1 **COMPREHENSIVE CHLOROPHYLL COMPOSITION IN THE MAIN**
2 **EDIBLE SEaweeds**

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13 **ABSTRACT**

14 Natural chlorophylls present in seaweeds have been studied regarding their
15 biological activities and health benefit effects. However, detailed studies regarding
16 characterization of the complete chlorophyll profile either qualitatively and
17 quantitatively are scarce. This work deals with the comprehensive spectrometric study
18 of the chlorophyll derivatives present in the five main coloured edible seaweeds. The
19 novel complete MS² characterization of five chlorophyll derivatives: chlorophyll *c*₂,
20 chlorophyll *c*₁, purpurin-18 *a*, pheophytin *d* and phytyl-purpurin-18 *a* has allowed to
21 obtain fragmentation patterns associated with their different structural features. New
22 chlorophyll derivatives have been identified and quantified by first time in red, green
23 and brown seaweeds, including some oxidative structures. Quantitative data of the
24 chlorophyll content comes to achieve significant information for food composition
25 databases in bioactive compounds.

26 **Keywords:** chlorophyll, seaweeds composition, mass spectrometry, pheophytin *d*,
27 seaweeds.

28

29 1. INTRODUCTION

30 The seaweed industry provides a wide variety of products that have an estimated
31 total annual value of US\$ 5.5-6 billion, and *ca.* 90% of the production comprises food
32 products for human consumption. They form part of the traditional dietary habits in
33 Asian countries, mainly Japan, China and Korea but recently, seaweeds have drawn the
34 interest of food industry in Western countries because of their nutritional value and as
35 raw materials for supply of components in functional foods (Shahidi, 2009). They are
36 excellent sources of polysaccharides, with relatively high protein content, including all
37 of the essential amino acids, a remarkable presence of essential fatty acids with a
38 nutritionally ideal n-6/n-3 fatty acid ratio, minerals, vitamins and trace elements. Edible
39 seaweeds are taxonomically grouped into three different groups depending on their
40 thallus colour, brown (Ochrophyta), red (Rhodophyta) and green (Chlorophyta). The
41 three most important seaweeds used for human consumption (FAO 2003) are species
42 from *Porphyra* (common Japanese name, Nori), *Laminaria* (Kombu) and *Undaria*
43 (Wakame). Among the green seaweeds, it is highly appreciated *Enteromorpha* (Aonori)
44 and *Ulva* (Sea Lettuce).

45 Several kinds of secondary metabolites with potential benefits to human health
46 have been described in seaweeds (Pangestuti & Kim, 2011). For example, it has been
47 shown that the administration of several green seaweeds increased the fecal excretion of
48 dioxins due to its chlorophyllic composition, and consequently the ingestion of green
49 seaweeds can be consider as a new approach in the treatments of patients exposed to
50 lipophilic xenobiotics. Specifically, the pheophytin *a* from *Porphyra* and *Enteromorpha*
51 species has been identified as a potent suppressor against genotoxin-induced *umu C*
52 gene expression in *S. typhimurium* (TA 1535/pSK 1002) probably associated with
53 carcinogenesis (Okai, Okai, Yano & Otani, 1996). According to this, pheophytin *a*

54 derivatives have been proposed to display a potent suppressive activity against
55 chemically induced mouse skin tumorigenesis. Pheophorbide *a* from brown seaweeds
56 has been also proposed as a candidate for treating neurodegenerative diseases such as
57 Alzheimer's disease (Ina, Hayashi, Nozaki & Kamei, 2007) due to its capacity to
58 promote the differentiation of PC12 cells. Occasionally, consumers have shown allergic
59 reactions after ingestion (Hwang et al., 2005), and pheophorbide *a* has been pointed as
60 the hypothetic responsible compound. Consequently, different chlorophyll derivatives
61 isolated from seaweeds have attracted considerable attention in the fields of food,
62 cosmetic and pharmacology (Pangestuti & Kim, 2011).

63 A difference with microalgae, whose detailed chlorophyll composition is known
64 (Garrido & Zapata, 1996; Garrido, Aires, Rodríguez, Van Heukelem, & Zapata 2011),
65 the "recalcitrant" extracellular material of seaweeds (macroalgae) has made difficult its
66 complete characterization. Analysis of chlorophyll compounds in seaweeds has been
67 restricted mainly to presence of chlorophyll *a*, chlorophyll *b* (Lin et al., 2011),
68 chlorophyll *c* (Fujii et al., 2012) and pheophorbide *a* (Ferraces-Casais, Lage-Yusty,
69 Rodríguez-Bernaldo de Quirós & López-Hernández, 2012; Amorim, Lage-Yusty &
70 López-Hernández, 2012; Hwang et al., 2005).

71 The present work deals with the detailed and complete analysis of the
72 chlorophyll fraction of the five seaweeds more consumed in the world. The
73 identification is made by HPLC-hrTOF-MS, using different sources, ESI or APCI, in
74 function of the polarity of compounds. Considering the biological activities and health
75 benefit effects of the different chlorophyll catabolites and the high intake levels of
76 seaweeds, it is essential to know exactly the composition of these sea foods.

77 **2. MATERIALS AND METHODS**

78 **2.1 Raw material.**

79 Green seaweeds, Aonori (*Enteromorpha* spp.) and Sea Lettuce (*Ulva* spp.), were
80 provided by Suralgae (Cádiz, Spain). Nori (*Porphyra umbilicalis*), Wakame (*Undaria*
81 *pinnatifida*) and Kombu (*Laminaria ochroleuca*) were provided by Algamar
82 (Pontevedra, Spain). The five seaweed species were collected on the Atlantic littoral
83 region on the south western part (Cádiz) and the north western part (Pontevedra) of
84 Spain. The dried material (25-45°C for 30-45 hours) is supplied in vacuum sealed bags.
85 *Acaryochloris marina* NBRC 102967 for chlorophyll *d* identification was supplied by
86 the Biological Resource Center of the National Institute of Technology and Evaluation
87 (Chiba, Japan). The culture procedure for *Acaryochloris marina* was adopted from
88 Swingley et al. (2005). Cultures were shaken at 125 rpm under a photon irradiance of 20
89 mol photons/m² s of light at 30°C during four weeks. Cultures were grown as 10 mL in
90 50-mL Erlenmeyer flasks. The used media (MGB-11) was supplemented with 20 mM
91 N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) adjusted to pH 8.35.
92 The medium was changed every one or two days.

