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Enzyme-assisted extraction (EAE) for the production of antiviral and antioxidant extracts from the green seaweed *Ulva armoricana* (Ulvales, Ulvophyceae)

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Abstract :

In order to develop innovative methods for the production of bioactive extracts with potential health benefits, enzyme-assisted extraction has been applied for the bioconversion of *Ulva armoricana* (Ulvales, Ulvophyceae). The extraction yields, chemical composition, the antioxidant and antiviral activities were determined to assess the efficiency of six commercial enzymatic preparations. Endo-protease treatments significantly increased the extraction yields compared to the control. The organic matter, neutral sugar and protein contents were increased in all extracts compared to an extraction with water, up to 2.0-fold, 2.7-fold and 1.75-fold, respectively. The samples prepared by a multiple-mix of glycosyl-hydrolases and an exo-*β*-1,3(4)-glucanase revealed activities against *herpes simplex virus* type-1 at the median effective concentration (EC_{50}) of 373.0 ± 20.7 and 320.9 ± 33.6 µg/ml, respectively. These activities were correlated to high amounts of rhamnose, uronic acids and sulfate groups which are the main constituents of ulvans. Free radical scavenging capacity at medium inhibition concentrations (IC_{50}) of 1.8 and 12.5 mg/ml was shown for the extracts produced with endo-protease treatments and 6.0 mg/ml for the sample resulting from the extraction with the multiple-mix of glycosyl-hydrolases. This study reports the antiviral and antioxidant activities of *Ulva armoricana* extracts produced by enzyme-assisted extraction.

Keywords : Ulvan, Antiviral activity, Carbohydrate, Endo-protease, Bioconversion, Biorefinery

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49 **1. Introduction**

50

51 Macroalgal blooms occur worldwide, mainly caused by species from the phyla
52 Rhodophyta [1] and Chlorophyta [2], but the most widespread are caused by the
53 Chlorophyta species, such as those within the genus *Ulva* Linnaeus [3]. During the last
54 few decades, the coasts of Brittany (France) have suffered from significant green tides
55 mainly constituted of non-attached populations of *Ulva armoricana* and *Ulva rotundata*
56 (Ulvophyceae) [2]. Many studies have addressed the ecological and environmental
57 consequences of green tides which include uncoupled biogeochemical cycles, decreased
58 water transparency, degradation of the intertidal environment, reduced biodiversity,
59 hypoxia or anoxia, destruction of the marine coastal habitat (e.g., seagrass beds) and
60 economic losses to marine industries [3]. However, these seaweed beachings also
61 represent a huge algal biomass which could be exploited, for applications as plant
62 elicitors, in animal health and in feed [4,5,6].
63 At this time, harvesting of these seaweeds remains the most cost-effective way to obtain
64 a high tonnage of biomass. However, as the resource is unpredictable, aquaculture can
65 be an alternative in order to ensure its availability and improve the quality and
66 homogeneity of the resource.
67 Over the last few years, there has been more investigations in the research into new
68 bioactive natural molecules and valuable metabolites from seaweeds having an
69 economic impact [4,7]. Several biological activities such as antiviral [6], antibacterial
70 [8], antioxidant [9], anticoagulant [10] and antitumoral [11] have been identified in red
71 (Rhodophyta) and brown (Phaeophyta) algae but few studies have been reported on

72 green algae (Chlorophyta). Therefore, the production of bioactive extracts could
73 represent a potential application for the valorization of algal blooms.
74 The algal cell wall is composed of two main classes of polysaccharides: (1) cellulose,
75 hemicelluloses, mannans and xylans which constitute the skeleton and (2) a species-
76 specific matrix phase of water soluble polysaccharides [12, 13]. In *Ulva* sp., the matrix
77 phase contains ulvans composed of sulfated (sulfation 17 %) xyloarabinogalactans and
78 sulfated (sulfation 22 %) glucuronoxylorhamnans containing 20 % of uronic acids
79 [14,15]. Lahaye and Robic [13] proposed that the two major repeating disaccharides of
80 the glucuronoxylorhamnans are aldobiuronic acids designated as type A: ulvanobiuronic
81 acid 3-sulfate (A3s) and type B: ulvanobiuronic acid 3-sulfate (B3s). Partially sulfated
82 xylose residues at O-2 can also occur in place of uronic acids. In addition, glucuronic
83 acid can branch at O-2 of rhamnose 3-sulfate [15].
84 Traditionally, hot water, acids, bases, organic solvents or ultrasounds were used to
85 extract molecules from most bioresources; however, the enzymatic hydrolysis, an
86 effective and nontoxic procedure, is currently applied in various industrial applications.
87 Manufactured proteases are used in animal feed to improve the digestibility of proteins
88 and in the detergent industries. Glycosyl hydrolases are applied in food/vegetable and
89 starch processing, brewing, paper and pulp industries.
90 For the biorefinery of macroalgae, there is a trend to isolate new specific enzymes from
91 marine organisms, for the degradation of polysaccharides, like ulvan-lyases,
92 glucuronan-lyases, laminarinases, agarases, carrageenases or porphyranases [16]. These
93 enzymes have been isolated and produced at laboratory scales but they are not yet
94 available for industrial purposes. In this study, in order to develop an industrially
95 applicable technology, commercial enzymatic preparations have been selected.

96 Enzyme-based processes, using non-selective enzymes, have already been applied for
97 the production of bioactive extracts from macroalgae. Anticoagulant activities,
98 antioxidant capacity and immunomodulation properties have been highlighted in
99 enzymatic extracts of green, brown and red seaweeds, respectively [17].
100 The aim of this work was to evaluate six non-selective commercial enzymatic
101 preparations, two proteases and four carbohydrases as tools for improving the extraction
102 efficiency and producing bioactive fractions from the French green seaweed *U.*
103 *armoricana*. The extracts were characterized by their respective biochemical
104 composition. Antiviral and antiradical activities were studied on both crude extracts and
105 their isolated polysaccharide fractions.
106
107 **2. Material and Methods**
108
109 ***2.1 Materials***
110
111 *Algal sample: Ulva armoricana* (Ulvales, Ulvophyceae) was collected on the beach in
112 Plestin-les-Grèves (48°39'28" N, 3°37'47" W), English Channel (Brittany, France), on
113 June 18th, 2012. Algae were then washed with tap water, ground and frozen at -25°C.
114 *Enzymes:* Six commercial enzymatic preparations, provided by Novozymes (Bagsværd,
115 Denmark), were used for the hydrolysis of seaweed, a neutral endo-protease (P1, EC
116 3.4.24.28), a mix of neutral and alkaline endo-proteases (P2, EC 3.4.24.28/EC
117 3.4.21.62), a multiple-mix of carbohydrases (C1, EC 3.2.1.6/EC 3.2.1.8/EC 3.2.1.4/ EC
118 3.1.1.73), a mix of endo-1,4-β-xylanase/endo-1,3(4)-β-glucanase (C2, EC 3.2.1.8/EC
119 3.2.1.6), a cellulase (C3, EC 3.2.1.4) and an exo-β-1,3(4)-glucanase (C4, 3.2.1.58). The

