SEPARATION AND CHARACTERIZATION OF PHLOROTANNINS FROM BROWN ALGAE *Cystoseira abies-marina* BY COMPREHENSIVE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY.

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Abbreviations: D1, first dimension; **D2**, second dimension; **DMBA,** 2,4-dimethoxybenzaldehyde; **LC × LC**, comprehensive two-dimensional liquid chromatography; $\mathbf{n_{c2D}}$, two-dimensional peak capacity; **PFP**, pentafluorophenyl; **PGE**, phloroglucinol equivalents; **PGU**, phloroglucinol units; **TOC**, total organic carbon.

Keywords: brown algae, Cystoseira abies-marina, LC × LC, polymeric polyphenols, phlorotannins.

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23 **ABSTRACT.**

24 Phlorotannins are an important class of polyphenolic compounds only found in brown
25 algae. The chemical analysis of these bioactive polyphenols is rather difficult due to the
26 great chemical variability and complexity of the natural composition of these
27 components in algae, forming large phloroglucinol polymers. In the present work, a new
28 approach based on the use of comprehensive two-dimensional liquid chromatography
29 (LC × LC) is shown to analyze this complex family of compounds. The developed LC ×
30 LC methodology is based on the coupling of a HILIC-based separation in the first
31 dimension and a RP-based separation in the second dimension. The employment of this
32 on-line coupling together with diode array detection (DAD) and tandem mass
33 spectrometry (MS/MS) allowed the separation and identification of more than 50
34 compounds in a *Cystoseira abies-marina* brown alga extract. Phlorotannins containing
35 from 5 to 17 phloroglucinol units were identified in this sample by HILIC × RP-DAD-
36 MS/MS. Besides, using the 2,4-dimethoxybenzaldehyde (DMBA) assay, it was possible
37 to determine that the total amount of phlorotannins present in the extract was 40.2 mg
38 phloroglucinol equivalents per g of extract. To our knowledge, this work is the first
39 demonstration of the usefulness of HILIC × RP-DAD-MS/MS for the determination of
40 phlorotannins.

41

42

43 **1. INTRODUCTION.**

44 Brown algae (Phaeophyceae) contain a typical kind of secondary metabolites which are
45 classified within the phenolic compounds family, called phlorotannins. These
46 compounds, that may reach a high percentage of the algae dry mass (up to 15 %) [1],
47 are formed as polymers of phloroglucinol (1,3,5-trihydroxybenzene) of different size
48 and composition. There exist four main classes of phlorotannins: fuhalols and
49 phlorethols, containing an ether linkage; fucols, containing a phenyl linkage;
50 fucophlorethols, with an ether and a phenyl linkage; and eckols, which possess a
51 benzodioxin linkage [2]. Besides, it is also possible to find quite complex chemical
52 variability with compounds containing different degree of polymerization as well as
53 structure (linearly linked or branched). It is widely accepted that phlorotannins are
54 components of the algal cell walls that may be forming complexes with alginic acid,
55 although it is not completely clear if they exert a chemical defense function or just an
56 influence on cell wall construction [2]. The genus *Cystoseira* comprises more than 30
57 species and it is one of the most-important brown algae genera found in the
58 Mediterranean Sea and Atlantic Ocean ecosystems. *Cystoseira abies-marina* is one of
59 the species already identified as possessing some interesting compounds, such as
60 meroterpenoids [3] and fucoxanthin [4], although it is also a good potential source of
61 phenolic compounds, including phlorotannins.

62 These latter compounds have recently raised attention as algae have been pointed out as
63 a potential source of bioactive compounds potentially useful for the food and
64 pharmaceutical industries [5]. In this regard, some researches have already hinted that
65 phlorotannins may confer with different bioactivities, including antibacterial [6,7], anti-
66 diabetic [8], anti-proliferative [9], anti-inflammatory [10], antioxidant [11-13] as well as
67 chemopreventive activity [14]. Consequently, there is a great interest in determining

68 these compounds in the different species of brown algae that may contain them. Due to
69 the huge chemical variability already mentioned, the analysis of these components is
70 quite complex, and it is rather common to roughly estimate the phlorotannins content in
71 algae by using colorimetric methods [10,15-17]. To partially solve the problems
72 associated to those methods, mainly, the little information on chemical composition that
73 they provide as well as the relatively low accuracy associated to most colorimetric
74 methods, some approaches involving the use of liquid chromatography-based methods
75 have been developed [17-20], frequently using MS-based detection. Considering the
76 high degree of hydrophilicity of these polymeric compounds, hydrophilic-interaction
77 liquid chromatography (HILIC) methods have also been recently applied [2,21] with the
78 aim to increase the resolution among the different phlorotannins contained in complex
79 algal samples. Despite these efforts, the chemical characterization of brown algae in
80 terms of phlorotannins composition is not well-known yet [22].
81 The use of multidimensional techniques, such as comprehensive two-dimensional LC
82 (LC × LC) may be an effective alternative to carry out this kind of characterization of
83 very complex samples. In fact, this technique has already been shown to possess a great
84 potential to analyze complex food and natural samples [23,24]. LC × LC is based on the
85 on-line coupling of two independent separation mechanisms through which the whole
86 sample is analyzed. In this sense, different couplings may be employed in the two
87 dimensions, including e.g., reversed phase, normal phase or HILIC-based separations
88 [25]. Our group has previously presented two novel LC × LC approaches to separate
89 and identify procyanidins, which are also polymeric phenolic compounds, from
90 complex food matrices such as grape seeds [26] and apples [27], combining the use of a
91 HILIC separation in the first dimension (D1) and a RP approach in the second
92 dimension (D2) together with the employment of tandem MS/MS detection. This type

93 of approach allowed the separation in terms of degree of polymerization in the D1 and
94 according to differential hydrophobicity in the D2. Following, this idea, the aim of the
95 present work is to develop a new method based on a HILIC × RP-DAD-MS/MS
96 coupling to separate and identify the phlorotannins present in *Cystoseira abies-marina*
97 brown algae. To the best of our knowledge, this is the first time that a LC × LC method
98 is developed and used to analyze phlorotannins.

99

100 **2. MATERIALS AND METHODS**

101 **2.1. Samples and chemicals**

102 *Cystoseira abies-marina* brown algae were obtained from the Spanish Bank of Algae
103 (Marine Biotechnology Center, University of Las Palmas de Gran Canaria, Gran
104 Canaria, Spain). Algae were sun-dried and stored protected from oxygen, light and
105 moisture until use.