93 **2.2 Reagents**

94 Selenium dioxide (sublimed for synthesis, 98%), ammonium sulphate (99.5%),
95 anhydrous sodium sulphate (99.0%) and silica gel 60 GF₂₅₄ for thin layer
96 chromatography were supplied by Merck (Darmstadt, Germany). Potassium phosphate
97 (98%), ammonium acetate (98%), Tris-HCl, Triton X-100, sodium hydroxide, sodium
98 chloride (99.5%), and the components of the MGB-11 were provided by Sigma-Aldrich
99 (St. Louis, MO, USA). Other reagents (pyridine, diethyl ether, acetone, HCl, KCl,
100 Na₂HPO₄, NaH₂PO₄, analysis grade) were supplied by Teknokroma (Barcelona, Spain).
101 Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka
102 (Zwijndrecht, The Netherlands). Bakerbond SPE C18 columns were supplied by J. T.
103 Baker (Deventer, Holland). *N,N*-dimethylformamide (DMF) PAR grade and LC/MS

grade solvents and water were supplied by Panreac (Barcelona, Spain), while acetone HPLC grade was supplied by Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). For MS calibrations a tuning mix (ESI-L low concentration tuning mix, Agilent Technologies, Santa Clara, CA, USA) was used for ESI interface and for APCI a homemade stock solution of polyethylene glycol 200, 400, 600, 1000 mix from Sigma-Aldrich (St. Louis, MO, USA) in MeOH:water (1:1).

2.3 Chlorophyll extraction

The raw material was mixed with liquid nitrogen and grinded into powder in a mortar. Then, this powder was sequentially passed through 576 meshes/cm² sieve, obtaining seaweed powder with particle size equivalent to 0.5 mm (Φ). Immediately, moisture content was determined by standard moisture analyser (OHAUS, MB35). For direct solvent extraction, 0.2 grams of seaweed powder were mixed with 30 mL of extraction solvent, DMF:water (9:1), vortex mixed (10 s), centrifuged (6000 rpm, 3 min) and filtrated (nylon, 0.45 μ m). Subsequently, one third of the final volume was mixed with the same volume of NaCl water solution (10% w/v) in a separation funnel, and the chlorophyll pigments were extracted with diethyl ether. The organic layer was isolated and concentrated to dryness in a rotary evaporator. The residue was dissolved in acetone. Samples were stored at -20°C until HPLC-MS analysis.

2.4 Description of chlorophyll standards and isolation of chlorophyll derivatives

Chlorophyll *a* and *b* were purchased from Wako (Neuss, Germany) and Sigma-Aldrich (Madrid, Spain), respectively. Chlorophyll *d* was obtained from *Acaryochloris marina* NBRC 102967 (former mbic11017). The culture sample was extracted three times by sonicating with acetone in an ice bath for 30 min, separated and concentrated as described earlier (Kashiyama et al., 2008). Purified chlorophyll *c₁* was a personal gift

129 from Prof. Garrido (Instituto de Investigaciones Marinas, CSIC) and chlorophyll *c*₂ was
130 purchased from DHI (Denmark). Chlorophyllide *a* was obtained by enzymatic de-
131 esterification of the corresponding parent chlorophyll *a*, using partially purified
132 chlorophyllase from *Ailanthus altissima* (Mill.) leaves. The reaction mixture contained
133 100 mM Tris-HCl (pH 8.5) with 0.24 % (w/v) Triton X-100, chlorophyll dissolved in
134 acetone and crude enzymatic extract in a 5:1:5 ratio (Roca, León & de la Rosa, 2011).
135 Mg-free derivatives (pheophytin *a* and *d*, and pheophorbide *a*) were obtained from their
136 corresponding parent chlorophylls (chlorophyll *a* and *d*, and chlorophyllide *a*) dissolved
137 in diethyl ether by acidification with two or three drops of 5 M HCl (Sievers &
138 Hynninen, 1977). Oxidation with selenium dioxide of chlorophyll *c*₂, *b* and *a*,
139 chlorophyllide *a*, pheophytin *a* and pheophorbide *a* in heated pyridine solution under
140 argon produced the corresponding 13²-hydroxy-derivatives (70°C for 3 h) and 15¹-
141 hydroxy-lactone-derivatives (70°C for 7 h) (Chen, Rios, Pérez-Gálvez & Roca, 2015a;
142 Chen, Rios, Roca & Pérez-Gálvez, 2015b). To produce pyro-pheophorbide *a* and pyro-
143 pheophytin *a*, parent pheophorbide *a* or pheophytin *a* dissolved in pyridine solution
144 blanketed with N₂ were directly heated at 80°C for 4 h (Chen et al., 2015a, b). Purpurin-
145 18 *a* and phytyl-purpurin-18 *a* were obtained by alkaline oxidation of pheophorbide *a*
146 and pheophytin *a* with 30% KOH in methanol (w/v) under atmospheric oxygen
147 (Hynninen, 1973).

148 Epimers at C13 of chlorophyll derivatives (chlorophyll *c*₁', 15¹-hydroxy-lactone-
149 pheophorbide *a*', 13²-hydroxy-pheophorbide *a*', pheophorbide *a*', 13²-hydroxy-
150 chlorophyll *b*', chlorophyll *b*', chlorophyll *a*', 13²-hydroxy-pheophytin *a*', and
151 pheophytin *a*') were prepared by treatment with chloroform for two hours in a
152 refrigerator (Watanabe, Hongu, Honda, Nakazato, Konno & Saitoh, 1984) from the
153 corresponding parent compounds.

154 2.5 Pigment identification by HPLC-API-hrTOF-MS/MS

155 The liquid chromatograph was Dionex Ultimate 3000RS U-HPLC (Thermo
156 Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed as
157 described by Chen et al. (2015a, b). The injection volume was 30 μ L and the flow rate
158 was 1 mL/min. A stainless steel column (200 $\times$ 4.6 mm, 3 μ m particle size) packed with
159 Mediterranea Sea18 (Teknokroma, Barcelona, Spain) was used. A split post-column of
160 0.4 mL/min was introduced directly on the mass spectrometer ion source. Mass
161 spectrometry was performed using a micrOTOF-QII High Resolution Time-of-Flight
162 mass spectrometer (UHR-TOF) with qQ-TOF geometry (Bruker Daltonics, Bremen,
163 Germany) equipped with an ESI or APCI source. The instrument was operated in
164 positive ion mode using a scan range of m/z 50-1200. Mass spectra were acquired
165 through broad-band Collision Induced Dissociation bbCID mode, providing MS and
166 MS/MS spectra, simultaneously. The instrument control was performed using Bruker
167 Daltonics HyStar 3.2. The *in-house* mass database created *ex professo* comprises
168 monoisotopic masses, elemental composition and optionally, retention time and
169 characteristic product ions for 164 chlorophyll derivatives, including the 32 known
170 chlorophyll derivatives (Chen et al., 2015a, b) and several other chlorophyll derivatives
171 reported in literature (Gauthier-Jaques, Bortlik, Hau & Fay, 2001; Walker, Jie & Keely,
172 2003). Data evaluation was performed with Bruker Daltonics DataAnalysis 4.1. From
173 the HPLC/TOF-MS acquisition data, an automated peak detection on the EICs expected
174 for the $[M+H]^+$ ion of each compound in the database was performed with Bruker
175 Daltonics TargetAnalysisTM 1.2 software. The software performed the identification
176 automatically according to mass accuracy and in combination with the isotopic pattern
177 in the SigmaFitTM algorithm (Ríos, Pérez-Gálvez & Roca, 2014). This algorithm
178 provides a numerical comparison of theoretical and measured isotopic patterns and it is