120 EAE were compared to a control (Blank), treated in the same conditions and without
121 enzyme. The blank corresponds to a classical water extraction at 50 °C for 3 h.
122
123 ***2.2 Enzyme-Assisted Extraction***
124
125 Extractions were performed in a bioreactor in which 500.0 g of crushed algae (8.3 %
126 dry matter, *dm*) and 500.0 g of Milli-Q water were mixed. The enzyme preparations
127 (6 % weight/dry weight, *w/dw*) were added to the algae and allowed to react during 3 h
128 at 50°C followed by denaturation at 90°C for 15 min. The pH of the reaction ranged
129 from 6.2 at the beginning of the reaction to 5.9 at the end. After extraction, the samples
130 were filtered, on a Buchner system using a synthetic cloth, and then centrifuged
131 (8,000*g*) for 20 min at 20°C. In the end, two fractions were obtained, a soluble extract
132 and an insoluble pellet. All samples were then freeze-dried and stored at 4°C. Every
133 extract consisted of the addition of the filtered soluble fraction plus the residual soluble
134 fraction retained in the pellet.
135 The addition of dry matter, proteins, carbohydrates etc. due to the addition of the
136 enzymatic preparations have been taken into account in the calculation both of
137 extraction yields and biochemical composition of the hydrolyzates.
138
139 ***2.3 Biochemical analyses***
140
141 An aliquot of dried seaweed (10.0 mg) was hydrolysed for 2 h at 100°C with 1 M
142 hydrochloric acid (1.0 ml) for the characterization of the raw material and then
143 neutralized with 1 M sodium hydroxide (1.0 ml). The methods used for the biochemical

144 analyses were the phenol sulfuric acid method of Dubois for neutral sugars, the meta-
145 hydroxy-di-phenyl (MHDP) method for uronic acids, the Azure A method for sulfate
146 groups and the bicinchinonic acid (BCA) protein assay kit for total proteins. All these
147 methods are described and detailed in Hardouin et al. [18]. The total phenolic content
148 was quantified by spectrophotometry according to the Folin Ciocalteu method [19] with
149 minor modifications. One milliliter aliquot of extract solution (concentration range from
150 1 to 5 mg/ml) was mixed with 5 ml of Folin Ciocalteu reagent (10 % in distilled water).
151 After 5 min, 4 ml of sodium carbonate (7.5 % in distilled water) were added. The
152 samples were incubated for 2 h at room temperature in darkness. The absorbance was
153 measured at 760 nm. A standard curve with serial gallic acid solutions (0 to 500 µg/ml)
154 was used for calibration. Results were expressed as g gallic acid equivalents (GAE) per
155 kg of extract.
156
157 ***2.4 Amino acids composition***
158
159 The total amino acid composition of freeze-dried extracts was determined after
160 hydrolysis in 6 M HCl at 118°C for 18 h. The hydrolyzed samples were completely
161 dried under a nitrogen atmosphere and diluted by adding 2.5 ml of water. The amino
162 acid samples were prepared according to the EZ faast™ (Phenomenex, Torrance,
163 California, U.S.A) procedure consisting of a solid phase extraction step followed by
164 derivatization and liquid/liquid extraction. The amino acid samples were analyzed on an
165 autosystem Gas chromatography–Flame ionization detection (Perkin Elmer, France;
166 GC-FID) system composed of a Zebron ZB-AAA GC column (10 m x 0.25 mm,
167 Agilent, CA, USA). 2 µl of samples were introduced in the injector (250°C) and

168 separated using the following program: 110°C to 320°C (increase of 32°C/min). The
169 detector was at 320°C and the gas pressure at 1.7 bar. The signals were registered using
170 the Galaxie software (Varian, CA, USA). The amino acids were quantified by their
171 response factor relative to the internal standard Norvaline added at a concentration of
172 200 µmol/l.
173
174 ***2.5 Sugar composition***
175
176 The composition of unitary sugars present in the polysaccharide chains was determined
177 by High Pressure Anion Exchange Chromatography (Dionex). Prior to analyses, 1.0 ml
178 (2 mg/ml) samples were hydrolysed for 48 h at 100°C with 110 µl of 1 M hydrochloric
179 acid in a sealed glass tube. Before filtration, the samples were neutralized with 1 M
180 sodium hydroxide. 30 µl were injected on an analytical column (CarboPac PA1, 4 x 250
181 mm) with elution, at 1 ml/min and 110 bars, performed in alkaline conditions. Solution
182 A consisted of Milli-Q water; solution B was 0.1 M sodium hydroxide and solution C
183 was 1 M sodium acetate/0.1 M sodium hydroxide. The elution program corresponded
184 to: 20 min isocratic conditions (solution A/solution B, 80:20), linear gradient (solution
185 C 100 %, 0 to 5 min), 5 min isocratic conditions (solution C, 100 %) and 20 min
186 isocratic conditions (solution A/solution B, 80:20). Sugars were detected by pulsed
187 amperometry with a detector composed of a silver standard electrode and a gold
188 working electrode. The chromatograms were treated using Chromeleon® software
189 provided by Dionex. Carbohydrates were identified and quantified by using calibration
190 curves of standards composed of ribose, glucose, rhamnose, galactose, arabinose,
191 xylose, mannose, fructose, guloheptose and glucuronic acid.

