1 **Extraction improvement of water-soluble compounds from *Arthrospira platensis* through the**
2 **combination of high-shear homogenization and pulsed electric fields**

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Abstract

In this work, the use of a mechanical pre-treatment such as high-shear homogenization (HSH) in combination with cell permeabilization by pulsed electric fields (PEF) was proposed to efficiently recover water-soluble proteins (WSP), carbohydrates (CH), and C-phycocyanin (C-PC) from *A. platensis* suspensions during water extraction. Efficiencies of combined treatment (HSH + PEF) were compared with those of individual HSH and PEF treatments.

HSH promoted the breakage of microalgal trichomes, whereas PEF induced only the permeabilization of cell membranes, without affecting cell size and morphology. Combined treatments synergically increased the extractability of WSP and C-PC from *A. platensis*, as compared with individually applied treatments, while an additive effect was detected in the recovery of CH. The synergistic effect of HSH + PEF allowed reducing the specific energy requirement for protein recovery to lower values than for individual treatments or full cell disruption techniques, such as high-pressure homogenization (HPH).

Keywords: *Arthrospira platensis*, high-shear homogenization, pulsed electric fields, hurdle approach, extraction.

24 **1. Introduction**

25 Microalgae are eukaryotic or prokaryotic microorganisms, with cell sizes ranging between 0.1 and
26 40 µm, which can grow rapidly and live in harsh environments due to their unicellular or simple
27 multi-cellular structure (Mata et al., 2010). Under specific cultivation conditions, they can synthesize
28 a significant amount of bioactive compounds, such as proteins, lipids, carbohydrates, carotenoids,
29 and vitamins, of potential interest for food, feed, pharmaceutical, and cosmetic sectors, as well as for
30 energy production (Carullo et al., 2018; Garcia et al., 2018; Pataro et al., 2019). Some of the most
31 biotechnologically relevant strains are the green algae (Chlorophyceae), namely *Chlorella vulgaris*,
32 *Nannochloropsis oceanica*, *Haematococcus pluvialis*, and *Dunaliella salina*, and the cyanobacteria
33 such as *Arthrospira platensis* (Gunerken et al., 2015).

34 In particular, *A. platensis* is capable of accumulating a large quantity of proteins (55-70% % dry
35 weight, (DW)), among which C-phycocyanin (≈ 20 % DW), a blue-colored compound well
36 recognized for its high antioxidant and therapeutic properties, in addition to carbohydrates (13 – 16%
37 DW), and lipids (6-10% DW) (Lupatini et al., 2016).

38 Traditionally, solvent extraction is utilized to recover bioactive compounds from biological matrices,
39 including microalgae (Poojary et al., 2016). However, the presence of a rigid cell wall/membrane
40 system surrounding the cytoplasmatic medium and internal organelles, where the valuable
41 compounds are stored (Postma et al., 2016; Vanthoor-Koopmans et al., 2013), dramatically limits the
42 mass transfer phenomena during conventional solvent extraction processes, generally characterized
43 by poor extraction yields and low selectivity (Carullo et al., 2018; Martinez et al., 2020).

44 In light of these drawbacks, cell permeabilization/disintegration pre-treatments, able to improve the
45 extractability of target compounds from microalgae by reducing the mass transfer resistances, are
46 generally required. Conventional methods are based on the application of high-pressure
47 homogenization (HPH), bead milling (BM), and freeze/thawing cycles which enable the penetration
48 of the solvent within the intracellular space and the subsequent increase in solubilization rates through

49 cell wall/membrane disintegration (Martinez et al., 2017; Postma et al., 2017; Safi et al., 2014; 2015).
50 However, despite their high degree of cell rupture, these techniques are typically energy-intensive
51 and may generate a huge amount of finely sized cell debris, as well as cause irreversible damages to
52 sensitive compounds, thus leading to a significant reduction in both quality and purity of the achieved
53 extracts (Carullo et al., 2018; Poojary et al., 2016).

54 For these reasons, novel tailored approaches for microalgae cell permeabilization have been
55 investigated, which aimed at enhancing the extraction efficiency of valuable compounds without
56 affecting their functionality, with the overall goal of pursuing better exploitation of the biomass with
57 reduced energy consumption (Günerken et al., 2015; Leonhardt et al., 2020; Vanthoor-Koopmans et
58 al., 2013).