179 used as an identification tool in addition to accurate mass determination. The calculation
180 of SigmaFit values includes generation of the theoretical isotope pattern for the assumed
181 protonated molecule and calculation of a match factor based on the deviations of the
182 signal intensities. Only those hits with mass accuracy and SigmaFit values within the
183 tolerance limits, which were set at 5 ppm and 50, respectively are considered as positive
184 matches. The interpretation of the MS/MS spectra was performed using the
185 SmartFormula3DTM module included in the DataAnalysis software (Ríos, Pérez-Gálvez
186 & Roca, 2014). This module includes an algorithm that estimates whether a formula for
187 a product ion is a subset of a formula for the precursor. The SmartFormula3D checks
188 the consistency highlighting the monoisotopic peaks with formula suggestion and the
189 related isotopic peaks. Mass FrontierTM 4.0 is a software package for the management,
190 evaluation and interpretation of mass spectra, including the automated generation of
191 possible fragments and rearrangement mechanisms, starting from a user-supplied
192 chemical structure (Ríos, Pérez-Gálvez & Roca, 2014).

193 **2.6 Pigment quantification by HPLC-UV-Visible**

194 The pigments were separated by reversed-phase HPLC using a Hewlett-Packard
195 HP 1100 liquid chromatograph. A Mediterranea Sea18 column (200×4.6 mm, 3 µm
196 particle size) was used (Teknokroma, Barcelona, Spain) protected by a guard column
197 (10×4.6 mm) packed with the same material. Separation was performed using the
198 elution gradient described by Roca, Gallardo-Guerrero, Mínguez-Mosquera & Gandul-
199 Rojas (2010). The on-line UV-Visible spectra were recorded from 350 to 800 nm with
200 the photodiode-array detector and sequential detection was performed at 410, 430, 450
201 and 666 nm. Data were collected and processed with a LC HP ChemStation
202 (Rev.A.05.04). Quantification of pigments was performed with the corresponding
203 calibration curves (amount versus integrated peak area). The calibration equations were

204 obtained by least-squares linear regression analysis over a concentration range
205 according to the observed levels of these pigments in the analysed samples. Injections in
206 duplicate were made for five different volumes at each standard solution.

207 **2.7 Statistical analysis**

208 Data were expressed as mean values $\pm$ standard deviation (SD) and were
209 analysed for significant differences using one-way analysis of variance (ANOVA). The
210 analysis was performed using Statistica for Windows (version 6, StatSoft, 2001)

211 **3. RESULTS AND DISCUSSION**

212 **3.1 HPLC-MS/MS identification of the chlorophyll derivatives present in the main** 213 **edible seaweeds.**

214 The three groups of seaweeds (green, red and brown) differ considerably in
215 many structural and biochemical traits including cell wall structure, which greatly
216 difficult the pigment extraction. Consequently, although there are several pigment
217 extraction protocols for microalgae materials (with less complex extracellular matrix),
218 there is not a common method suitable for the three seaweed groups. After evaluation of
219 the efficiency of the two more frequent extraction strategies: solvent extraction and cell
220 disruption (Castle, Morrison & Barger, 2011; Samarasinghe, Fernando, Lacey &
221 Faulkner, 2012), the mixture DMF and water at 9:1 (v/v) was selected as the effective
222 method regarding pigment extraction. Figure 1 shows the structure and numbering
223 system of the chlorophyll derivatives identified in the five analysed seaweed species,
224 while Table 1 shows their retention time, high accuracy measurements and main
225 product ions obtained from the HPLC-MS/MS analyses. Mass spectrometry
226 measurements were performed with an APCI or ESI source for the phytylated or
227 dephytylated chlorophyll derivatives, respectively (Chen et al., 2015a, b) as described in
228 Materials and Methods. The identification of the chlorophyll derivatives was made

229 considering retention time, the UV-visible spectrum features from liquid
230 chromatography, exact mass, isotopic pattern from the mass spectrometry
231 measurements and characteristics of the product ions from the MS²-based reactions. As
232 established above, the chlorophyll profile identified in seaweed has been restricted to
233 the main pigments so far: pheophorbide *a*, pheophytin *a*, chlorophyll *a* and chlorophyll
234 *b*. Table 1 shows a total number of twenty seven chlorophyll derivatives identified by
235 first time in seaweeds. Thus, sixteen chlorophyll derivatives (assigned with *a*, and *c*
236 superscripts in Table 1) fit with the results reported previously in literature (Chen et al.,
237 2015a, b). An extra number of nine chlorophyll derivatives were identified as epimers at
238 the C13² position of the parent compounds (chlorophyll *c*_l¹, 13²-hydroxy pheophorbide
239 *a*¹, pheophorbide *a*¹, 13²-hydroxy chlorophyll *b*¹, chlorophyll *b*¹, chlorophyll *a*¹, 13²-
240 hydroxy pheophytin *a*¹, and pheophytin *a*¹) or at the C15¹ (15¹-hydroxy lactone
241 pheophorbide *a*¹) of the earlier described compounds (Table 1). Although mass
242 spectrometry data are available for chlorophyll *a*¹ and *b*¹ epimers (Gauthier-Jaques,
243 Bortlik, Hau & Fay, 2001; Walker, Jie & Keely, 2003), the characteristic product ion for
244 additional seven chlorophyll epimers is reported here by first time. These epimers
245 display the same UV-visible and mass spectra as their parental compounds, fulfil with
246 the requirements to mass error and mSigma values, and show the same main product
247 ion, but present small differences in retention time (Table 1). Minute amounts of
248 epimers of chlorophyll derivatives have been described in photosynthetic organisms
249 (Nakamura, Akai, Yoshida, Taki & Watanabe, 2003), while they are very frequently
250 observed when pigments are isolated from natural sources or produced in the laboratory
251 (Roca, León & de la Rosa, 2011).