192

193 ***2.6 Polysaccharides molecular weight (*Mw*) distribution***

194

195 The polysaccharide molecular weight (*Mw*) distribution was determined by High
196 Performance Steric Exclusion Chromatography (HPSEC). The analytical chain was
197 composed of an auto sampler (ASI 100, Dionex), an analytical pump (P680, Dionex)
198 and two detectors connected in series, a UV detector set at 280 nm (UVD 400, Dionex)
199 and a refractometer (Iota 2, Precision Instrument). Samples were prepared at 1.0 mg/ml
200 in the eluent and filtered at 0.45 µm. The separation system consisted of a TSKgel®
201 GMPW standard Guard Column (TOSOH) and a TSKgel® $GMPW_{XL}$ column (TOSOH
202 300 x 7.8 mm, 13 µm). Elution was carried out at 30°C, by using 0.1 M sodium nitrate
203 ($NaNO_3$) at a flow rate of 0.5 ml/min. Dextran standards with molecular weights of 5.3
204 kDa, 17.9 kDa, 129 kDa and 636.4 kDa, 1 % (*w:v*), were used for calibration. Sodium
205 chloride (NaCl) was used to determine the maximum retention time (total volume, V_t)
206 of the column.

207

208 ***2.7 Polysaccharide isolation***

209

210 The isolation of polysaccharides was performed by ethanol precipitation. 450 ml of
211 ethanol were added to 50 ml of extract and stored at 4°C over-night. Precipitates were
212 recovered by centrifugation (8,000*g*) at 4°C for 20 min, washed twice with cold ethanol
213 and freeze-dried.

214

215 ***2.8 Evaluation of biological activities***

216

217 *In vitro antiviral and cytotoxicity evaluation by cell viability:* The *in vitro* antiviral and
218 cytotoxicity evaluation were performed according to the method used by Hardouin et al.
219 [18] on the model *Vero* cells and *Herpes simplex* virus-type 1 (HSV-1).
220 *DPPH free radical scavenging assays:* The antiradical activities of extracts were
221 measured with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [20]. A solution of
222 DPPH was prepared at 0.25 mM (0.1 g/l) by diluting 10 mg of DPPH in 100 ml of
223 methanol. Butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) were
224 used as standards (2 to 50 µg/ml). The samples were dissolved in water at 1, 5, 12.5 and
225 25 mg/ml and aliquots of 1 ml were mixed with 1 ml of DPPH reagent. The samples
226 were incubated for 30 min at 40°C and the OD measured at 517 nm was compared to a
227 blank of methanol and water. The inhibitory concentration (scavenging) was calculated
228 by the formula: Scavenging (%) = $[(OD_{control} – OD_{sample}) / OD_{control}] \times 100$. The IC_{50}
229 was determined with the curve and correspond to 50% inhibition.

230

231 ***2.9 Statistical analysis***

232

233 Results are expressed as means ± standard deviation (SD) (n = 3). The statistical
234 analysis was carried out on SPSS (IBM, Armonk, NY, USA) using the one-way
235 analysis of variance (ANOVA) followed by a Duncan test at the 5% level ($p < 0.05$) to
236 evaluate differences between the samples. For each series of values, the significant
237 differences are labelled by superscript letter.

238

239 **3. Results and discussion**

240

241 ***3.1 Characterization of the* Ulva armoricana *raw sample***

242

243 *Ulva armoricana* samples contained 15.9 ± 1.2 % ash, 23.4 ± 1.4 % neutral sugars, 21.1
244 ± 0.1 % uronic acids, 24.4 ± 0.1% proteins, 4.0 ± 0.1% total nitrogen, 20.9 ± 0.9 %
245 sulfate groups, 0.6 ± 0.1 % of total phenolic compounds and 0.6 ± 0.0 % lipids. The
246 total carbohydrate content (64.4 %) was calculated by the addition of neutral sugars,
247 sulfate groups and uronic acids values. Lipids and phenolic compounds represented a
248 very small part of the algal composition. These levels are in agreement with those
249 reported by Holdt and Kraan [7] for the genus *Ulva* sp. This composition also confirmed
250 that polysaccharides are the main constituent of the *Ulva* sp. thallus. The respective
251 contents of each metabolite in algae vary greatly depending on season, state of growth,
252 geographical area and environment quality [21].

253

254 ***3.2 Enzymatic hydrolysis yields***

255

256 Figure 1 shows the amount of dry matter in the extracts, expressed as a percentage of
257 the total seaweed dry weight (% total *dw*). The proportion of the dry matter solubilized
258 by grinding and incubation was estimated by the blank at 44.3 ± 0.5 % of the initial
259 seaweed dry weight. The difference between this value and values obtained after
260 enzymatic treatment correspond to the benefits of the enzyme-assistance. The extraction
261 yields, 44.9 ± 1.7 % for C1, 44.6 ± 0.6 % for C2, 45.1 ± 2.7 % for C3, were not
262 significantly different from the blank and show that enzymes C1 (mix of
263 carbohydrases), C2 (endo-1,4-β-xylanase/endo-1,3(4)-β-glucanase) and C3 (endo-1,4-β-

264 glucanase) did not improve the extraction. The enzymes C4 (exo-1,3(4)-β-glucanase),
265 P1 (neutral endo-protease) and P2 (neutral and alkaline endo-proteases) induced a
266 significant increase in the amount of solubilized dry matter, with extraction yields of
267 70.7 ± 1.1 %, 76.7 ± 0.8 % and 88.4 ± 0.2 %, respectively. These three enzymes
268 provided an extraction gain of 60 %, 73 % and 100 %, respectively, compared to the
269 control (blank).
270 Previous studies have reported the extraction of water-soluble compounds from
271 different edible seaweeds belonging to the genus *Ulva* sp [14,22,23,24]. The yields and
272 the chemical nature of the isolated compounds are greatly dependent on the extraction
273 procedures.
274 Aqueous and chemical extractions were applied in order to produce fractions enriched
275 in different metabolites. Co-extraction of the water-soluble polysaccharides (ulvans and
276 glucuronans), using oxalate solution at 85°C, led to an 8-29 % extraction yield for *Ulva*
277 sp. [12,13,22]. Xyloglucans and glucuronans, two minor parietal polysaccharides, have
278 also been reported to be selectively isolated in an alkali-soluble phase [13,14].
279 The application of a basic (0.1 M NaOH) solution containing β-mercaptoethanol
280 produced the highest protein yield for the species *Ulva rigida* and *Ulva rotundata* [22].
281 In this latter study, the use of enzymatic preparations (cellulase and polysaccharidases
282 mixture) appeared to be less efficient than aqueous solutions procedures. In this work,
283 the lack of effect of the three carbohydrase preparations C1, C2 and C3 agrees with data
284 observed by Fleurence et al. [22]. The enzymatic preparation C4 has been reported to
285 exhibit a proteolytic side-activity [25] that may explain the improved yield, only slightly
286 below those of the proteases. This is the first report of enzymatic degradation of *Ulva*
287 sp. using proteases. The high efficiency of the proteolytic activities can be explained by