59 In this line, pulsed electric fields (PEF) technology has been proposed as a mild and sustainable cell
60 disruption method to significantly improve the extractability of high-purity valuable compounds from
61 biomass of vegetable and microbial origin (Carullo et al., 2018; Martinez et al., 2020; Pataro et al.,
62 2018), through the electroporation of cell membranes while avoiding the formation of cell debris, and
63 consequent extract contamination (Carullo et al., 2020). Moreover, PEF technology offers the
64 advantages of wet biomass processing and easy scale-up to great production volumes (Golberg et al.,
65 2016; Käferböck et al., 2020).

66 In the last decade, the capability of PEF pre-treatment to enhance the extractability of target
67 intracellular compounds has been widely demonstrated for several microalgal strains, mostly
68 belonging to the Chlorophyceae family (Gunerken et al., 2015; Martinez et al., 2020), whereas, as per
69 literature survey, only few works focused on *A. plantesis* cyanobacteria (Carullo et al., 2020;
70 Chittapun et al., 2020; Jaeschke et al., 2019; Käferböck et al., 2020; Martinez et al., 2017).

71 In particular, it has been highlighted the capacity of PEF to efficiently release small components, such
72 as carbohydrates and water-soluble proteins of small molecular weight, while, for example, most
73 proteins, which are likely larger and more bounded to intracellular structure, could require the
74 application of intense PEF processing conditions (high field strengths and energy input), especially

75 in the case of "hard-structured" microalgal cells (Pataro et al., 2019; Postma et al., 2016).
76 Nevertheless, it must be remarked that the extraction yield of specific target compounds (e.g.,
77 proteins) from PEF-treated microalgae was found significantly lower than those achieved with highly
78 effective cell disruption techniques, such as BM (Postma et al., 2016) or HPH (Carullo et al., 2018;
79 Safi et al., 2017).
80 Therefore, in order to reduce the operative costs and to maximize the extraction efficiency of high-
81 added-value components up to levels comparable to those obtainable with powerful mechanical
82 disintegration methods (e.g., HPH, BM), the use of PEF in a hurdle approach has been suggested. For
83 example, the recent work of 't Lam et al. (2017a) demonstrated that the release of proteins from the
84 hard-structure *C. reinhardtii* microalgae, at comparable yields to those obtained with BM (70%),
85 could be reached using PEF in combination with an enzymatic pre-treatment for cell wall removal.
86 Instead, the combination of PEF with moderate heating (35°C) resulted in a synergistic effect on the
87 extraction of water-soluble compounds from *A. platensis* microalgae (Carullo et al., 2020).
88 Grimi et al. (2014) investigated for the first time the application of PEF in a sequential cascade mode
89 with other disruption techniques (e.g., high voltage electrical discharges, ultrasound, and high-
90 pressure homogenization) for the efficient recovery of proteins from *Nannochloropsis sp*. microalgae.
91 However, to the best of our knowledge, this is the first attempt to investigate the effect of PEF
92 technology, applied in combination with high-shear homogenization (HSH), on the extraction
93 improvement of intracellular compounds from microalgae.
94 The main objective of this work was to assess the potential of applying an HSH pre-treatment before
95 PEF processing for the intensification of the extraction and the selective recovery of valuable
96 compounds from wet *A. platensis* biomass in a continuous-flow system. In particular, the effect of
97 either single or combined technologies on algal cell morphology, as well as on the release of target
98 molecules (e.g., water-soluble proteins, C-phycocyanin, and carbohydrates), was investigated.
99
100

101 **2. Materials and Methods**

102 *2.1. Microalgal strain and cultivation conditions*

103 Biomass of *A. platensis* (PHC 8005) was gently supplied by ATI Biotech Srl, an algae producer
104 located in Castel Baronia (Avellino, Italy). *A. platensis* was cultivated in open pond systems, in which
105 a maximum biomass concentration of about 0.4 % DW was achieved at the end of the exponential
106 growth phase. After harvesting, the biomass was concentrated using a dewatering system consisting
107 of vibrating screen filters, which allowed to furtherly increase the biomass concentration up to 12%
108 DW. The microalgae paste was subsequently packed in polyethylene bags and immediately
109 transported in an EPS box under refrigerated conditions to the laboratory of ProdAl Scarl (University
110 of Salerno, Fisciano, Italy), where it was stored at 4°C until use, within two days from the delivery
111 date.