252 In addition, seven chlorophyll derivatives were identified for the first time in the
253 seaweed species analysed in this work including 13²-hydroxy-chlorophyllide *c*₂,

254 chlorophyll c_2 , chlorophyll c_1 , chlorophyll c_1' , purpurin-18 a , pheophytin d and phytol-
255 purpurin-18 a , (b and e superscripts in Table 1). The lack of a previous complete MS²
256 characterization of these compounds, led us to perform such study. We obtained the
257 MS/MS spectra of the corresponding standards (section 2.4) and compared their product
258 ions with those obtained for the chlorophyll derivatives present in the seaweeds,
259 reviewing the *in silico* prediction of theoretical product ions obtained with the software
260 Mass FrontierTM. Hence, comparison between theoretical product ions and the
261 experimental MS/MS data for the isolated standards and the chlorophyll derivatives
262 identified in the seaweed species allowed their identification. Table 2 contains the
263 experimental product ions identified for five chlorophyll derivatives and their structural
264 configuration. The epimer chlorophyll c_1' exhibited the same fragmentation pattern as
265 chlorophyll c_1 while for 13²-hydroxy-chlorophyllide c_2 it was impossible to perform the
266 MS² analysis due to the low concentration of the pigment in the sample.

267 The chromatographic characteristics of chlorophyll c_1 and c_2 have been
268 described in detail (Jeffrey, 1972; Garrido & Zapata, 1996; Garrido, Airs, Rodríguez,
269 Van Heukelem & Zapata), but only the m/z of few product ions after a MSⁿ study are
270 available (Goericke, Storm & Bell, 2000). According to our results (Table 2), both
271 chlorophyll c series follow a very similar MS/MS fragmentation behaviour, showing the
272 main product ion as the one derived from the complete loss of carboxymethoxy group,
273 signals at m/z 551.1861 Da and m/z 549.1720 Da for chlorophyll c_1 and c_2 (B
274 fragmentation), respectively, and some other product ions arising from the loss of water,
275 signals at m/z 593.1996 Da and m/z 591.1851 Da for chlorophyll c_1 and c_2 , respectively,
276 and the fragmentation of both carboxymethoxy and keto groups from V ring, signals at
277 m/z 535.1856 Da and m/z 533.1752 Da for chlorophyll c_1 and c_2 , respectively (B+Y
278 fragmentation). These m/z signals were found by Gozericke, Storm & Bell (2000),

279 although in this paper it is proposed in addition their structural configuration. In
280 comparison with the product ions arising from the MS/MS analysis of the structurally
281 related chlorophyll derivative, the chlorophyllide *a* (Chen, Rios, Roca & Pérez-Gálvez,
282 2015b), any of the fragmentations at the C17 unit were significant. Chlorophyll *c*₁ and
283 *c*₂ present an acrylic acid unit (unsaturation at C17¹-C17² bond) instead of the propionic
284 one of chlorophyllide *a*, and the C17-C18 bond is unsaturated (Figure 2A). Both
285 unsaturated positions are crucial to diminish the extent of MS²-based reactions that may
286 arise from the acrylic acid unit, because the McLafferty rearrangement mechanism,
287 which would yield those fragments, works with the transfer of hydrogen atoms from
288 those positions to the adjacent ones generating a new radical site that follows an α -
289 cleavage reaction. Consequently, product ions observed for chlorophyllide *a* along the
290 propionic chain (Van Breemen, Canjura & Schwartz, 1991; Chen, Rios, Roca & Pérez-
291 Gálvez, 2015b) are not accounted in the MS² spectra of chlorophyll *c*₁ and *c*₂, but
292 fragmentations involving functional groups of V ring are thermodynamically favoured.

293 Surprisingly, chlorophyll derivatives from *d* series were also observed in
294 seaweeds. To the best of our knowledge, chlorophyll *d* derivatives have been scarcely
295 characterized (Kashiyama et al., 2008). But, it has been detected as a chlorophyll
296 derivative with the exact mass and elemental composition of pheophytin *d* (Table 1).
297 The co-injection of the standard compound confirmed our hypothesis. The UV-Visible
298 spectrum of pheophytin *d* with maxima at 380, 420 and 690 nm, agrees with the
299 previous published description (Kashiyama et al., 2008). The MS² spectrum of
300 pheophytin *d* (Table 2) showed the loss of phytyl chain (product ion C, *m/z* 595.2485
301 Da) as the main product ion in accordance with Kashiyama et al. (2008). Additionally,
302 new product ions were observed including fragmentation E (*m/z* 535.2381 Da) and
303 combined loss of phytyl chain, rearrangement of carboxylic group, and methanol from

304 the carboxymethoxy group (m/z 559.2378 Da). The product ion C follows a secondary
305 reaction process that continuously loses the keto group at V ring (m/z 567.2500 Da)
306 with low intensity signal value (<5%) (C+Y fragmentation).

307 The term ‘purpurin-18’ includes a set of compounds with a cyclic anhydride at
308 the V ring (structure E Figure 1). Mg-purpurin-18 *a* phytyl esters have been identified
309 by NMR in several senescent leaves (Sakdarat, Shuyprom, Ayudhya, Waterman &
310 Karagianis, 2006) and by MS in sediments of contemporary lakes (Naylor & Keely,
311 1999). It seems that this catabolite is a product of oxidative transformation (by
312 enzymatic or chemical mechanisms) during senescence. In fact, Hynninen, Leppakasesa
313 & Mesilaakso (2006) have demonstrated that the oxidative allomerization of 13²-
314 hydroxy-chlorophyll *a* generates Mg-purpurin-18 *a* phytyl ester. A derivative from this
315 catabolite, purpurin-18 *a* (without central magnesium nor phytol) has attracted much
316 interest as it could be used as efficient photosensitizer (PS) for mediating photodynamic
317 therapy (Drogat, Barrière, Granet, Sol & Krausz, 2011). Purpurin-18 *a* in combination
318 with light induces rapid apoptotic cell death in the human leukemia cell line and
319 necrosis (Stefano, Ettorrea, Sbranab, Giovaniab & Ner, 2001). Consequently, several
320 methods have been developed to improve its synthesis (Drogat et al., 2011), although
321 there was no information about its MS-fragmentation behaviour. The purpurin-type
322 chlorophyll derivatives present an interesting rearrangement structurally related to the
323 15¹-hydroxy-lactone ones (structures E and B in Figure 1), as both classes of derivatives
324 share the lactone function at the V ring but the former lack the carboxymethoxy group
325 and include a keto function at the C15¹. This rearrangement changes the outcome of
326 MS²-based reactions (Table 2). Thus, for phytyl purpurin-18 *a*, and consecutive to the
327 main signal corresponding to the product ion C at m/z 565.2465 Da (loss of the phytyl
328 chain), it follows the signal at m/z 503.2446 Da that was found to correspond to a