288 the ubiquitous nature of proteins and illustrates the importance of proteins for the
289 integrity of the cell wall.
290 Finally, the nature of enzymatic activity greatly influenced the extraction yields which
291 are higher than those obtained by chemical methods [26,27]. According to these results,
292 enzymes can be classified in two categories: enzyme preparations C1, C2, C3, which
293 have low efficiencies and enzyme preparations C4, P1 and P2 which have a higher
294 extraction power. These last three enzymatic preparations are also more efficient than
295 classical water and chemical methods. The selectivity of the enzyme-assisted extraction
296 was assessed by the characterization of the biochemical composition.
297

298 ***3.3 Biochemical characterization of crude enzymatic extracts***

299

300 Table I shows the biochemical composition of extracts, after deducting the input from
301 the enzymatic preparations. The biochemical composition is expressed as the percentage
302 in the dry hydrolyzates and by the absolute weights (g) solubilized by the process.
303 The ash contents (% *dw*) ranged between 16.7 ± 2.1 and 18.6 ± 2.1 and were consistent
304 with the content found in the raw material. These levels correspond to the inorganic part
305 of the seaweed. They were increased in samples C4, P1 and P2 with a good correlation
306 with the extraction yields. The same trend was observed for total phenolic compounds,
307 indicating that the extraction of non-structural components (ash, total phenols) is
308 correlated with the extraction yields with no apparent effect of the extraction procedure.
309 The neutral sugars contents were higher in samples C1 and C3, 18.4 ± 0.8 and 18.3 ±
310 3.3 % *dw*, respectively. Conversely, the blank and the extract C2 contained the lowest
311 amounts, respectively 9.0 ± 1.2 and 8.1 ± 1.6 % *dw*. Meanwhile, the extracts obtained

312 from the enzymes C4, P1 and P2 contain intermediate contents between 11.3 ± 2.3 and

313 14.7 ± 0.5 % *dw*. However, the absolute amount of neutral sugars solubilized by these

314 three enzymes is higher with respectively 3.7 ± 0.2, 4.1 ± 0.2 and 3.7 ± 0.8 g. The

315 neutral sugars contents were lower than those determined after chemical extraction

316 methods from *Ulva clathrata* [27] and after aqueous extraction from *Ulva rotundata*

317 [26].

318 The blank, C2 and P1 contained the highest protein concentrations with 9.4 ± 0.4, 8.8 ±

319 0.9 and 10.1 ± 0.3 % *dw* respectively, but the highest total protein weights were

320 observed in samples C4 (2.2 ± 0.1 g), P1 (2.8 ± 0.1 g) and P2 (2.1 ± .0.1 g). The

321 increase in total protein weights of these samples is directly correlated with the increase

322 in extraction yields.

323 From all extracts, samples C1 and C3 containing 53.7 % and 55.6 % *dw* of total

324 carbohydrates (neutral sugars, uronic acids and sulfate groups), respectively, were the

325 most concentrated. On the opposite, the samples C4, P1 and P2 contained the lowest

326 concentrations, 46 %, 46 % and 36 % *dw*, respectively. Enzymatic preparations C1 and

327 C3 appear to be the most selective for the extraction of total carbohydrates. However,

328 due to the significant increase in extraction yields, enzymes C4, P1 and P2 solubilized a

329 larger amount (g) of total sugars and protein, and are the most efficient. The proteolytic

330 side-activity of the enzymatic preparation C4 [25] is confirmed by the selective

331 extraction of proteins.

332 Due to the mainly polysaccharidic nature of the cell wall of *U. armoricana*, these results

333 may seem paradoxical, but they confirm that non-selective industrial carbohydrases do

334 not hydrolyze the matricial polysaccharides of the seaweed cell wall and therefore do

335 not improve the extraction of seaweed components. Conversely, to explain the efficient

effect of proteases on seaweeds, the first assumption is that proteases have a destabilizing effect on the cell wall, which causes the release and solubilization of a higher amount of compounds. These results also suggest that proteins are attached to polysaccharides and act as a cement in the structure of the cell wall [13]. However, there is a second hypothesis which is that proteases hydrolyze the membrane cell proteins causing a release of intracellular material and not exclusively molecules from the seaweed cell wall.

3.4 Amino acid composition of extracts

Gas chromatography-Flame ionization detection (GC-FID) was used to identify and quantify 16 amino acids (AA). Cysteine, arginine and tryptophan were not determined by this method and glutamine and asparagine were quantified as glutamic and aspartic acids, respectively. The results were expressed as a percentage of the total AA measured. The average rates of AA released in the extracts are given in Table II. The characterization of the AA profile revealed minor differences between extracts. AA can be divided into three categories according to their content in samples, high (< 10 %), medium (3-10 %) and low (> 3 %).

These results provided information on the protein composition of the algae. Four AAs were identified as major, alanine, glycine, aspartic acid and glutamic acid as they constituted more than 10 % of the extracted proteins. Methionine, hydroxyproline, lysine, histidine and tyrosine were present in very small amounts, ranging from 0.9 to 2.9 %. The content of the remaining AAs was average, between 3 and 10 %. Although the composition of the samples was quite similar, there were still some differences

