophyll-type (see **Table 4**). Peak 24 has been identified as chlorophyll a' in

478 agreement with its MS and UV-Vis spectra, similar to those of chlorophyll a, but
479 possessing longer retention time. Chlorophyll a' presented the protonated molecule and lost
480 the phythyl group ($C_{20}H_{38}$, *m/z* 278), showing a fragment at *m/z* 616.3. Chlorophyll a or
481 chlorophyll a' derivatives tentatively identified in the extracts have been named as
482 chlorophyll a derivatives.
483 Chlorophyllide a (peak 2) was tentatively identified by its molecular ion ($[M+H]^+$ *m/z*
484 613.7) and the dehydrated molecule ($[M+H-H_2O]^+$, *m/z* 596.8). Peak 17 was tentatively
485 identified as chlorophyll a derivative I by its protonated molecule (*m/z* 889.7) [Pacini et al.,
486 2015]. Peak 18 was identified as hydroxychlorophyll a by its $[M+H]^+$ (*m/z* 909.9) and its
487 main fragment (*m/z* 631.8) [Bijttebier et al., 2014]. Hydroxychlorophyll a was present in a
488 higher amount in MAE extracts, and was found partially coeluting with a chlorophyll
489 derivative compound that could not be identified. Following a similar approach, peak 16
490 was tentatively identified as pheophorbide a, as it presented the protonated molecule (*m/z*
491 594.1) and a characteristic fragment (*m/z* 533.4) [van Breemen et al., 1991] of this
492 compound. Pheophorbide a was only detected in PLE extracts, while peak 15 was only
493 detected in MAE extracts. This latter peak was tentatively identified as a compound related
494 to chlorophyll c_1 structure. Chlorophyll c_1 was the tentative identification of peak 13, based
495 on the presence of its protonated molecule (*m/z* 612.1).
496 **<Figure 2 and Table 4>**
497 As aforementioned, E-fucoxanthin was the main pigment and the most valuable compound
498 in the extracts due its bioactivities. Thus, it was further quantified in the extracts. The
499 amount of fucoxanthin recovered in PLE at 170ºC and 97% EtOH was 5.81 mg g^{-1} algae,
500 while the extracted fucoxanthin at 50ºC and 100% EtOH reached 7.73 mg g^{-1} algae,
501 pointing out the sensitivity of this compound to temperature. The same trend was observed

502 for MAE. Extracted fucoxanthin at 170ºC with 100% EtOH during 6 min was 2.97 mg g^{-1}
503 algae, while 4.59 mg of fucoxanthin g^{-1} algae were recovered at 30ºC with 100% EtOH in
504 2 minutes.
505
506 **3.4. Comparative analysis of the results**
507 Extraction conditions at low temperature (50°C and 30°C for PLE and MAE, respectively)
508 were considered the best conditions, allowing the attainment of extracts concentrated two-
509 fold in fucoxanthin. Total phenols content, total carotenoids and antioxidant activity were
510 higher in MAE optimum extracts, although PLE was able to provide higher extraction
511 yields. The concentration of fucoxanthin in MAE and PLE extracts was similar (31.60 and
512 32.29 mg g^{-1} extract, respectively), but the recovery of this valuable carotenoid from the
513 initial biomass was higher in PLE extracts because of their higher extraction yield. To
514 calculate the recoveries, *P. tricornutum* was macerated in acetone during 24 h at room
515 temperature and the average concentration of fucoxanthin in these extracts (three replicates)
516 was calculated to be 59.5 ± 4.7 mg g^{-1} extract. This amount was used as reference value for
517 total fucoxanthin in the raw biomass.
518 **Table 5** shows the calculated recoveries for fucoxanthin in MAE and PLE optimum
519 extracts; as can be seen, the recovery of fucoxanthin in PLE extracts is 54%, while the
520 recovery of fucoxanthin in MAE extracts is 32%, due to the lower extraction yield
521 obtained.
522 The amount of fucoxanthin extracted in the present study was by far higher than those
523 reported by other authors using also PLE and MAE. For instance, Xiao et al., 2012 and
524 Shang et al., 2011 used, respectively, MAE at 60ºC, 100% EtOH, 10 min and PLE at