product ion generated after phytol fragmentation and charge remote rearrangement at the propionic unit, and subsequent inductive cleavage at V ring involving the lactone and one keto function (either from 13¹ or 15¹ position, pathway 1 in Figure 2B). The loss of the lactone group was not thermodynamically favoured for the 15¹-hydroxy-lactone derivatives as described before (Chen et al., 2015a, b). Additionally, this reaction starting from the [M+H]⁺ ion takes place in parallel with a very similar process involving the same keto-lactone function pair but originates from the dephytylated product ion C at *m/z* 565.2465 Da reported above (pathway 2 in Figure 2B). The extent of this second process is minor as the intensity of the signal for the product ion (at *m/z* 521.2591 Da) is lower (<7%). The difference between both processes is the inclusion of a water loss in the first reaction from the propionic unit while the configuration of the acid at C17¹ is preserved in the second one (Figure 2B). Product ion F with lower intensity value (<2%) was also detected (*m/z* 491.2064 Da), a typical fragmentation of phytolated chlorophyll derivatives (Van Breenmen, Canjura & Schwartz, 1991; Chen, Rios, Roca & Pérez-Gálvez, 2015b). In this sense, the MS² behaviour of purpurin-18 *a* is parallel to phytol purpurin-18 *a*, with the exception of the two products ions arising specifically from phytolated chlorophyll derivatives (C and F fragmentation, Table 2). The analysis by first time of the fragmentation products of different purpurin-18 *a* derivatives has shown the existence of alternative reactions affecting the V ring indicating that the double keto rearrangement at the E ring easily displaces the ion charge from remote positions and allows the fragmentation of the keto-lactone group.

3.2 Characterization of the chlorophyllic profile of the main seaweeds

Table 3 shows the identification by HPLC-MS/MS of the chlorophyll derivatives present in the five seaweeds analysed plus their quantification by HPLC-UV-Visible. For few derivatives, the low content in the extracts made unable their quantification

354 although the combined system HPLC-hrTOF-ESI/APCI-MS/MS allowed their detection
355 in the samples.

356 *Porphyra* spp. as red seaweed belonging to the phylum Rhodophyta only
357 contains chlorophyll *a* derivatives. A total amount of 2719 mg/kg of chlorophyll
358 pigments (dry weight basis) was obtained, in accordance with previous results
359 (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós & López-Hernández,
360 2012; Hwang et al., 2005). The chlorophyll profile is composed by *ca.* 50% of
361 pheophorbide *a* and 50% of pheophytin *a*, with small amounts of chlorophyll *a* and
362 traces of chlorophyllide *a*. The literature reports opposite effects for the chlorophyll
363 profile of *Porphyra umbilicalis*. The specific work of Hwang et al. (2005) attributes a
364 rare example of food toxicity to the presence of pheophorbide *a*, meanwhile several
365 papers point to pheophytin *a* as anti-mutagenic agent in *Porphyra umbilicalis* extracts
366 (Okai, Okai, Yano & Otani, 1996). The obtained data show that also oxidative
367 derivatives as 13²-OH- and 15¹-lactone derivatives are present in the seaweeds. With a
368 further grade of oxidation are the purpurin-18 *a* derivatives, both phytylated and
369 dephytylated, also described by first time in this seaweeds. A surprising finding is the
370 presence of chlorophyll derivatives of *d* series, pheophytin *d*. Chlorophyll *d* was
371 discovered in red seaweeds (Rhodophyta) several decades ago (Manning & Strain,
372 1949) but as there were no reports describing chlorophyll *d* in freshwater alga before,
373 and there was a possibility that this pigment might be produced from breakdown
374 reactions during pigment extraction, the existence of chlorophyll *d* was circumvent for
375 years. Miyashita, Ikemoto, Kurano, Adachi, Chihara & Miyachi (1996) discovered the
376 cyanobacterium *Acaryochloris marina* as the only oxygenic photoautotroph that uses
377 the pigment chlorophyll *d* as the predominant photosynthetic pigment, with only trace
378 amounts of chlorophyll *a*. Since then, several papers have outlined the presence of

379 chlorophyll *d* in other cyanobacteria (Airs, Temperton, Sambles, Farnham, Skill &
380 Llewellyn, 2014). In algae, Kashiya et al. (2008) have described its presence not only
381 in red seaweeds but also in brown and even in green species. Although the exact origin
382 of chlorophyll *d* is an open question (Swingley et al., 2008) we report here by first time
383 content of pheophytin *d* in *Porphyra umbilicalis* seaweed species.

384 The chlorophyll fraction of the green seaweeds included in the phylum
385 Chlorophyta is identical to that of terrestrial plants: chlorophylls *a* and *b*. This is the
386 case of *Enteromorpha* and *Ulva* seaweeds species analyzed in this work. In fact, the
387 stoichiometric ratio among both series (2-3, *a* to *b* series) is the typical of a green
388 chloroplast. Among the analyzed samples, green seaweeds showed the maximum
389 content in chlorophyll derivatives (higher than 9 mg/g d.w.), similar to previous reports
390 (El-Baky, El-Baz & El-Baroty, 2009). The chlorophyll derivatives described in green
391 seaweeds are chlorophyll *a* and *b* (Lin et al., 2011), pheophytin *a* (Okai, Okai, Yano &
392 Otani, 1996) and pheophorbide *a* (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de
393 Quirós & López-Hernández, 2012). Our study comes to further clear up the picture of
394 the chlorophyll derivatives in *Ulva* and *Enteromorpha* seaweeds species (Table 3).
395 Here, we show that the profiles are more intricate, with oxidative derivatives as 13²-
396 hydroxy- and 15¹-hydroxy-lactone derivatives of chlorophylls, pheophorbides and
397 pheophytins. *Ulva* even contains phytyl-purpurin-18 *a* and pyropheophytin *a*. In
398 general, the profile of both species is almost analogous, with *ca.* 90% of chlorophylls (*a*
399 and *b*) and less than 10% of de-chelated chlorophylls (pheophytins and pheophorbides).
400 Any chlorophyllide derivatives were detected, what means that chlorophyllase enzyme
401 is not very active in these species (Roca, León & de la Rosa, 2011). It is interesting to
402 highlight that *Enteromorpha* spp. species seems to be more resistant to oxidation, as it

403 does not contain oxidative derivatives from pheophorbide *a*, or 15¹-hydroxy-lactone
404 pheophytin *a*, pyropheophytin and purpurin-18 *a*, as *Ulva* does.