106 Acetonitrile, methanol, dichloromethane and 2-propanol were of HPLC-grade and
107 acquired from VWR Prolabo (Barcelona, Spain), whereas acetone was from Lab-Scan
108 (Dublin, Ireland). Acetic acid, formic acid and 2,4-dimethoxybenzaldehyde (DMBA)
109 were obtained from Sigma Aldrich (Madrid, Spain). Hydrochloric acid was acquired
110 from Probus (Barcelona, Spain), whereas ammonium acetate was supplied from Panreac
111 (Barcelona, Spain). Ultra-pure water quality (resistivity of 18.2 MΩcm at 25 °C) with
112 1–5 ppb total organic carbon (TOC) was produced in-house using a laboratory water
113 purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA, USA).
114 Phloroglucinol and quercetin rutinoside reference standards were purchased from
115 Extrasynthèse (Genay, France).

116

117 **2.2. Sample preparation.**

118 The extraction of the phenolic compounds from *Cystoseira abies-marina* was carried
119 out employing a previously described protocol slightly modified [21]. Briefly, the alga
120 was freeze-dried (LyoBeta 15, Telstar, Terrassa, Spain) and ground. 30 g of the dried
121 powder were extracted with 300 mL of acetone/water (70:30, *v/v*) by magnetic stirring
122 during 45 min in darkness. Afterwards, the supernatant was decanted and the remaining
123 residue was extracted three times more with 100 mL of solvent. The supernatants were
124 pooled and the acetone was removed by rotary evaporation (Rotavapor R-210, Buchi
125 Labortechnik AG, Flawil, Switzerland). Next, the aqueous extract was defatted three
126 times with dichloromethane (1:1, *v/v*), collecting the aqueous phases. The phenolic
127 fraction of the aqueous extract was concentrated using Discovery DSC-18 6 mL solid
128 phase extraction (SPE) cartridges (Supelco, Bellefonte, PA, USA). SPE cartridges were
129 conditioned with 12 mL of methanol and with 18 mL of water. Then, 12.5 mL of
130 sample were loaded in the SPE cartridge, rinsed with 20 mL of water, and finally, the
131 polyphenols were eluted with 30 mL of acetone/water (70:30, *v/v*). Lastly, acetone was
132 evaporated again by rotary evaporation, and the remaining aqueous extract was
133 lyophilized.
134
135 **2.3. Determination of total phlorotannins.**
136 To estimate the amount of total phlorotannins content in the brown alga, the 2,4-
137 dimethoxybenzaldehyde (DMBA) colorimetric assay was employed [10]. Briefly, a
138 DMBA solution was prepared just prior use by mixing equal volumes of 2% DMBA
139 reagent in acetic acid (*m/v*) and 6% hydrochloric acid in acetic acid (*v/v*). 50 µL of
140 sample (0.075 mg mL^{-1}) were mixed with 250 µL of DMBA solution in a 96-well
141 microplate. The reaction was conducted at room temperature in the dark for 60 min.
142 After this time, the absorbance was read at 515 nm using a microplate

143 spectrophotometer reader Powerwave XS (Bio Tek, Winooski, VT). Blanks with 50 μL
144 of water instead of sample and a control samples without DMBA solution were also
145 included. All samples, blanks and controls were prepared in triplicate. The
146 concentration of total phlorotannins was estimated from a calibration curve using
147 phloroglucinol (0.98 – 62.5 μg mL^{-1}). Data were presented as the average of triplicate
148 analyses expressed as mg phloroglucinol equivalents (PGE) g^{-1} dry matter.

149

150 **2.4. Comprehensive two-dimensional liquid chromatography (LC × LC) analysis of**
151 **phlorotannins.**

152 *2.4.1. Instruments.*

153 LC × LC analyses were carried out on an Agilent 1200 series liquid chromatograph
154 (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector and an
155 autosampler. A Protecol flow splitter (SGE Analytical Science, Milton Keynes, UK)
156 was placed between the first dimension pumps and the autosampler in order to have
157 robust and reproducible low flow rates and gradients in the D1. Besides, an additional
158 LC pump (Agilent 1290 Infinity) was coupled to this instrument to perform the second
159 dimension separations, hyphenated through an electronically-controlled two-position
160 ten-port switching valve. An Agilent 6320 Ion Trap mass spectrometer equipped with
161 an electrospray interface was coupled on-line and operated in negative ionization mode
162 using the following conditions: dry temperature, 350ºC; mass range, m/z 90–2200 Da;
163 dry gas flow rate, 12 L min^{-1}; nebulization pressure, 40 psi. The LC data were
164 elaborated and visualized in two and three dimensions using LC Image software
165 (version 1.0, Zoex Corp., Houston, TX).

166

167 *2.4.2. LC × LC separation conditions.*

168 Samples were prepared at 12 mg mL^{-1} of the extract obtained as described in section 2.2
169 in MeOH/ACN, 3:7 (*v/v*) and filtered through 0.45 μm nylon syringe filters (Análisis
170 vínicos, Tomelloso, Spain) before injection.
171 In the D1, a Lichrospher diol-5 (150 × 1.0 mm, 5 μm d.p., HiChrom, Reading, UK)
172 column was employed with a precolumn with the same stationary phase. The flow rate
173 employed was 15 μL min^{-1}. The mobile phases were (A) acetonitrile/acetic acid (98:2,
174 *v/v*) and (B) methanol/water/acetic acid (95:3:2, *v/v/v*) used according to the following
175 gradient: 0 min, 0% B; 3 min, 0% B; 5 min, 7% B; 30 min, 15% B; 70 min, 15% B; 75
176 min, 25% B; 85 min, 25% B. The injection volume was 20 μL.
177 In the D2, two different columns were tested, namely, an Ascentis Express C_{18} partially
178 porous column (50 × 4.6 mm, 2.7 μm d.p., Supelco, Bellefonte, CA) with a C_{18}
179 precolumn, and a Kinetex PFP partially porous particles column (50 × 4.6 mm, 2.6 μm
180 d.p., Phenomenex, Torrance, CA). During the whole LC × LC separation, 78 s-
181 repetitive D2 gradients were employed, being also 78 s the modulation time
182 programmed in the switching valve. The wavelength used to monitor the separations
183 was 280 nm, although UV–Vis spectra were collected from 190 to 550 nm during the
184 whole analysis using a sampling rate of 20 Hz in the DAD. The MS was operated under
185 negative ESI mode. The mobile phases employed in the D2 analysis of both columns
186 consisted of water (0.1% formic acid, A) and acetonitrile (B) eluted according to the
187 following gradients: 0 min, 0% B; 0.1 min , 10% B; 0.6 min , 30% B; 0.8 min , 50% B;
188 0.9 min , 70% B; 1 min , 90%; 1.01 min , 0% B; 1.3 min , 0% B for the C_{18} column, and
189 0 min , 0% B; 0.1 min , 5% B; 0.3 min , 20% B; 0.8 min , 40% B; 0.9 min, 70% B; 1
190 min, 90%; 1.01 min, 0% B; 1.3 min , 0% B for de PFP column. The flow rate employed
191 was always 3 mL min^{-1}. The flow eluting from the D2 column was splitted before