360 against the blank. The alanine content was between 12.1 % and 14.1 % against 16.3 %
361 in the blank. The results were similar for glycine with rates between 8.7 % and 9.1 % in
362 the extracts against 11.1 % in the control. The trend was reversed for glutamic acid and
363 aspartic acid as their rates in the negative control, 8.1 % and 11.1 % respectively, were
364 lower than in the extracts, 9.3 to 14.3 % and 12.9 to 14.5 %, respectively. No difference
365 appeared between extracts and the blank for the other AAs. Therefore, due to the
366 differences in the amounts of several amino acids, the proteins solubilized by the
367 enzymatic treatment seem to have a different composition from those solubilized by
368 incubation in water. The nature of the enzymatic preparation did not affect the amino
369 acid profile of the extracts.
370 These results matched with those obtained by Fleurence et al. [28], who had shown the
371 enriched composition, in alanine (5.51 – 7.01 % proteins), glycine (6.34 – 7.53 %
372 proteins), aspartic acid (6.09 – 11.84 % proteins) and glutamic acid (11.70 – 23.35 %
373 proteins), of proteins from *U. armoricana* collected in October 1997. However, some
374 slight differences appear in the respective amino acids contents. These differences could
375 be due to the seasonal variability and the life cycle of seaweed which do not produce the
376 same types of proteins during the year. These differences could also be explained by the
377 extraction method used. Thus, enzyme-assisted extraction might selectively extract
378 different types of protein. The seasonal variability of the proteins and amino acids
379 composition was also highlighted by Fleurence et al. [28].
380

381 ***3.5 Sugar composition***

382

383 The results from the compositional analyzes of carbohydrates by High Pressure Anion
384 Exchange Chromatography (HPAEC) are given in Table III. Values represent the mean
385 of the percentage of the different sugars relative to the total carbohydrate content
386 (g/100 g total carbohydrate). Fructose and ribose were identified in very low
387 concentration (< 3% total sugars) while some unidentified and minor carbohydrates are
388 included in 'other sugars'. Two unidentified and charged carbohydrates were added to
389 uronic acids (UAc) due to their retention time, close to the glucuronic acid. These were
390 probably galacturonic and iduronic acids [13].
391 Analyzes showed the predominance of glucose, rhamnose and uronic acids. Lahaye and
392 Robic [13] described the composition of ulvans and showed that it is composed mainly
393 of rhamnose (16.8 - 45.0 % *dw*), xylose (2.1 - 12.0 %), glucose (0.5 - 6.4 %), uronic
394 acid (6.5 - 19.0 %), and sulfate groups (16.0 - 23.2 %). The results obtained in our study
395 correspond to these values and show the presence of ulvans in the extracts. Lahaye and
396 Robic [13] also reported the presence of β-glucans, polymers of glucose, which could
397 explain the high levels of glucose in crude samples (Table III a).
398 The isolation step aimed to selectively separate the polysaccharide fractions from the
399 extracts. The sugar compositions of these fractions (Table III b) differed from those of
400 the crude hydrolyzates. The precipitation led to a decrease of the glucose amounts in all
401 samples, highly amplified (-47 to -72 %) in $C1_p$, $C2_p$, $C3_p$, and $C4_p$. The isolation also
402 corresponded to a high increase in the rhamnose and uronic acids contents in the same
403 samples. These variations were lower in the blank and in samples $P1_p$ and $P2_p$.
404 These results suggest that the proteases and the control led to the solubilization of
405 neutral (β-glucans) and matricial (ulvans) carbohydrates in their native forms, without
406 degradation. Regarding the loss of glucose after precipitation, β-glucans might be

407 degraded by carbohydrases C1, C2, C3 and C4 (which contain β-glucanases) in
408 oligosaccharides and in monosaccharides which were not precipitated, while the ulvans
409 were recovered in their native forms. Combined with the extraction yields, these results
410 show that carbohydrases have a selective action on the glucans of the *U. armoricana*
411 cell wall, without improving the efficiency of the extraction, whereas proteases, which
412 were more efficient, solubilized native carbohydrates.
413 The samples obtained with the enzymatic preparation C4, which was one of the most
414 efficient enzyme mixtures, show a decrease in glucose and an increase in rhamnose and
415 uronic acid contents, confirming the simultaneous effect of the β-glucanase and the
416 protease previously observed in this study.
417
418 ***3.7 Polysaccharides molecular weight (Mw) distribution***
419
420 The molecular weight distribution of poly- and oligosaccharides in extracts has been
421 determined by High Performance Steric Exclusion Chromatography (HPSEC). Figure 2
422 is a representative example of the chromatograms obtained for the different samples,
423 comparing the samples C4 and $C4_p$, respectively, before and after ethanol precipitation.
424 The dotted line represents the crude extract C4 and the solid line the polysaccharide
425 fraction $C4_p$. The figure shows four peaks in C4 and three in $C4_p$. The last peak (C4)
426 was the total volume of the column and corresponded to salts. This peak does not
427 appear on chromatogram $C4_p$, confirming that salts and monosaccharides were
428 eliminated by the precipitation. The high mass polysaccharide fractions increased in
429 isolated samples while the low mass polysaccharides decreased. These results
430 confirmed the efficiency of ethanol precipitation for the isolation of polysaccharides.

431 Chromatograms also show that there were two groups of polysaccharides, with average
432 molecular weights of 2,000 kDa and 600 kDa, and a group of oligosaccharides (3 kDa).
433 The two groups of polysaccharides confirmed the presence of ulvans in samples and
434 correspond to the results reported by Robic et al. [29] describing the structure of ulvans.
435 The same study also described the molecular weights of ulvans ranging from 300 to 500
436 kDa for the first group and from 140 to 180 kDa for the second group. These values are
437 below those obtained in our study. This difference may be due to the extraction method
438 or HPSEC conditions. In their study, Robic et al. [29] used water extraction in sodium
439 oxalate at 85°C for 2 h, which is described as the optimal condition for ulvan extraction.
440 The increase of polysaccharides and the corresponding carbohydrate compositions
441 confirm the enrichment in ulvans in the isolated fractions.
442

443 ***3.8 Screening of in vitro antiviral activity by cell viability of extracts***

444

445 The crude extracts and the isolated polysaccharide fractions were tested *in vitro* for their
446 cytotoxicity on *Vero* cells and their antiviral activity against *herpes simplex virus* type-1
447 (Table IV).
448 Acyclovir, used as reference molecule, presented an effective concentration (EC_{50}) of
449 0.3 µg/ml and a cytotoxicity (CC_{50}) above 500 µg/ml. No crude extracts exhibited
450 cytotoxic effect to *Vero* cells for concentrations up to 500.0 µg/ml. The microscopic
451 examination and the assessment of cell viability showed that all cells were viable after
452 72 h of contact with the extracts at $MOI_{0.001}$. The crude extracts did not show activity
453 against the virus for concentrations under 500.0 µg/ml. After isolation, the
454 polysaccharide fractions still did not show cytotoxic activity. $C1_p$ and $C4_p$ fractions