405 *Undaria pinnatifida* and *Laminaria ochroleuca* belong to the brown seaweeds.
406 The chlorophyll profile of the brown seaweeds included in the Phylum Ochrophyta (or
407 the Heterokontophyta), class Phaeophyceae contains chlorophylls *a* and *c* (Fujii et al.,
408 2012). *Undaria pinnatifida* showed higher contents of chlorophyll derivatives than
409 *Laminaria ochroleuca* (Table 3), as reported previously (Amorim, Lage-Yusty &
410 López-Hernández, 2012). Table 3 shows that in addition to the presence of series *a*
411 chlorophylls, *c*₁ and *c*₂ pigments are characteristic in both seaweeds, and surprisingly
412 pheophytin *d* is present in *Undaria pinnatifida*. Although for both species pheophytin *a*
413 derivatives are the main chlorophyll pigments and the presence of other chlorophyll
414 derivatives is minimal, some traits make different these profiles. *Laminaria ochroleuca*
415 chlorophyll profile contains almost 90% of pheophytin *a* and *ca.* 10% of pheophorbide
416 *a* derivatives, while *Undaria pinnatifida* extracts contain around 60% of pheophytin *a*
417 and 35% of pheophorbide *a*. Earlier characterizations of chlorophyll profile in
418 *Laminaria ochroleuca* usually showed pheophytin *a* and chlorophyll *a* as unique
419 chlorophyll pigments (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós &
420 López-Hernández, 2012; Amorim, Lage-Yusty & López-Hernández, 2012). In both
421 green seaweeds, as in the other seaweeds analyzed (Table 3), and independently of the
422 seaweeds group, the 13²-hydroxy-derivatives represent around 10% while 15¹-hydroxy-
423 lactone derivatives reach almost 5%. To the best of our knowledge, only Kataura,
424 Komiya, Koseki, Fuse & Tateno (2000) has described the presence of 13²-hydroxy-
425 chlorophyll *a* in *Laminaria japonica*.

426 4. CONCLUSION

427 Although *Porphyra umbilicalis*, *Ulva*, *Enteromorpha* spp., *Undaria pinnatifida*
428 and *Laminaria ochroleuca* are daily consumed in the world, there was no detailed
429 characterization of the chlorophyll profile of these seaweeds. This is at odds with the
430 increasing number of reports showing beneficial effects of several chlorophyll
431 derivatives. Structural studies based on spectroscopic properties allow to progress in the
432 knowledge of the chlorophyll structure and behavior of functional arrangements. The
433 spectrometric behavior of pheophytin *d*, from the "intriguing" *d* series, has provided the
434 fragmentation patterns for this new group of chlorophyll derivatives. One further step
435 has been the proposal of different mechanisms for MS²-based reactions, including
436 inductive cleavages or McLafferty rearrangement related to the in-depth study of the
437 diverse chlorophyll structures (series *a*, *b*, *c* and *d*, purpurins, and oxidized derivatives)
438 present in the different seaweeds species. To gain information from the chemical
439 behavior of different chlorophyll structures is a fundamental knowledge for future
440 applications.

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574

575 **FIGURE CAPTIONS**

576 **Figure 1:** Structural configuration names and numbering system for all the chlorophylls
577 derivatives identified by HPLC-ESI/APCI-hrTOF- MS/MS in the different seaweeds
578 analyzed.

579 **Figure 2:** (A) Scheme for chlorophyll *c* MS²-based reactions: lack of hydrogen atom at
580 C17 position in chlorophyll *c*₁ (or *c*₂) restrains McLafferty rearrangement and
581 fragmentation(s) at the propionic unit. (B) Scheme for phytyl-purpurin-18*a* MS²-based
582 reactions: fragmentation involve phytyl chain (1,) or phytyl chain loss (2) and inductive
583 cleavage of the keto-lactone pair from ring V.

584 **TABLES**

585 **Table 1:** Retention time and high accuracy measurements of chlorophyll derivatives
586 present profile in *Porphyra umbilicalis*, *Ulva* spp., *Enteromorpha* spp., *Laminaria*
587 *ochroleuca* and *Undaria pinnatifida* extract analyzed by HPLC-ESI/APCI-hrTOF-MS²

588 **Table 2:** Product ions (*m/z*) in the ESI/APCI-hrTOF-MS² spectra of new chlorophyll
589 derivative standards

590 **Table 3:** Chlorophyll derivatives identified and quantified in *Porphyra umbilicalis*,
591 *Ulva* spp., *Enteromorpha* spp., *Laminaria ochroleuca* and *Undaria pinnatifida*
592 commercialized seaweeds (mg/Kg dw±SD)

593

Table 1.- Retention time and high accuracy measurements of chlorophyll derivatives present in *Porphyra umbilicalis*, *Ulva*, *Enteromorpha* spp., *Laminaria ochroleuca* and *Undaria pinnatifida* analyzed by HPLC-ESI/APCI-hrTOF-MS²