192 entering the MS instrument, so that the flow rate introduced in the MS detector was 600
193 μL min^{-1}.
194
195 **3. RESULTS AND DISCUSSION**
196 Phorotannins estimation is commonly carried out by using colorimetric methods
197 [10,15,16]. In this work, the DMBA assay was used as a starting point to determine the
198 phlorotannins content in the *Cystoseira abies-marina* extract (see section 2.3). Using
199 this approach, the amount of phlorotannins determined was 40.2 mg phloroglucinol
200 equivalents g^{-1} extract. As mentioned above, comprehensive two-dimensional coupling
201 using a HILIC-based separation in the D1 and a RP-based separation in the D2 could
202 potentially solve many of the problems commonly encountered when analyzing
203 phlorotannins. This combination is characterized by providing a high degree of
204 orthogonality [26] at the same time that completely miscible mobile phases are
205 employed in the two dimensions. Besides, the capabilities of HILIC followed by RP-LC
206 to separate complex mixtures of polymeric phenolic compounds have been already
207 shown, both using on-line [26,27] and off-line couplings [28,29]. Moreover, the on-line
208 approach followed by the direct hyphenation to several detectors, such as DAD and MS
209 detectors, gives rise to a powerful analytical system whose use is mandatory if complex
210 samples have to be analyzed. In this work a HILIC × RP-DAD-MS/MS approach is
211 proposed to characterize the phlorotannins composition from brown algae. Since the
212 sample as well as the target compounds have not been previously studied using this
213 approach, a complete optimization of the separation and coupling conditions was firstly
214 needed.
215
216 **3.1. HILIC-based D1 separation optimization.**

217 The LC × LC instrument set-up employed in this work is based on the use of two
218 identical injection loops installed in a 10-port 2-position switching valve that is used as
219 modulator. This device makes possible the physical coupling between both dimensions.
220 Under this configuration, one of the injection loops injects the eluate collected from the
221 D1 to the D2 whereas the other loop is collecting new eluate, so that the complete
222 collection and transfer of the whole effluent from the D1 to the D2 is performed. This
223 implies that each D2 separation should be completely finished before the collecting
224 injection loop is filled with the D1 eluate. For this reason, in this kind of couplings, the
225 use of microbore columns in the first dimension is highly recommended. By using this
226 kind of columns, a very low D1 flow rate can be employed, in order to give enough time
227 for the D2 separation to be completed while the transfer volume is maintained as small
228 as possible. To perform the HILIC separation in the first dimension, a microbore
229 column with diol particles was selected.

230 To carry out the optimization of the separation, the whole phlorotannins purified extract
231 from *Cystoseira abies-marina* was injected and the conditions previously employed to
232 separate the complete profile of apple polyphenols were used [27]. As expected, under
233 these conditions, using a flow rate of 15 μl min^{-1}, the separation obtained was not
234 satisfactory. Next, different new gradients using the same mobile phases (A,
235 ACN/acetic acid 98:2, *v/v*; B, methanol/water/acetic acid 95:3:2, *v/v/v*) as well as other
236 different mobile phases were tested. Namely, 10 mM ammonium acetate pH 9 was also
237 tested as mobile phase B as well as other different proportions of methanol and water in
238 that mobile phase. After a close study to the profiles obtained, the mobile phases
239 ACN/acetic acid 98:2, *v/v* (A) and methanol/water/acetic acid 95:3:2, *v/v/v* (B), were
240 selected optimizing next the gradient in order to obtain a better separation of this
241 complex sample (see section 2.4.2.). In Figure S1 (supplementary information), a

242 comparison is shown between the initial (Figure S1A) and the final selected conditions
243 for the D1 analysis (Figure S1B). As can be observed, the separation of the complex
244 profile obtained is not completely resolved. It is interesting to mention that, at this stage
245 of the LC × LC optimization, a complete baseline separation of all the components in
246 the D1 is not aimed, but to obtain a good distribution of all of them in the time, in order
247 to be able to collect those peaks and be injected and their components separated in the
248 D2.

249

250 **3.2. RP-based D2 separation optimization.**

251 For the second dimension, two different types of stationary phases and columns were
252 studied with the aim to compare their performances under LC × LC conditions. Namely,
253 a partially porous C_{18} short column which had already shown its potential in LC × LC
254 [26,27,30,31] and a partially porous pentafluorophenyl (PFP) short column, that has
255 been pointed out as a possible new alternative for the efficient separation of phenolic
256 compounds [32, 33]. In this regard, the optimization of the final analytical conditions in
257 each case was carried out separately. To do that, the whole sample was directly injected
258 in the second dimension column. Although these analytical conditions are not exactly
259 equal to those taking place during the LC × LC analysis, in which only fractions of the
260 sample will be separated in each D2 analysis, this step injecting the whole sample will
261 provide important information on the D2 conditions that produce a better separation of
262 the sample components. Once these conditions are selected, further confirmation or fine
263 tuning of the separation conditions are needed directly performing two-dimensional
264 analyses.
265 The mobile phases selected were water (0.1% formic acid, A) and acetonitrile (B) for
266 both C_{18} and PFP columns, although different gradients were chosen. The use of other

solvents in the mobile phase B, such as 2-propanol in different proportions or mixtures between acetonitrile and methanol did not improve the separations obtained and significantly increased the backpressure obtained (mainly when 2-propanol was used).