112 Prior to being processed, the algae paste was diluted with distilled water up to a final concentration
113 (*Cx*) of 2% DW with an initial conductivity (σ) of about 2.7 mS/cm at 25°C (Conductivity-meter HI
114 9033, Hanna Instrument, Milan, Italy). The biomass concentration was assessed using the method
115 described by Goettel et al. (2013), with the paste dried in a circulating air-drying oven for 24 h at
116 80°C.

117

118 *2.2. Combination of HSH and PEF treatments*

119 The proposed disruption process of *A. platensis* microalgae through the sequential application of HSH
120 and PEF technologies is described as follows. Freshly prepared microalgal suspensions (0.5 L) were
121 subjected to an HSH batch pre-treatment using an Ultra Turrax T25 apparatus (IKA Werke GmbH &
122 Co., DE) equipped with S25-N18-G probe and working at a nominal power of 800 W. The aim of
123 this step was disaggregating *A. platensis* trichome clusters in order to avoid their spontaneous
124 separation from the liquid medium, thus inducing a certain degree of trichome cell disruption.
125 Temperature control during treatment was achieved by immersing the flasks containing the

126 biosuspensions in a water bath set at 25°C. From preliminary results (data not shown) it was found
127 that the application of an HSH treatment at 20000 rpm for 1 min of processing time (t) was capable
128 of ensuring a high extent of *A. platensis* cell clusters disruption and further processing caused only a
129 marginal improvement in the disruptive effect. Under these conditions, the estimated energy
130 consumed per unit mass of biosuspension (W_{HSH}) was 96 kJ/kg_{SUSP}.
131 Subsequently, the microalgal suspensions were treated by PEF in a bench-scale continuous flow
132 system, previously described in detail by Postma et al. (2016) and Carullo et al. (2018). During the
133 experiments, pre-stirred algae suspensions (2% DW) were circulated through the PEF system at a
134 controlled flow rate of 2 L/h by a peristaltic pump (model PU-2080, Jasco Europe, Cremella (LC),
135 Italy). The inlet temperature of samples to the treatment chamber was controlled using a stainless-
136 steel coiled tube submerged in a water heating bath (Thermo Haake DC 10, Henco Srl, Italy) set at
137 25°C. The treatment chamber consisted of two modules, each made of two co-linear treatment
138 chambers, hydraulically connected in series, with an inner radius of 1.5 mm and a gap distance of 4
139 mm. Monopolar square wave electric pulses were delivered to the microalgal suspension by a high
140 voltage pulsed power (20 kV - 100 A) generator (Diversified Technology Inc., Bedford, WA, USA).
141 In all the experiments, the pulse width was fixed at 5 μs, while the electric field strength (E) and total
142 specific energy input (W_T) were set at 20 kV/cm and 100 kJ/kg, respectively, which were previously
143 identified as optimal PEF treatment conditions to achieve the maximum release of intracellular
144 compounds from *A. platensis* cell suspensions with the minimum treatment intensity (Carullo et al.,
145 2020). T-thermocouples were used to measure the product temperature at the inlet and outlet of each
146 module of the PEF chamber. Under the selected operating conditions, the maximum temperature
147 increase of the samples, detected at the exit of the PEF treatment chamber, never exceeded 10°C.
148 At the exit of the treatment chamber, samples of treated algae suspensions were collected in plastic
149 tubes, immediately placed in an ice-water bath to be rapidly cooled up to a final temperature of 25°C,
150 and then undergoing the aqueous extraction process for 3 h at 25°C under gentle agitation (160 rpm),
151 according to the protocol described in a previous work (Carullo et al., 2020).

152 At the end of the extraction process, aqueous supernatants were separated from the spent pellet by
153 centrifugation of cell suspensions for 10 min at 5700×*g* (PK121R model, ALC International, Cologno
154 Monzese, Milan, IT) and stored under refrigerated conditions (T=4°C) until further analysis.
155 For the sake of comparison, untreated samples (control), and samples treated individually by either
156 HSH or PEF were also collected and subjected to water extraction by following the same protocol as
157 for HSH + PEF treated biosuspensions.
158