Compound	k	Error (mDa)	mSigma	Molecular formula	[M+H] ⁺ (m/z)		MS ²
					MW. calc.	MW. meas.	Main product ion
13 ² -Hydroxy-chlorophyllide <i>a</i>	0.99	1.94	34.6	C ₃₅ H ₃₄ MgN ₄ O ₇	647.2351	647.2331	571.2139 ^a
13 ² -Hydroxy-chlorophyllide <i>c</i> ₂	1.08	0.60	42.3	C ₃₅ H ₂₈ MgN ₄ O ₆	625.1932	625.1938	^e
Chlorophyll <i>c</i> ₂	1.13	4.40	38.2	C ₃₅ H ₂₈ MgN ₄ O ₅	609.1982	609.1939	549.1793 ^b
Chlorophyllide <i>a</i>	1.16	2.46	21.0	C ₃₅ H ₃₄ MgN ₄ O ₅	615.2452	615.2434	555.2251 ^a
15 ¹ -Hydroxy-lactone pheophorbide <i>a</i>	1.31	0.60	8.5	C ₃₅ H ₃₆ N ₄ O ₇	625.2657	625.2661	537.2425 ^a
Chlorophyll <i>c</i> ₁	1.50	2.61	33.5	C ₃₅ H ₃₀ MgN ₄ O ₅	611.2139	611.2109	551.1861 ^b
Chlorophyll <i>c</i> ₁ '	1.58	1.90	34.4	C ₃₅ H ₃₀ MgN ₄ O ₅	611.2139	611.2120	551.1831 ^b
15 ¹ -Hydroxy-lactone pheophorbide <i>a</i> '	1.63	1.23	31.1	C ₃₅ H ₃₆ N ₄ O ₇	625.2657	625.2667	537.2397 ^b
13 ² -Hydroxy-pheophorbide <i>a</i>	1.85	0.83	4.7	C ₃₅ H ₃₆ N ₄ O ₆	609.2708	609.2695	503.2410 ^a
13 ² -Hydroxy-pheophorbide <i>a</i> '	2.21	2.84	0.7	C ₃₅ H ₃₆ N ₄ O ₆	609.2708	609.2679	503.2445 ^b
Pheophorbide <i>a</i>	2.29	4.30	7.5	C ₃₅ H ₃₆ N ₄ O ₅	593.2759	593.2802	533.2526 ^a
Pheophorbide <i>a</i> '	2.63	0.08	11.0	C ₃₅ H ₃₆ N ₄ O ₅	593.2759	593.2760	533.2532 ^b
Pyro-pheophorbide <i>a</i>	2.89	2.03	30.4	C ₃₃ H ₃₄ N ₄ O ₃	535.2704	535.2683	447.2145 ^a
Purpurin-18 <i>a</i>	3.04	0.36	8.20	C ₃₃ H ₃₂ N ₄ O ₅	565.2446	565.2438	503.2435 ^b
15 ¹ -Hydroxy-lactone chlorophyll <i>b</i>	5.29	1.69	35.2	C ₅₅ H ₇₀ MgN ₄ O ₈	939.5117	939.5085	585.1899 ^c
13 ² -Hydroxy-chlorophyll <i>b</i>	5.79	5.00	49.9	C ₅₅ H ₇₀ MgN ₄ O ₇	923.5168	923.5118	569.4874 ^c
13 ² -Hydroxy-chlorophyll <i>b</i> '	5.96	4.90	49.9	C ₅₅ H ₇₀ MgN ₄ O ₇	923.5168	923.5119	569.4869 ^b
15 ¹ -Hydroxy-lactone chlorophyll <i>a</i>	6.38	3.76	18.8	C ₅₅ H ₇₂ MgN ₄ O ₇	925.5324	925.5332	553.2018 ^c
Chlorophyll <i>b</i>	6.42	3.67	32.9	C ₅₅ H ₇₀ MgN ₄ O ₆	907.5219	907.5252	569.2065 ^c

Chlorophyll <i>b</i> ′	6.71	3.20	35.4	C ₅₅ H ₇₀ MgN ₄ O ₆	907.5219	907.5251	569.2032 ^d
13 ² -Hydroxy-chlorophyll <i>a</i>	6.75	1.13	35.7	C ₅₅ H ₇₂ MgN ₄ O ₆	909.5375	909.5259	555.2202 ^c
Chlorophyll <i>a</i>	7.08	3.89	12.9	C ₅₅ H ₇₂ MgN ₄ O ₅	893.5426	893.5381	555.2247 ^c
Chlorophyll <i>a</i> ′	7.42	1.82	18.7	C ₅₅ H ₇₂ MgN ₄ O ₅	893.5426	893.5407	555.2187 ^d
15 ¹ -Hydroxy-lactone-pheophytin <i>a</i>	7.88	2.00	7.1	C ₅₅ H ₇₄ N ₄ O ₇	903.5630	903.5612	549.2518 ^c
13 ² -Hydroxy-pheophytin <i>a</i>	8.67	3.32	15.6	C ₅₅ H ₇₄ N ₄ O ₆	887.5681	887.5649	593.2712 ^c
13 ² -Hydroxy-pheophytin <i>a</i> ′	8.92	2.81	11.1	C ₅₅ H ₇₄ N ₄ O ₆	887.5681	887.5656	593.2714 ^b
Pheophytin <i>d</i>	9.17	0.10	15.4	C ₅₄ H ₇₂ N ₄ O ₆	873.5525	873.5526	595.2485 ^b
Pheophytin <i>a</i>	9.50	4.07	16.3	C ₅₅ H ₇₄ N ₄ O ₅	871.5732	871.5685	593.2796 ^c
Pheophytin <i>a</i> ′	9.71	2.00	8.5	C ₅₅ H ₇₄ N ₄ O ₅	871.5732	871.5749	593.2732 ^b
Phytol-purpurin-18 <i>a</i>	10.50	2.25	11.2	C ₅₃ H ₇₀ N ₄ O ₅	843.5419	843.5399	565.2465 ^b
Pyro-pheophytin <i>a</i>	10.58	0.30	43.1	C ₅₃ H ₇₂ N ₄ O ₃	813.5677	813.5675	535.2782 ^c

k: retention (capacity) factor. MW. Cal. means molecular weight calculated for [M+H]⁺. MW meas. means molecular weight measured for [M+H]⁺. Error and mSigma correspond to high accuracy measurements of each chlorophyll standard derivative that should be within the tolerance limits (<±5 ppm and <50. respectively). ^aIn agreement with Chen et al., 2015a. ^bProduct ions described by first time in this work (except for chlorophyll c₁ and c₂, whose *m/z* signals were found by Goericke et al., (2000), and in this paper it is proposed by first time their structural configuration). ^cIn agreement with Chen et al., 2015b. ^dAccording to Gauthier-Jaques et al., 2001 and Walker et al., 2003 ^eIt has been impossible to develop the MS² analysis due to the low concentration of the pigment in the sample.

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Table 2
Product ions (m/z) in the ESI/APCI-hrTOF-MS² spectra of chlorophyll derivative standards.

Compound ^a	Structure ^a	Fragmentations ^b									H ₂ O
		B	B+Y	C	E	J+MeOH	C+Y	C+Z	J+Z	F	
Chlorophyll <i>c</i> ₁	C	551.1861	535.1856								593.1996
Chlorophyll <i>c</i> ₂	D	549.1793	533.1752								591.1851
Pheophytin <i>d</i>	A			595.2485	535.2381	559.2378	567.2500				
Phytol-purpurin-18 <i>a</i>	E			565.2465				521.2591	503.2446	491.2064	
Purpurin-18 <i>a</i>	E							521.2551	503.2435		547.2314

^aSee Figure 1. ^bFollowing Van Breemen et al., 1991 and Chen et al. 2015a,b.