3.3. Overall HILIC × RP optimization and method performance.

Once the two dimensions were optimized separately, the fine tuning of the coupling conditions was performed. To do that, two identical 30 μl internal volume injection loops were installed in the 10-port 2-position switching valve acting as modulator. This internal volume allowed the complete transfer of eluate from the D1 to the D2 in each modulation period (1.3 min, 19.5 μl). In this regard, it is worth to mention that although 20 μl loops would have been enough to collect all the effluent in the modulation time, we have previously observed a beneficial influence when the injection volume in the D2 of HILIC × RP methods was slightly increased [26]. These observations were also confirmed in the present approach. In fact, the dilution of the eluent from the D1 with D2 mobile phase (up to 30 μl) just before the injection permitted to minimize the negative effects on band broadening and retention derived of using as injection solvent a stronger solvent than the initial mobile phase. It is necessary to remark that under LC × LC conditions, being an on-line system, the injection solvent in the D2 is fixed by the D1 and cannot be modified. Moreover, the set-up was completed by coupling a MS detector at the exit of the DAD after the D2 separation. Considering the high flow rates used in the D2, the inclusion of a flow splitter was necessary in order to reduce the flow rate entering the ESI interface to 600 μl min^{-1}, which is more suitable for a proper ionization of the target compounds.

Subsequently, the *Cystoseira abies-marina* phlorotannins extract was injected and analyzed using the two different optimized set-ups, namely diol × C_{18} and diol × PFP

292 configurations. Figure 1 shows the obtained 2D-plots corresponding to both
293 configurations. As it can be observed in that figure, both set-ups were able to provide
294 adequate separations of such a complex mixture. The separation in the first dimension
295 (same conditions for both configurations) was produced according to the degree of
296 polymerization of phlorotannins, whereas in the D2, the separation was obtained in
297 terms of relative hydrophobicity. It is possible to observe in the figures how the use of
298 LC x LC permits the resolution of co-elutions in both dimensions, such as the coelution
299 of peaks 12, 13, 14 and 17 in the first dimension, or the coelution of peaks 21, 30, 33
300 and 44 in the second dimension (see Figure 1A), making possible the separation of
301 components that, otherwise, would not be separated using monodimensional
302 separations.

303 Although the separation mode and mobile phases were the same, it can be clearly
304 observed how the two tested columns produced different profiles under the selected
305 conditions, showing differential retention. This is mainly due to the use in the PFP short
306 column of fluorine atoms in the periphery of a phenyl ring which are highly
307 electronegative, in contrast to the long C_{18} alkyl chain. As it can be appreciated in the
308 figure, using the C_{18} column a better separation among the different peaks could be
309 obtained in the D2 (Figure 1A), compared to the PFP column (Figure 1B). To the best
310 of our knowledge this is the first application of a PFP column in LC × LC, showing
311 acceptable capabilities for its coupling to HILIC separations. Comparing the two
312 separations, different peak capacities values can be obtained. It is important to remark
313 that this value is just a theoretical measure of the performance of the system that does
314 not necessarily describes what actually happens in practice. In fact, although there are
315 several methods for measuring peak capacity in a LC × LC system, normally, it is
316 assumed that the peaks are homogeneously distributed across the 2D plane, which is

317 obviously a great source of error. Anyhow, this value helps to compare different LC ×
318 LC set-ups or methods. In this regard, the peak capacity (n_{c2D}) was measured for the two
319 instrumental set-ups under the optimized conditions, obtaining values of theoretical
320 peak capacity [34] of 1248 and 902 for the diol × C_{18} and diol × PFP configurations,
321 respectively. Following the approach developed by Li et al. [35] that considers the D2
322 time cycle as well as the influence of undersampling of the D1 eluate, the values
323 obtained for the diol × C_{18} and diol × PFP were 992 and 739, respectively, showing the
324 great potential capabilities of both developments. Peak capacity values also show the
325 better performance of the C_{18} column in the D2 compared to the PFP column under the
326 selected conditions.
327

328 **3.4. *Cystoseira abies-marina* phlorotannins characterization.**

329 As already mentioned, the main difficulty to analyze phlorotannins is the great
330 heterogeneity and chemical variability within this family of compounds due to the
331 differential degree of polymerization as well as the diverse bonds between monomers.
332 In this regard, the use of LC × LC allows the attainment of a distribution along the D1
333 in order to separate smaller groups of components into the D2. Table 1 summarizes the
334 information corresponding to the assigned compounds. As it can be observed in Table 1,
335 phlorotannins from a degree of polymerization of 5 phloroglucinol units (PGU) up to 17
336 PGU were separated and assigned. In total, 43 different phlorotannins were tentatively
337 identified according to their MS and MS/MS spectra as well as their position in the 2D
338 plane. Besides, a flavonoid, quercetin rutinoside, was also found in the extract.
339 Additionally, 8 other compounds were detected in the sample, although no proper
340 identification of these compounds was possible. The highest number of compounds of a
341 same degree of polymerization corresponded to 5 PGU (peaks 1-8), whereas the most-

342 intense compound was a phlorotannin containing 7 PGU (peak 13). Phorotannins of less
343 than 5 PGU were not detected, in line with the phlorotannins composition in other
344 brown algae, where just high degree of polymerization phlorotannins were found [21].
345 Phlorotannins assignment was performed thanks to the detection of typical $[M-H]^-$ ions
346 together with MS/MS fragments corresponding to phlorotannin structures.
347 Phlorotannins containing 5 PGU were detected as $[M-H]^-$ at m/z 621, with typical
348 fragments of m/z 603 corresponding to the loss of water, m/z 495, in agreement with the
349 loss of a phloroglucinol, m/z 373 assigned to the loss of 2 PGU and m/z 228 that
350 corresponded to a dehydrated fragment containing 2 PGU. Figure 2 shows the MS
351 spectra as well as the MS/MS fragmentation pattern of peak 3 as well as its tentative
352 chemical structure (branching not unequivocally confirmed). Similar losses and
353 fragments were detected for phlorotannins with 6-11 PGU, except in the compounds
354 containing 7 PGU from which no fragments were clearly produced beyond the loss of a
355 water molecule.
356 On the other hand, phlorotannins having 13-17 PGU were detected as doubly charged
357 ions, as can be observed in Table 1. The different ion charge states were detected thanks
358 to the presence of specific ions in the MS spectra, as it is highlighted in Figures 2 and 3
359 (see in the MS spectra the isotopes of the molecular ion separated by a difference of 1 or
360 0.5 for the singly-charged or doubly-charged, respectively). These latter multi-charged
361 compounds were also identified according to the detection of different fragments
362 corresponding to phlorotannin fragments of smaller PGU, which allow confirming the
363 identifications. For example, in the case of phlorotannins of 13 PGU, a doubly-charged
364 ion was detected at m/z 807 indicating the possible presence of this type of polymer.
365 The fragmentation pattern of that ion produced ions at m/z 1365 (11 PGU), 1115 (9
366 PGU), 993 (8 PGU), 975 (8 PGU dehydrated), 867 (7 PGU), 745 (6 PGU), 619 (5 PGU)