455 exhibited activities against HSV-1 with a respective EC_{50} of 373.0 ± 20.7 µg/ml and
456 320.9 ± 33.6 µg/ml. The polysaccharides obtained from other extracts did not show
457 activity in the range of concentration.
458 The antiviral activity of the samples $C1_p$ and $C4_p$ is correlated to the high amounts of
459 rhamnose, the main component of ulvans, coupled with a higher purity. The antiviral
460 activity of sulfated polysaccharides from seaweed had already been shown in previous
461 studies [6, 30]. However, the EC_{50} are higher than those obtained for other sulfated
462 polysaccharides.
463 This work is the first reporting the antiviral activity of enzyme-assisted extracts
464 containing ulvans from *U. armoricana*. For many years, there has been an increasing
465 demand from the pharmaceutical industry for the discovery of new antiviral molecules.
466 The huge *Ulva* sp. biomass might therefore respond to this demand [6].
467

468 ***3.9 DPPH scavenging - Antiradical activity***

469

470 The free radical scavenging of the blank and the enzymatic extracts of *Ulva armoricana*
471 was estimated by the decrease in absorbance due to the reduction of the DPPH radical
472 by the extracts (Table IV). The standards BHA and BHT present an inhibiting
473 concentration (IC_{50}) of 4.8 and 6.8 µg/ml, respectively. Samples obtained with P1, C1
474 and P2 presented positive results with an IC_{50} of 1.8, 6.0 and 12.5 mg/ml, respectively.
475 The other samples did not have a significant antiradical effect in the concentration range
476 used. As previously reported [31], the antioxidant power of the extract is generally
477 related to the presence of phenolic compounds in seaweed and/or seaweed extracts.
478 Some studies also reported that sulfated polysaccharides (ulvans) could also have an

479 antiradical effect [32]. According to the biochemical composition, the three bioactive
480 extracts are also the most concentrated in polyphenols, with respective concentrations of
481 0.8 %, 0.6 % and 1.0 %. Secondly, no significant differences appear, between these
482 samples and the others, in the ulvan content. Therefore, the antiradical activity could be
483 related to phenolic compounds but some further analyses, before and after specific
484 purification, would need to be realized in order to confirm this hypothesis.
485 Despite its low levels of antioxidant compounds, *Ulva armoricana* could be an
486 interesting and novel bioresource for these compounds. As with antiviral compounds,
487 the pharmaceutical, cosmetic and food industries, which currently use mainly synthetic
488 molecules, are constantly looking for new sources of natural sources of antiradical
489 molecules. Antioxidant compounds are used in order to limit the organoleptic
490 degradation of food, as anti-aging in cosmetics or as drugs in the treatment of oxidative
491 stress [9, 18].
492
493 **4. Conclusion**
494
495 This study demonstrates the potential of Enzyme-Assisted Extraction for the
496 valorization of *Ulva* sp. biomass. The improvement of extraction yields using proteases
497 and the selective degradation of β-glucans using carbohydrases have been demonstrated.
498 This is the first study reporting the efficiency of proteases for the liquefaction without
499 degradation of *Ulva armoricana* components. These extraction procedures and the
500 isolation step allowed the preparation of samples enriched in ulvans. Finally, the
501 relationship between ulvans and antiviral activity has been established.
502

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504

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596
597

598 **Figure captions**

599

600 Figure 1: Percent dry weight (% total *dw*) in extracts after the enzyme-assisted
601 extraction (EAE).
602 The respective enzymatic preparations inputs have been deducted from the dry samples.
603 Values are means ± SD (n=3). Values with different superscript letters are significantly
604 different ($p < 0.05$). Proteases are indicated with black bars; Carbohydrases by grey
605 bars; Water extract by a white bar.
606 Blk: control, C1: multiple-mix of carbohydrases, C2: mix of endo-1,4-β-xylanase/endo-
607 1,3(4)-β-glucanase, C3: cellulase, C4: exo-β-1,3(4)-glucanase, P1: neutral endo-
608 protease, P2: mix of neutral and alkaline endo-proteases.

609

610 Figure 2: High pressure steric exclusion chromatogram (RI) of samples C4 and $C4_p$,
611 before and after ethanol precipitation.
612 Flow: 0.5 ml/min; Pressure: 9-13 bars; Eluent: 0.1M NaNO3; Sample: 1 mg/ml; Inject.
613 vol.: 100 µL, detection: refractive index. C4: dotted line; $C4_p$: solid line.

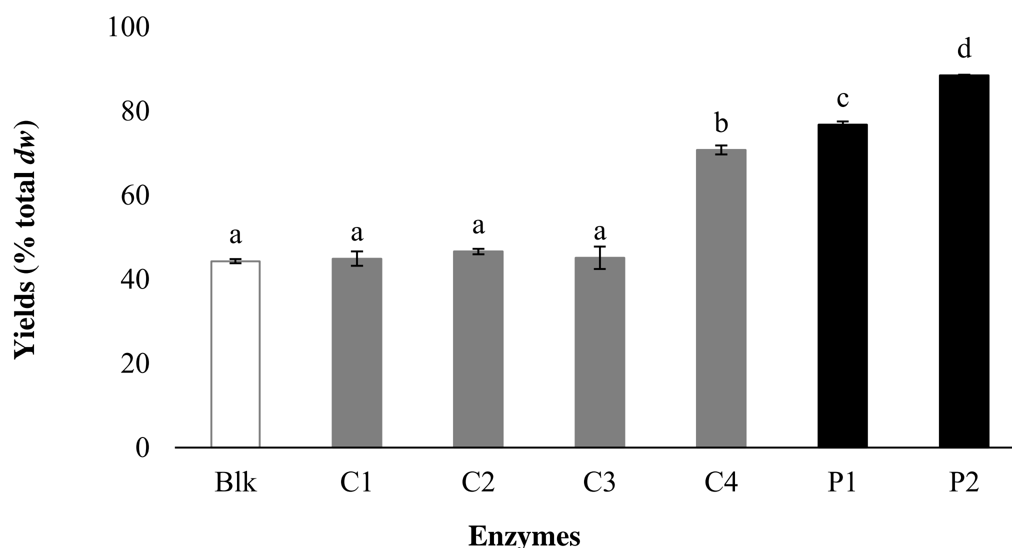
614

615

616

617 **Tables and Figures**

618



619

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628 protease, P2: mix of neutral and alkaline endo-proteases.