159 *2.3. Complete cell disruption by HPH treatment*

160 HPH treatment was used as a benchmark for full disruption of *A. platensis* cells, for the estimation of
161 the total content of target intracellular compounds. HPH treatments were carried out by using an in-
162 house developed laboratory scale high-pressure homogenizer (Carullo et al., 2018). The *A. platensis*
163 suspensions, at the same concentration as for HSH - PEF treatment tests (2% DW), were forced to
164 pass through a 100 μm diameter orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt,
165 Germany) upon pressurization at 150 MPa through an air-driven Haskel pump (model DXHF-683,
166 EGAR S.r.l., Milan, Italy). According to preliminary tests (data not shown), the full disruption of *A.*
167 *platensis* cells and, hence, the complete release of intracellular compounds, was achieved at a pressure
168 drop (P) across the orifice of 150 MPa and after 3 homogenization passes (n_p).
169

170 *2.4. Energy analysis*

171 To enable the comparison in terms of energy efficiency among the different investigated extraction
172 processes (i.e., HSH, PEF, HSH + PEF, and HPH), the energy consumed (EC) to extract 1 kg DW of
173 target intracellular compounds, namely water-soluble proteins (WSP), C-phycocyanin (C-PC) and
174 total carbohydrates (CH), from *A. platensis* cell suspension, was calculated according to Eqs. (1-3)
175 for the individual treatment of HSH, PEF, and HPH, respectively.

176 $$EC_{HSH} = \frac{W_{HSH}}{C_X \cdot 3600 \cdot Y_i} \quad (1)$$

177 $$EC_{PEF} = \frac{W_T}{C_X \cdot 3600 \cdot Y_i} \quad (2)$$

178 $$EC_{HPH} = \frac{P \cdot n_p}{C_X \cdot \eta_{PUMP} \cdot 3600 \cdot \rho_{BIOMASS} \cdot Y_i} \quad (3)$$

179 where EC is expressed in kWh/kgDW, η_{PUMP} is the overall efficiency of HPH pumping system (0.87)
180 (Carullo et al., 2018), $\rho_{BIOMASS}$ is the density of microalgal suspensions (~ 1000 kg/m^3), 3600 is the
181 conversion factor between kJ and kWh, and Y_i is the recovery yield (in kg/kg of DW microalgae) of
182 the target compounds (i=WSP, C-PC, CH) achieved upon the different extraction processes.

183

184 *2.5. Analytical methods*

185 *2.5.1. Particle size distribution (PSD)*

186 PSD of untreated and treated (PEF, HSH, HSH + PEF) algae suspensions were analyzed in a
187 MasterSizer 2000 particle size analyzer (Malvern, United Kingdom) at 25°C. The size distribution of
188 biosuspensions was evaluated by using the Fraunhofer approximation, and the volume moment mean
189 diameter ($D_{4,3}$) was calculated for each processing condition. The parameters used in the
190 determination of the PSD were the properties of water at 25 °C (refraction index = 1.33), which was
191 used as a dispersant medium.

192

193 *2.5.2. Optical microscopy and Scanning Electron Microscopy*

194 The effect of different treatments (PEF, HSH, or HSH + PEF) on morphological features of algal
195 cells was evaluated either by optical or Scanning Electron Microscopy (SEM). Micrographs were
196 obtained with an inverted optical microscope (Nikon Eclipse TE2000-S) at 20 × magnification. SEM
197 analysis was performed according to the method previously illustrated elsewhere (Carullo et al.,
198 2018). Metalized dried pellets from untreated and treated (HSH, PEF, HSH + PEF) algae suspensions