367 and 228 (2PGU dehydrated) that completed the identification. The same behavior was
368 observed for one of the phlorotannins containing 12 PGU (peak 39), as shown in Figure
369 3. Moreover, as previously indicated, the relative position of each peak in the 2D plane
370 helped to conclude the identification; as can be observed in Figure 1B, the different
371 polymers were clearly separated according to their degree of polymerization along the
372 D1 analysis time, being the compounds of a similar size grouped together.

373

374 **4. CONCLUDING REMARKS.**

375 This contribution shows the first application of LC × LC to analyze phlorotannins, a
376 family of complex algal polyphenolic compounds. The coupling between a HILIC-
377 based separation in the D1 and a RP-based separation in the D2 provides a high degree
378 of orthogonality at the same time that produces a distribution according the degree of
379 polymerization of phlorotannins in the D1 that facilitates their separation in the D2. The
380 optimized HILIC × RP-DAD-MS/MS approach has been demonstrated to be useful for
381 the separation and identification of more than 50 compounds in a *Cystoseira abies-*
382 *marina* brown alga extract. Besides, two different set-ups, involving different D2
383 columns were tested. Although partially porous C_{18} column produced the best results in
384 terms of separation capabilities, a partially porous PFP column was also applied for the
385 first time in a LC × LC development, with acceptable results. Once this method has
386 been developed and its applicability demonstrated, it can be expected its future use for
387 the determination of phlorotannins in different brown algae as well as to determine the
388 influence of the algal growing conditions on the composition of these bioactive
389 compounds, which currently is an important analytical challenge.

390

391

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398

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400 **5. REFERENCES.**

401 [1] Koivikko, R., Loponen, J., Pihlaja, K., Jormalainen, V., *Phytochem. Anal.* 2007, *18*,
402 326-332.

403 [2] Kim, S. M., Kang, S. W., Jeon, J. S., Jung, Y. J., Kim, W. R., Kim, C. Y., Um, B.
404 H., *Food Chem.* 2013,*138*, 2399-2406.

405 [3] Gouveia, V. L. M., Seca, A. M. L., Barreto, M. C., Neto, A. I., Kijjoa, A., Silva, A.
406 M. S., *Phytochem. Lett*. 2013, *6*, 593-597.

407 [4] Moraes, E. P., Ruperez, F. J., Plaza, M., Herrero, M., Barbas, C., *Electrophoresis.*
408 2011, *32*, 2055-2062.

409 [5] Ibañez, E., Cifuentes, A., *J. Sci. Food Agric*. 2013, *93*, 703-709.

410 [6] Sandsdales, E., Haug, T., Stensvag, K., Styrvold, O. B., *World J. Microbiol.*
411 *Biotechnol.* 2003, *19*, 777-782.

412 [7] Lopes, G., Pinto, E., Andrade, P. B., Valentao, P., *PLOS One* 2013, *8,* e72203.

413 [8] Lee, S. H., Jeon, Y. J., *Fitoterapia* 2013, *86*, 129-136.

414 [9] Nwosu, F., Morris, J., Lund, V. A., Stewart, D., Ross , H. A., McDougall, G. J.,
415 *Food Chem.* 2011, *126,* 1006-1012.

416 [10] Lopes, G., Sousa, C., Silva, L. R., Pinto, E., Andrade, P. B., Bernardo, J., Mouga,
417 T., Valentao, P., PLOS One 2012, *7*, e31145.

418 [11] Wang, T., Jonsdottir, R., Liu, H., Gu, L., Kristinsson, H. G., Raghavan, S.,
419 Olafsdottir, G., *J. Agric. Food Chem.* 2012, *60*, 5874-5883.

420 [12] Tierney, M. S., Smyth, T. J., Hayes, M., Soler-Vila, A., Croft, A. K., Brunton, N.,
421 *Int. J. Food Sci. Technol.* 2013, *48*, 860-869.

422 [13] Kang, S. M., Lee, S. H., Heo, S. J., Kim, K. N., Jeon, Y. J., *Nutr. Res. Pract.* 2011,
423 5, 495-502.

424 [14] Parys, S., Kehraus, S., Krick, A., Glombitza, K. W., Carmeli, S., Klimo, K.,
425 Gerhauser, C., Konig, G. M., *Phytochem.* 2010, *71*, 221-229.

426 [15] Stern, J. L., Hagerman, A. E., Steinberg, P. D., Winter, F. C., Estes, J. A., *J. Chem.*
427 *Ecol.* 1996, *22*, 7.

428 [16] Parys, S., Rosenbaum, A., Kehraus, S., Reher, G., Glombitza, K., König, G. M., *J.*
429 *Nat. Prod.* 2007, *70,* 1865-1870.

430 [17] Koivikko, R., Eränen, J. K., Loponen, J., Jormalainen, V., *J. Chem. Ecol.* 2008, *34*,
431 54-64.

432 [18] Ferreres, F., Lopes, G., Gil-Izquierdo, A., Andrade, P.B., Sousa, C., Mouga, T.,
433 Valentao, P., *Mar. Drugs* 2012, *10*, 2766-2781.

434 [19] Liu, H., Gu, L., *J. Agric. Food Chem.* 2012, *60*, 1326-1334.

435 [20] Tierney, M. S., Soler-Vila, A., Rai, D. K., Croft, A. K., Brunton, N. P., Smyth, T.
436 J., *Metabolomics,* in press, DOI: 10.1007/s11306-013-0584-z.

437 [21] Steevenz, A. J., Mackinnon, S. L., Hankinson, R., Craft, C., Connan, S., Stengel,
438 D. B., Melanson, J. E., *Phytochem. Anal.* 2012, *23*, 547-553.

439 [22] Isaza Martinez, J. H., Torres Castañeda, H. G., *J. Chromatogr. Sci.* 2013, *51*, 825-
440 838.