629

TABLE I: Biochemical composition of raw material and enzymatic extracts (% *dw* and g).

Values are means ± SD (n=3). Values with different superscript letters are significantly different ($p < 0.05$). Organic matter was determined by the difference between the total dry matter and the ash content.

		Ash	Organic matter	Neutral sugars	Uronic acids	Sulfate	Proteins	Total phenols
Raw algae	**% *dw***	**15.9 ± 1.2**	**84.1 ± 1.2**	**23.2 ± 1.4**	**21.1 ± 0.1**	**20.1 ± 0.9**	**24.4 ± 0.1**	**0.6 ± 0.1**
	g	6.0 ± 0.1	31.5 ± 0.4	8.7 ± 0.1	7.9 ± 0.1	7.5 ± 0.1	9.2 ± 0.1	0.3 ± 0.1
Blank	**% *dw***	**18.0 ± 1.2^{a}**	**82.0 ± 1.2^{a}**	**9.0 ± 1.2^{a}**	**21.5 ± 4.2^{e}**	**17.2 ± 0.1^{d}**	**9.4 ± 0.4^{cd}**	**0.5 ± 0.0^{ab}**
	g	3.1 ± 0.3^{a}	13.9 ± 0.2^{a}	1.5 ± 0.3^{a}	3.6 ± 0.7^{bc}	2.9 ± 0.1^{a}	1.6 ± 0.1^{c}	0.1 ± 0.0^{a}
C1	**% *dw***	**17.3 ± 1.6^{a}**	**82.7 ± 1.6^{a}**	**18.4 ± 0.8^{d}**	**16.2 ± 0.9^{cd}**	**19.1 ± 0.3^{e}**	**8.8 ± 0.9^{c}**	**0.7 ± 0.2^{b}**
	g	3.0 ± 0.2^{a}	14.4 ± 0.7^{a}	3.2 ± 0.2^{b}	2.8 ± 0.2^{b}	3.3 ± 0.2^{c}	1.5 ± 0.2^{c}	0.1 ± 0.0^{a}
C2	**% *dw***	**17.0 ± 2.7^{a}**	**83.0 ± 2.7^{a}**	**8.1 ± 1.6^{a}**	**10.9 ± 1.3^{ab}**	**17.4 ± 0.2^{d}**	**6.2 ± 0.4^{a}**	**0.6 ± 0.1^{ab}**
	g	2.8 ± 0.6^{a}	13.9 ± 0.2^{a}	1.4 ± 0.6^{a}	1.8 ± 0.2^{a}	2.9 ± 0.1^{a}	1.0 ± 0.1^{a}	0.1 ± 0.0^{a}
C3	**% *dw***	**17.7 ± 1.8^{a}**	**82.3 ± 1.8^{a}**	**18.3 ± 3.3^{d}**	**19.9 ± 1.2^{de}**	**17.4 ± 0.1^{d}**	**7.3 ± 0.7^{b}**	**0.4 ± 0.1^{a}**
	g	3.1 ± 0.5^{a}	14.5 ± 1.0^{a}	3.2 ± 0.8^{b}	3.5 ± 0.2^{bc}	3.1 ± 0.1^{b}	1.3 ± 0.2^{b}	0.1 ± 0.0^{a}
C4	**% *dw***	**18.6 ± 2.1^{a}**	**81.4 ± 2.1^{a}**	**14.1 ± 0.5^{bc}**	**13.7 ± 2.3^{bc}**	**14.3 ± 0.4^{a}**	**8.5 ± 0.4^{c}**	**0.7 ± 0.1^{b}**
	g	4.8 ± 0.8^{b}	21.2 ± 0.4^{b}	3.7 ± 0.2^{bc}	3.5 ± 0.6^{bc}	3.7 ± 0.3^{d}	2.2 ± 0.1^{d}	0.2 ± 0.0^{b}
P1	**% *dw***	**16.7 ± 2.1^{a}**	**83.3 ± 2.1^{a}**	**14.7 ± 0.5^{c}**	**15.6 ± 0.6^{c}**	**15.7 ± 0.1^{c}**	**10.1 ± 0.3^{d}**	**0.9 ± 0.1^{c}**
	g	4.6 ± 0.9^{b}	23.1 ± 0.3^{c}	4.1 ± 0.2^{c}	4.3 ± 0.8^{c}	4.4 ± 0.1^{e}	2.8 ± 0.1^{e}	0.2 ± 0.0^{c}
P2	**% *dw***	**16.7 ± 0.2^{a}**	**83.3 ± 0.2^{a}**	**11.3 ± 2.3^{ab}**	**9.6 ± 2.3^{a}**	**14.8 ± 0.3^{b}**	**6.5 ± 0.3^{ab}**	**1.1 ± 0.2^{c}**
	g	5.5 ± 0.1^{c}	27.6 ± 0.1^{d}	3.7 ± 0.8^{bc}	3.6 ± 0.2^{b}	4.9 ± 0.3^{f}	2.1 ± 0.1^{d}	0.4 ± 0.1^{d}

636 TABLE II: Amino-acids composition of extracts (g/100 g proteins)

637 Ala: alanine; Asp : aspartic acid; Glu : glutamic acid; Gly: glycine; His : histidine;

638 Hyp : hydroxyproline; Ile: isoleucine; Leu: leucine; Lys : lysine; Met : methionine;

639 Phe : phenylalanine; Pro : proline; Ser : serine; Thr : threonine; Tyr : tyrosine; Val:

640 valine.