525 110ºC, 90% EtOH, 5 min, for the extraction of fucoxanthin from brown algae. They
526 obtained concentrations of fucoxanthin lower than 1 mg g^{-1} algae, while average
527 concentration of fucoxanthin from *P. tricornutum* obtained in the present study for 2 min
528 MAE at 30 ºC, 100% EtOH was 4.59 mg g^{-1} algae. This supports the idea that microalgae
529 are a good source of valuable compounds, even better than macroalgae in some cases. In
530 this sense, the extraction of fucoxanthin from *P. tricornutum* was recently performed by
531 Kim et al., 2012, reporting a maximum concentration of fucoxanthin of 16.33 mg g^{-1} algae
532 obtained by 1 h maceration in EtOH at room temperature; this value is similar to the
533 amount of fucoxanthin obtained by acetone maceration, and approximately 2-fold higher
534 the concentration obtained by PLE in this study (7.73 mg g^{-1} algae).
535 On the other hand, the extraction of lipids from *P. tricornutum* is of interest because this
536 alga is rich in the polyunsaturated w-3 fatty acid EPA (C20:5). Therefore, the total amount
537 of lipids was determined in each extract, and also in the raw alga. Considering the
538 extraction yield, recoveries of total lipids were calculated in a similar way than for
539 fucoxanthin. As it is detailed in **Table 5**, PLE extracts recovered 79% of total lipids present
540 in the raw algae, while MAE extracts recovered 45% of total lipids.
541 **<Table 5>**
542 The method employed for the analysis of lipids profile by HPLC-ELSD allows not only the
543 separation of lipid classes, but also a further separation of polar lipids (e.g.: phospholipids)
544 in the same run. Non-polar lipids, mainly triacylglycerides (TAGs) eluted in the first part of
545 the analysis, up to 18-20 min. Medium polar lipids as mono- (MAG) and diacylglycerides
546 (DAG) eluted between 20 and 25 min, together with free fatty acids (FFA) and pigments
547 (carotenoids, chlorophylls and their derivatives); finally, polar lipids eluted in the third
548 segment of the chromatogram (from 25 min onwards). Both MAE and PLE optimum

549 extracts of *P. tricornutum* showed similar chromatographic profiles (depicted in **Figure 3**),
550 which demonstrated that lipids of all polarity ranges were extracted. The presence of EPA
551 in neutral lipids, phospholipids and glycolipids of *P. tricornutum* has been recently reported
552 by Ryckebosch et al., 2014. The reported relative abundances of EPA within the total fatty
553 acids content in the different fractions were 31.7, 12.1 and 27.2%, respectively. From these
554 results, we can expect the presence of EPA within the lipid content of our extracts, which
555 reinforces the beneficial effects provided by the presence of fucoxanthin.
556 **< Figure 3>**
557 Despite of the differences among the quantitative extraction of bioactive compounds from
558 *P. tricornutum* by MAE and PLE, both are valuable technologies for the development of
559 green processes using microalgae as biomass. Both developed methods meet the principles
560 of green extraction [Chemat et al., 2012], as the use of a renewable plant resource such as
561 commercial microalgae and ethanol as a non-toxic solvent; moreover, the proposed
562 methods provide with short extraction times at low temperatures, thus requiring low energy
563 consumption. In addition, the by-products generated (residue) were rich in proteins and
564 sugars, also valuable compounds that may be the target of different processes that can be
565 integrated with the proposed in the present work for microalgae biorefinery, a concept that
566 has been gaining interest in the recent years [Rombaut et al., 2014; Herrero & Ibáñez,
567 2015].
568

569 ## Concluding remarks

570 In this work, two different green extraction processes (MAE and PLE) have been tested to
571 study the optimum conditions leading to valuable extracts in terms of bioactivity and

572 composition. Similar results were obtained using either MAE or PLE regarding to the
573 composition of the extracts. However, as extraction yields were higher using PLE,
574 recoveries of valuable compounds such as lipids and the carotenoid fucoxanthin were
575 higher using PLE than using MAE. In both cases, ethanol was the best solvent to obtain
576 extracts with high antioxidant activity and rich in fucoxanthin, which was favorably
577 extracted at low temperatures. The use of an experimental design based on RSM was
578 confirmed as a useful tool to optimize extraction conditions. The amount of fucoxanthin
579 extracted either by PLE or MAE in the present study was higher than those reported from
580 brown algae, which confirms microalgae as a better source of fucoxanthin than macroalgae.
581 In addition, the presence of different lipid classes containing EPA in the extracts reinforced
582 the beneficial effects provided by the presence of fucoxanthin.
583

584 ## Acknowledgements

585 The authors acknowledge funding from EU MIRACLES project (7th Framework Program –
586 Grant Agreement No. 613588). B.G.L. thanks MINECO (Ministerio de Economía y
587 Competitividad) for her *Juan de la Cierva* postdoctoral research contract. M.H. thanks
588 MINECO for his *Ramón y Cajal* postdoctoral research contract. Authors would like to
589 thank Anton-paar GmbH for access to Monowave EXTRA instrument.
590

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