441 [23] Dugo, P., Kumm, T., Cacciola, F., Dugo, G., Mondello, L., *J. Liq. Chromatogr.*
442 *Rel. Technol.* 2008, *31*, 1758-1807.

443 [24] Herrero, M., Ibañez, E., Cifuentes, A., Bernal, J., *J. Chromatogr. A* 2009, *1216*,
444 7110-7129.

445 [25] Donato, P., Cacciola, F., Tranchida, P. Q., Dugo, P., Mondello, L., *Mass Spectrom.*
446 *Rev.* 2012, *31*, 523-559.

447 [26] Montero, L., Herrero, M., Prodanov, M., Ibáñez, E., Cifuentes, A., *Anal. Bioanal.*
448 *Chem.* 2013, *405*, 4627-4638.

449 [27] Montero, L., Herrero, M., Ibáñez, E., Cifuentes, A., *J. Chromatogr. A* 2013, *1313*,
450 275-283.

451 [28] Kalili, K. M., de Villiers, A., *J. Chromatogr. A* 2009, *1216*, 6274-6284.

452 [29] Kalili, K. M., de Villiers, A., *J. Chromatogr. A* 2013, *1289*, 58-68.

453 [30] Dugo, P., Cacciola, F., Herrero, M., Donato, P., Mondello, L., *J. Sep. Sci.* 2008, *31*,
454 3297-3308.

455 [31] Dugo, P., Cacciola, F., Donato, P., Airado-Rodriguez, D., Herrero, M., Mondello,
456 L., *J. Chromatogr. A* 2009, *1216,* 7483-7487.

457 [32] Regos, I., Treutter, D., *J. Chromatogr. A* 2010, *1217*, 6169-6177.

458 [33] Manns, D. C., Mansfield, A. K., *J. Chromatogr. A* 2012, *1251*, 111-121.

459 [34] Neue, U. D., *J. Chromatogr. A* 2005, *1079*, 153-161.

460 [35] Li, X., Stoll, D. R., Carr, P. W., *Anal. Chem.* 2009, *81*, 845-850.

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463 **FIGURE LEGENDS.**

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465 **Figure 1.** Two-dimensional plot (280 nm) of the *Cystoseira abies-marina* phlorotannins
466 extract obtained using the optimized diol × C_{18} set-up (A) and the diol × PFP set-up (B).
467 Areas marked correspond to phlorotannins containing the same number of
468 phloroglucinol units (PGU). For peak identification, see Table 1.

469

470 **Figure 2**. MS spectrum and MS/MS fragmentation pattern of peak 3 (phlorotannin with
471 5 PGU) as well as the tentatively proposed chemical structure.

472

473 **Figure 3.** MS spectrum and MS/MS fragmentation pattern of peak 39 (phlorotannin
474 with 12 PGU) as well as the tentatively proposed chemical structure.

475

476 **Figure S1 (Supplementary information).** Chromatograms (280 nm) corresponding to
477 the *Cystoseira abies-marina* phlorotannins extract obtained in first dimension under
478 initial (A) and optimized (B) HILIC conditions.

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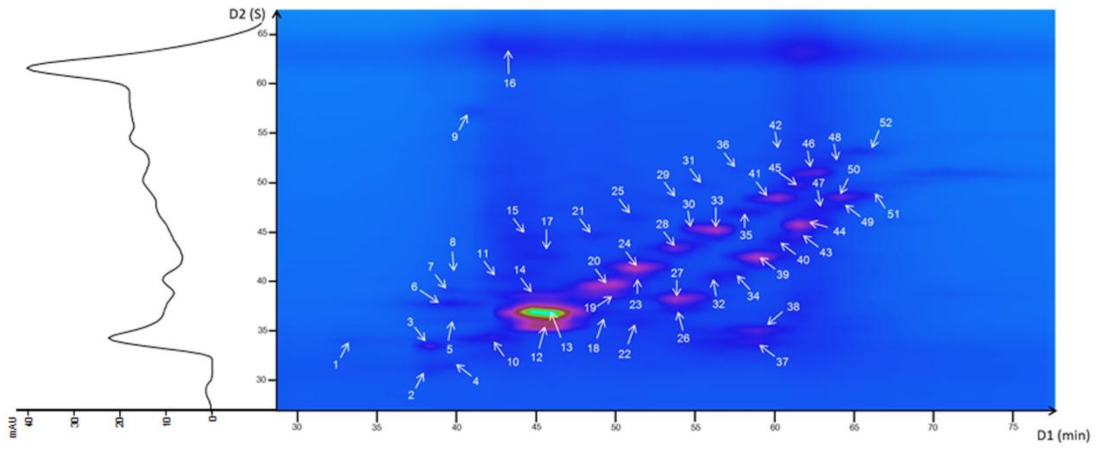

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489 Figure 1A.

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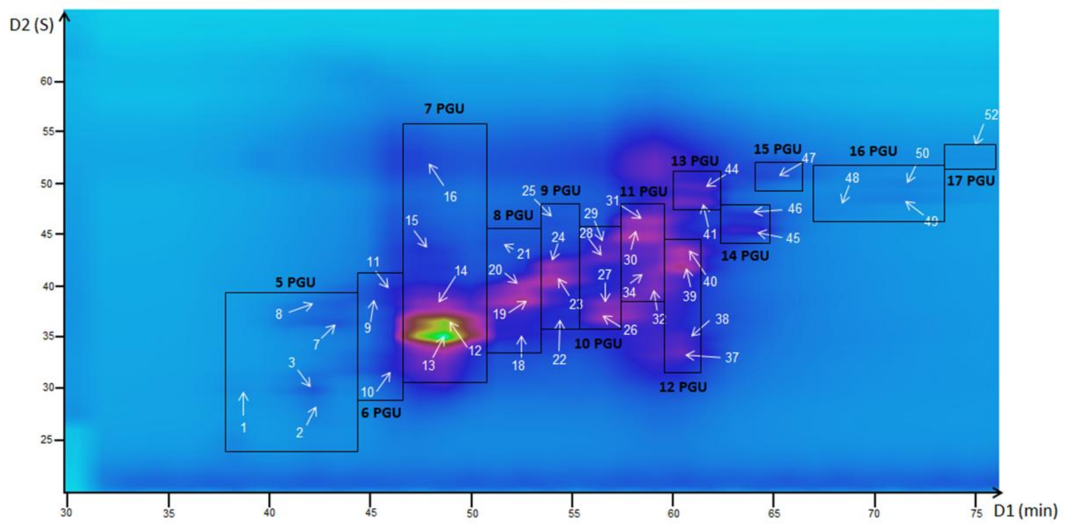

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493 Figure 1B.