	Blank	**C1**	**C2**	**C3**	**C4**	**P1**	**P2**
Ala	16.3	13.6	13.7	14.1	13.0	12.1	12.2
Asp	11.1	14.5	14.1	14.4	14.0	13.6	12.9
Glu	8.1	13.2	13.1	14.3	12.8	11.0	9.3
Gly	11.1	9.0	9.0	8.9	8.7	8.7	9.1
His	1.1	0.9	1.1	1.0	1.1	1.2	1.3
Hyp	2.3	2.3	2.1	2.0	1.7	1.4	1.7
Ile	5.2	5.2	5.3	5.1	5.3	5.4	5.2
Leu	8.4	7.1	7.3	7.0	7.6	8.3	8.8
Lys	2.6	3.0	3.2	3.1	2.9	2.8	2.5
Met	0.8	0.8	0.8	0.8	1.0	1.0	1.1
Phe	4.9	4.3	4.0	3.9	4.6	5.5	6.3
Pro	6.6	5.9	6.3	6.0	6.3	6.7	6.9
Ser	5.6	5.4	5.1	4.8	5.4	6.4	6.8
Thr	6.9	6.4	6.3	6.0	6.7	7.2	7.4
Tyr	1.5	1.2	1.3	1.2	1.4	1.4	1.4
Val	7.4	7.1	7.3	7.2	7.3	7.2	7.0

641

642 TABLE III: Simple sugars composition of extracts (a) and isolated polysaccharide

643 fractions (b) (g/100g total sugars).

644 Rha: rhamnose; Gal: galactose; Glc: glucose; Xyl: xylose; UAc: Uronic Acids. 'Other

645 sugars' represents the sum of low concentrated and non-identified sugars.

(a)	*Rha*	*Gal*	*Glc*	*Xyl*	*UAc*	*Other Sugars*
Blk	40.0	6.7	26.2	4.4	13.6	9.2
C1	33.5	5.0	36.4	4.5	11.3	9.3
C2	27.2	3.8	32.5	4.0	8.8	23.5
C3	26.9	3.6	36.4	3.8	9.2	20.1
C4	26.1	3.9	48.8	2.5	10.1	8.6
P1	30.6	3.9	28.1	2.8	11.9	22.6
P2	35.1	4.3	31.7	4.2	14.1	10.4

(b)	*Rha*	*Gal*	*Glc*	*Xyl*	*UAc*	*Other Sugars*
Blk $_p$	45.8	4.3	23.6	5.4	12.6	8.2
C1 $_p$	53.7	4.4	15.5	4.1	13.4	8.7
C2 $_p$	53.4	5.1	9.2	4.3	16.5	11.5
C3 $_p$	55.6	4.4	11.4	3.7	13.7	11.3
C4 $_p$	42.3	4.7	26.0	5.4	13.1	8.5
P1 $_p$	41.9	3.9	26.2	5.1	13.1	9.7
P2 $_p$	47.9	4.5	20.8	4.6	13.6	8.7

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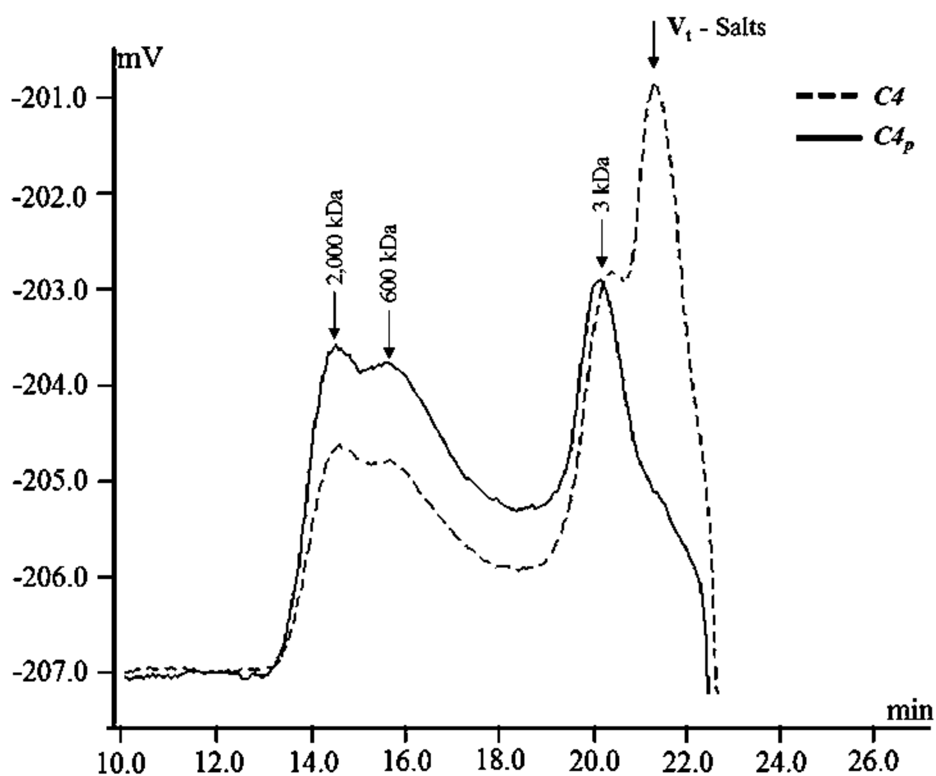

648

649 Figure 2: High pressure steric exclusion chromatogram (RI) of samples C4 and C4$_p$,

650 before and after ethanol precipitation.

651 Flow: 0.5 ml/min; Pressure: 9-13 bars; Eluent: 0.1M NaNO3; Sample: 1 mg/ml; Inject.

652 vol.: 100 µL, detection: refractive index. C4: dotted line; C4$_p$: solid line.

653

TABLE IV: Screening of *in vitro* cytotoxic, antiviral and antioxidant activities of crudes extracts and polysaccharides fractions.

* BHA : Butylated HydroxyAnisole; ** BHT : Butylated HydroxyToluene

	Crude extracts				**Polysaccharides**	
	Cytotoxicity *CC_{50} (µg/ml)*	**Antiviral** *EC_{50} (µg/ml)*	**Antioxidant** *IC_{50} (µg/ml)*		**Cytotoxicity** *CC_{50} (µg/ml)*	**Antiviral** *EC_{50} (µg/ml)*
Acyclovir	> 500.0	0.3 ± 0.1	-		> 500.0	0.3 ± 0.1
BHA*	-	-	0.0048		-	-
BHT**	-	-	0.0069		-	-
Blk			> 25.0	**Blk_p**		> 500.0
C1			6.0	**$C1_p$**		373.0 ± 20.7
C2			> 25.0	**$C2_p$**		> 500.0
C3	> 500.0	> 500.0	> 25.0	**$C3_p$**	> 500.0	> 500.0
C4			> 25.0	**$C4_p$**		320.9 ± 33.6
P1			1.8	**$P1_p$**		> 500.0
P2			12.5	**$P2_p$**		> 500.0