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Table 3.- Chlorophyll derivatives identified and quantified in *Porphyra umbilicalis*, *Ulva*, *Enteromorpha* spp., *Laminaria ochroleuca* and *Undaria pinnatifida* (mg/Kg d.w.±SD)

	<i>Porphyra umbilicalis</i>	<i>Ulva</i>	<i>Enteromorpha spp.</i>	<i>Laminaria ochroleuca</i>	<i>Undaria pinnatifida</i>
13 ² OH chlide <i>a</i>	+				
13 ² OH chl <i>c</i> ₂				+	
Chl <i>c</i> ₂				+	4.13±0.26
Chlide <i>a</i>	+				
15 ¹ Lact-pheo <i>a</i>	+				
Chl <i>c</i> ₁				33.17±2.67	129.86±5.32
Chl <i>c</i> ₁ '					10.34±0.21
15 ¹ Lact- pheo <i>a</i> '	+				
13 ² OH pheo <i>a</i>	33.95±4.00	9.90±0.57		21.27±3.04	95.8±10.80
13 ² OH pheo <i>a</i> '	57.23±7.56				
Pheo <i>a</i>	1086.32±146.20	101.63±10.70	364.88±15.65	177.84±18.17	1579.29±175.42
Pheo <i>a</i> '	183.17±17.10	10.63±2.23	27.90±1.24		467.72±39.71
Pyro pheo <i>a</i>	+				
Purpurin-18 <i>a</i>	6.21±1.87				
15 ¹ Lact-chl <i>b</i>		398.60±43.44	296.44±38.52		
13 ² OH chl <i>b</i>		253.27±10.88	315.02±23.58		
13 ² OH chl <i>b</i> '			86.57±13.24		
15 ¹ Lact- chl <i>a</i>	5.14±0.85			18.5±1.51	
Chl <i>b</i>		1907.47±178.35	1548.32±57.12		
Chl <i>b</i> '		371.44±3.91	517.53±10.90		
13 ² OH chl <i>a</i>	+	972.89±68.36	585.57±6.86	26.97±1.00	
Chl <i>a</i>	17.84±1.91	4440.71±271.39	4061.70±16.26	99.16±6.66	46.39±5.66
Chl <i>a</i> '	+	407.81±7.09	973.46±114.47	17.70±1.48	+
15 ¹ Lact- phy <i>a</i>	+	33.87±2.45		67.49±7.03	65.59±2.57
13 ² OH phy <i>a</i>	191.10±15.60	100.16±16.39	54.94±4.70	183.77±15.81	166.00±19.86
13 ² OH phy <i>a</i> '	9.46±0.97	76.71±6.19	25.50±2.24	146.90±12.64	146.58±17.96
Phy <i>d</i>	+			+	+
Phy <i>a</i>	836.44±66.81	443.44±62.70	545.14±41.50	1824.58±146.96	2711.47±252.00
Phy <i>a</i> '	165.80±11.20	87.82±8.80	7.72±1.61	355.98±37.74	557.61±61.33
Phytol -purp-18 <i>a</i>	14.35±1.28	7.34±1.44		11.47±1.10	24.69±2.17
Pyro phy <i>a</i>	111.98±9.59	8.29±1.29		21.02±3.60	37.7±3.32
Total chls	2718.98±242.52	9631.98±741.39	9410.71±151.75	3006.04±248.62	6043.15±576.07
<i>a/b</i> series	-	2.29±0.03	2.40±0.00	-	-
<i>a</i> series (%)	100.00±0.00	69.57±0.25	70.63±0.03	98.90±0.03	97.61±0.53
<i>b</i> series (%)	-	30.43±0.25	29.37±0.03	-	-
<i>c</i> series (%)	-	-	-	1.10±0.03	2.39±0.53
Chlorophylls (%)	0.85±0.10	90.87±0.95	89.10±0.01	6.52±0.32	3.29±0.32

Pheophorbides (%)	50.04±2.35	1.27±0.35	4.17±0.11	6.62±0.27	35.46±3.00
Pheophytins (%)	48.36±2.38	7.79±1.28	6.73±0.64	86.49±0.32	60.98±3.36
Purpurins (%)	0.76±0.07	0.08±0.02	-	0.38±0.01	0.41±0.03

⁺Chlorophyll compounds identified by HPLC-MS but below the quantification limit of HPLC-UV/Visible.

Compound	Structure	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
13 ² -Hydroxy-chlorophyllide <i>a</i>	A	CH ₂	CH ₃	OH	COOCH ₃	H	Mg
13 ² -Hydroxy-chlorophyll <i>c</i> ₂	D	CH ₂	CH ₃	OH	COOCH ₃	H	Mg
Chlorophyll <i>c</i> ₂	D	CH ₂	CH ₃	H	COOCH ₃	H	Mg
Chlorophyllide <i>a</i>	A	CH ₂	CH ₃	H	COOCH ₃	H	Mg
15 ¹ -Hydroxy-lactone pheo <i>a</i>	B	CH ₂	CH ₃	OH	(15 ²)COOCH ₃	H	2H
Chlorophyll <i>c</i> ₁	C	CH ₂	CH ₃	H	COOCH ₃	H	Mg
13 ² -Hydroxy-pheophorbide <i>a</i>	A	CH ₂	CH ₃	OH	COOCH ₃	H	2H
Pheophorbide <i>a</i>	A	CH ₂	CH ₃	H	COOCH ₃	H	2H
Pyro-pheophorbide <i>a</i>	A	CH ₂	CH ₃	H	H	H	2H
Purpurin-18 <i>a</i>	E	CH ₂	CH ₃	-	-	H	2H
15 ¹ -Hydroxy-lactone chlorophyll <i>b</i>	B	CH ₂	CHO	OH	(15 ²)COOCH ₃	phytyl	Mg
13 ² -Hydroxy chlorophyll <i>b</i>	A	CH ₂	CHO	OH	COOCH ₃	phytyl	Mg
15 ¹ -Hydroxy-lactone chlorophyll <i>a</i>	B	CH ₂	CH ₃	OH	(15 ²)COOCH ₃	phytyl	Mg
Chlorophyll <i>b</i>	A	CH ₂	CHO	H	COOCH ₃	phytyl	Mg
13 ² -Hydroxy-chlorophyll <i>a</i>	A	CH ₂	CH ₃	OH	COOCH ₃	phytyl	Mg
Chlorophyll <i>a</i>	A	CH ₂	CH ₃	H	COOCH ₃	phytyl	Mg
15 ¹ -Hydroxy-lactone pheophytin <i>a</i>	B	CH ₂	CH ₃	OH	(15 ²)COOCH ₃	phytyl	2H
13 ² -Hydroxy pheophytin <i>a</i>	A	CH ₂	CH ₃	OH	COOCH ₃	phytyl	2H
Pheophytin <i>d</i>	A	O	CH ₃	H	COOCH ₃	phytyl	2H
Pheophytin <i>a</i>	A	CH ₂	CH ₃	H	COOCH ₃	phytyl	2H
Phytyl-purpurin-18 <i>a</i>	E	CH ₂	CH ₃	-	-	phytyl	2H
Pyro-pheophytin <i>a</i>	A	CH ₂	CH ₃	H	H	phytyl	2H

A **B**