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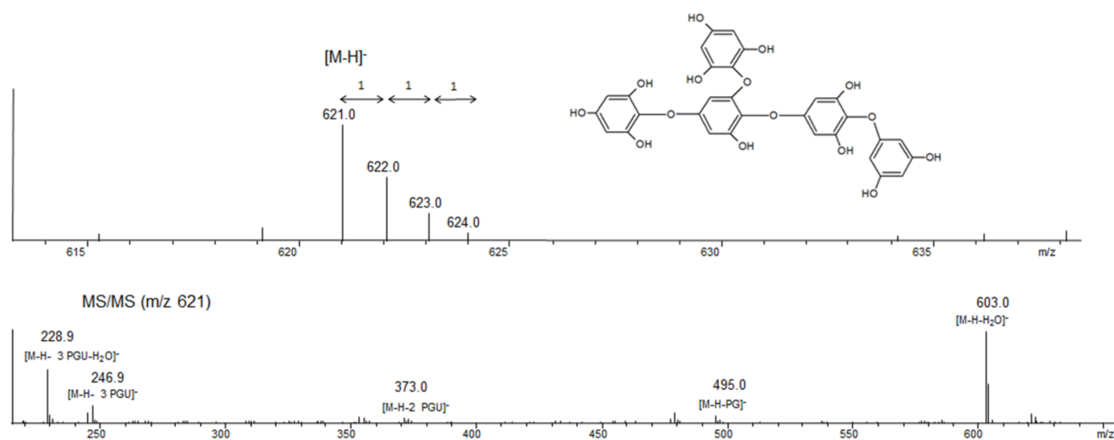

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501 Figure 2.

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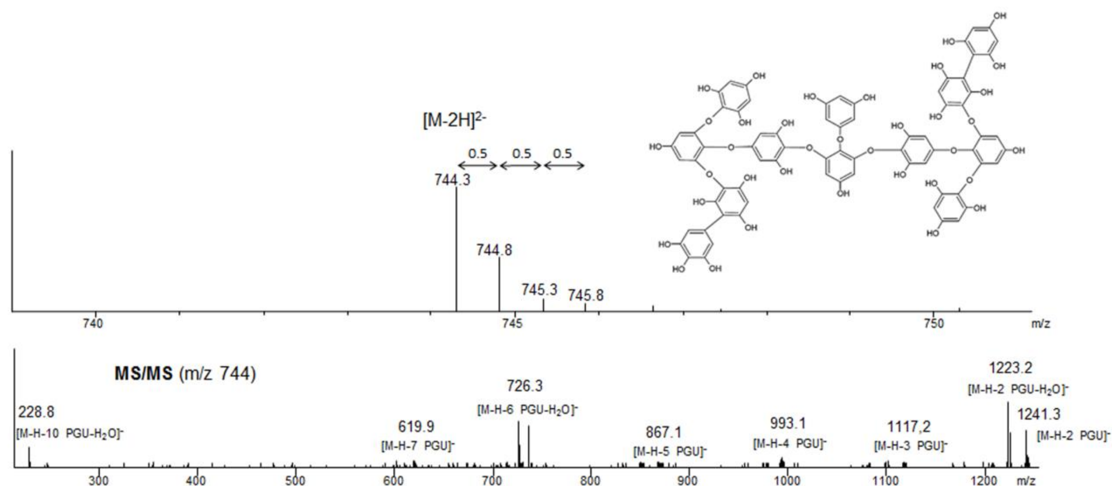

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520 Figure 3.

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538 **Figure S1 (Supplementary information).**

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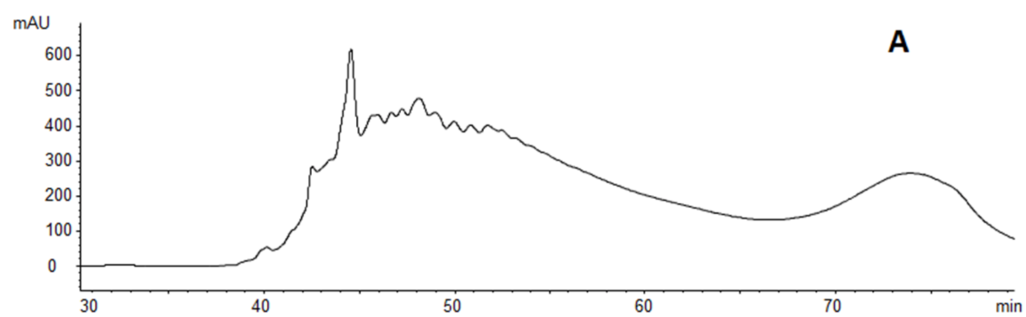

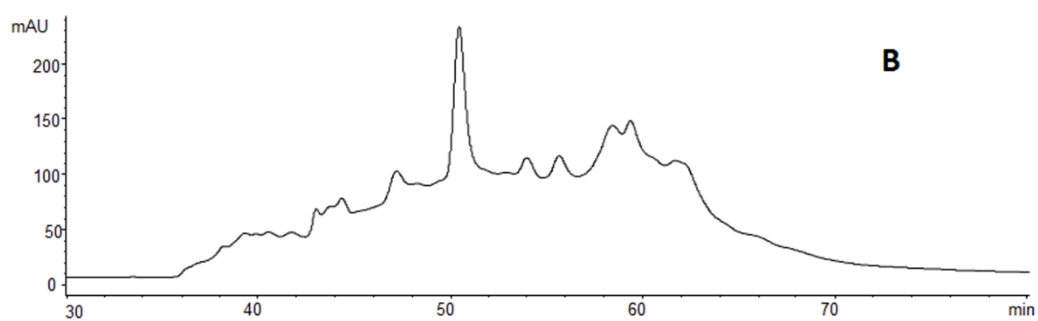

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541 **Table 1.** Peak assignments of the purified *Cystoseira abies-marina* phlorotannins extract analyzed using the diol x C_{18} set-up under optimized conditions. For peak numbers,
542 see Figure 1. PGU, phloroglucinol units.

Peak	Identification	Total t_R (min)	D2 t_R(s) ± sd	$[M-H]^-$	$[M-2H]^{2-}$	Main MS/MS fragments
1	Phlorotannin - 5 PGU	33.07	34.10 ± 0.22	621.6		603, 495, 373, 229
2	Phlorotannin - 5 PGU	38.22	31.08 ± 0.14	621.4		603, 479, 353, 247, 229
3	Phlorotannin - 5 PGU	38.26	33.33 ± 0.13	621.0		603, 495, 373, 247, 229
4	Phlorotannin - 5 PGU	39.52	31.17 ± 0.15	621.7		603, 246, 229
5	Phlorotannin - 5 PGU	39.60	35.83 ± 0.16	621.8		603, 495, 230
6	Phlorotannin - 5 PGU	39.63	37.60 ± 0.15	621.3		601, 495, 371, 229
7	Phlorotannin - 5 PGU	39.65	38.90 ± 0.10	621.3		601, 495, 371, 229
8	Phlorotannin - 5 PGU	39.67	40.30 ± 0.10	621.5		601, 495, 229
9	*Not identified*	41.25	56.98 ± 0.32	941.3		897, 855, 693 , 400, 319
10	Phlorotannin - 6 PGU	42.17	34.02 ± 0.26	745.4		728, 229
11	Phlorotannin - 6 PGU	43.57	40.13 ± 0.15	745.2		727, 601, 479, 353, 229
12	Phlorotannin - 7 PGU	44.79	34.88 ± 0.64	869.4		853
13	Phlorotannin - 7 PGU	44.81	36.63 ± 0.10	869.4		852
14	Phlorotannin - 7 PGU	44.84	38.45 ± 0.10	869.2		851
15	Quercetin rutinoside	44.94	44.23 ± 0.14	609.2		301, 270, 178
16	*Not identified*	45.26	63.50 ± 0.18	955.9		937, 849, 794
17	Phlorotannin - 7 PGU	46.21	42.30 ± 0.10	869.2		852
18	Phlorotannin - 8 PGU	48.71	36.70 ± 0.13	993.5		975, 849, 743
19	Phlorotannin - 8 PGU	48.74	38.33 ± 0.13	993.9		975, 849
20	Phlorotannin - 8 PGU	48.76	39.48 ± 0.13	993.5		975, 849, 743, 621
21	Phlorotannin - 8 PGU	48.84	44.38 ± 0.08	993.3		975, 849, 743, 621
22	Phlorotannin - 9 PGU	51.29	35.58 ± 0.08	1117.5		1099, 1081, 869, 851, 727, 603

23	Phlorotannin - 9 PGU	51.37	40.20 ± 0.10	1117.9		1099, 869, 727
24	Phlorotannin - 9 PGU	51.39	41.25 ± 0.00	1117.5		1099, 869, 727, 619
25	Phlorotannin - 9 PGU	51.47	46.33 ± 0.03	1117.4		1099, 869, 727, 619
26	Phlorotannin - 10 PGU	53.92	36.88 ± 0.23	1241.6		1223, 1205, 993, 975, 603
27	Phlorotannin - 10 PGU	53.93	38.15 ± 0.09	1241.8		1223, 1205, 975, 833
28	Phlorotannin - 10 PGU	54.02	43.32 ± 0.03	1241.9		1223, 993, 975, 869, 744
29	Phlorotannin - 10 PGU	54.10	48.05 ± 0.00	1241.5		1223, 1099, 975, 849, 726, 601
30	Phlorotannin - 11 PGU	55.36	45.15 ± 0.17	1365.8		1347, 1117, 991, 868, 727, 618
31	Phlorotannin - 11 PGU	55.43	49.52 ± 0.19	1365.5		1347, 1117, 991, 867, 723
32	Phlorotannin - 11 PGU	56.58	40.53 ± 0.13	1365.4		1329, 1099, 975, 849
33	Phlorotannin - 11 PGU	56.65	45.15 ± 0.05	1365.7		1347, 1117, 973, 867, 727
34	*Not identified*	57.89	40.55 ± 0.10	1043.8		1025, 925, 907
35	Phlorotannin - 12 PGU	57.98	46.88 ± 0.08	1489.6		1453, 1223, 1100
36	Phlorotannin - 12 PGU	58.05	50.92 ± 0.24	1490.2		1471, 1453, 1241, 1223, 1115, 867
37	*Not identified*	59.06	33.65 ± 0.13	1017.3		999, 909, 869, 851
38	*Not identified*	59.08	34.93 ± 0.08	1018.0		999, 909, 869, 851
39	Phlorotannin - 12 PGU	59.21	42.35 ± 0.00		744.3	1241, 1223, 1117, 993, 867, 726, 619, 229
40	*Not identified*	60.53	43.85 ± 0.17	1017.4		999, 981, 927, 909, 869 , 851, 621 , 305
41	Phlorotannin - 13 PGU	60.61	48.25 ± 0.05		808.4	1453, 1365, 1242, 1116, 1099, 993, 975, 867, 745, 619
42	Phlorotannin - 13 PGU	60.69	52.80 ± 1.39		807.6	1365, 1223, 1115, 993, 975, 867, 745, 619, 350, 229
43	*Not identified*	61.84	44.52 ± 0.14	1142.6		1123, 1051, 1033, 975, 891, 755, 495
44	*Not identified*	61.86	45.67 ± 0.15	1141.6		1123, 1106, 1051, 1033, 975, 849, 769, 745, 648, 478
45	Phlorotannin - 14 PGU	61.93	49.63 ± 0.16	1737.0		1493, 1243, 975, 852
46	Phlorotannin - 14 PGU	62.01	53.62 ± 0.73		869.4	1489, 1471, 1241, 991, 850
47	Phlorotannin - 15 PGU	63.19	46.65 ± 0.82		930.8	1613, 1594, 1366, 975, 921, 795, 744
48	Phlorotannin - 16 PGU	64.49	47.35± 0.10		992.6	1737, 1594, 1239, 974, 477

49	Phlorotannin - 16 PGU	64.51	48.42 ± 0.08	992.6	1738, 1719, 1598, 1469, 1239, 1095, 975, 354	543
50	Phlorotannin - 16 PGU	64.57	51.85 ± 0.15	992.6	1737, 1720, 1594, 1239, 974, 477	
51	Phlorotannin - 16 PGU	65.81	48.52 ± 0.15	992.6	1737, 1720, 1490, 974, 931, 622, 494	
52	Phlorotannin - 17 PGU	65.89	52.95 ± 0.15	1054.0		

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