199 were analyzed in a high-resolution ZEISS HD15 Scanning Electron Microscope (Zeiss, Oberkochen,
200 Germany) at 5000 × magnification.
201
202 ### 2.5.3. *Protein analysis*
203 The water-soluble protein content of supernatants was evaluated by using the method of Lowry et al.
204 (1951), with some modifications as described in Carullo et al. (2018). Specifically, the reactive
205 system consisted in 0.5 mL of diluted (1/2, v/v in ultra-pure water) Folin-Ciocalteau reactive (Folin
206 & Ciocalteau, 1927), to which 1 mL of fresh sample (supernatant), previously mixed with 5.0 mL of
207 the reactive "C" [50 volumes of reactive "A" (2% Na_2CO_3 + 0,1 N NaOH) + 1 volume of reactive
208 "B" (1/2 volume of 0.5% $CuSO_4 \cdot 5H_2O$ + 1/2 volume of 1% $KNaC_4H_4O_6 \cdot 4H_2O$)] (Sigma Aldrich,
209 Milan, Italy) were added. Absorbance was measured at 750 nm against a blank (5 mL reactive "C" +
210 1 mL deionized water + 0.5 mL Folin-Ciocalteau reactants), 35 min after the start of the chemical
211 reaction by using a V-650 Spectrophotometer (Jasco Inc. Easton, MD, USA). Bovine serum albumin
212 (BSA) (A7030, Sigma Aldrich, Milan, Italy) was used as standard and the results were expressed as
213 mg equivalent of BSA per g of dry biomass (mg_{BSA}/g_{DW}).
214
215 ### 2.5.4. *Carbohydrate analysis*
216 The total carbohydrate concentration of the supernatants was analyzed according to the phenol-
217 sulfuric acid method previously described by DuBois et al. (1956). 0.2 mL of 5% (w/w) phenol and
218 1 mL of concentrated sulfuric acid (Sigma Aldrich, St. Louis, USA) were added to 0.2 mL of diluted
219 supernatant (Dilution Factor = 5). Samples were incubated at 35 °C for 30 min before reading the
220 absorbance at 490 nm against a blank of 0.2 mL 5% (w/w) phenol, 1 mL concentrated sulfuric acid,
221 and 0.2 mL of deionized water. D-Glucose (G8270, Sigma-Aldrich, Milan, Italy) was used as a
222 standard and the results were expressed as equivalent mg of D-glucose per g of dry biomass (mg_{D-}
223 $_{Glu}/g_{DW}$).

224

225 *2.5.5. C-phycocyanin and protein extract purity*

226 The quantification of C-PC content of the supernatants was performed according to the method of
227 Bennet & Bogorad (1973), which is based on the measurements of the absorbance (A) of the samples
228 at two fixed wavelengths (λ_1=615 nm, and λ_2=652 nm). The C-phycocyanin concentration, expressed
229 as mg/g_{DW} of supernatant, was evaluated according to Eq. (4):

230 $$C - PC = \frac{(A_{615\ nm} - 0.474\ A_{652\ nm})}{5.34 \cdot C_x} \quad (4)$$

231 The purity of C-PC extract was monitored spectrophotometrically and calculated by the Eq. (5)
232 (Abelde et al., 1998; Martinez et al., 2017):

233 $$EP = \frac{A_{615\ nm}}{A_{280\ nm}} \quad (5)$$

234 where EP is the protein extract purity, A_{615nm} absorbance represents the maximum absorption of the
235 C-phycocyanin peak, proportional to its concentration in the supernatant, and A_{280nm} is the absorbance
236 at 280 nm, indicating the total concentration of proteins in the supernatant.

237

238 *2.5.6. UV-Vis Spectrum measurements*

239 UV – Vis spectra of aqueous supernatants obtained after water extraction were plotted as a function
240 of the investigated range of wavelengths (λ = 200 – 800 nm). Characteristics peaks of water-soluble
241 proteins (λ = 280 nm), carotenoids (λ = 435 nm), C-phycocyanin (λ = 615 nm), and chlorophyll (λ =
242 675 nm) were determined from spectra measurements and used to compare the effect of HSH and
243 PEF technologies, applied individually or in combination, on the achieved degree of selectivity
244 towards the above-mentioned compounds.

245

246

247 *2.6. Statistical analysis*

248 All treatments and analyses were carried out in triplicate and the results were reported as mean values
249 ± standard deviations. The statistical analysis was performed with the IBM SPSS Statistics 20.0
250 (SPSS Inc., Chicago, USA) software using one-way analysis of variance (ANOVA). Tukey's test was
251 executed at a fixed significance level ($p \leq 0.05$), for the determination of any statistical difference
252 among the untreated and processed samples.

253

254 **3. Results and Discussion**

255 *3.1.Effect of HSH and PEF treatments on A. platensis morphology*

256 The particle size distribution (PSD) analyses and microscopic observations, reported in Figures 1 and
257 2, respectively, highlight the different effects of HSH and PEF treatments, applied individually or in
258 combination, on the size and microstructure of *A. platensis* trichomes.
259 Remarkably, PSD curves show that intact *A. platensis* trichomes (Control) exhibited a multimodal
260 size distribution curve, characterized by two main peaks detected in the range between 20 μm and
261 100 μm, together with a smaller peak at around 500 μm, which could be likely associated to the
262 presence of trichome clusters. PEF processing (20 kV/cm – 100 kJ/kg) of *A. platensis* suspensions
263 did not induce appreciable changes in the PSD curve, as also testified by the statistically similar value
264 ($p > 0.05$) of the volume-weighted mean diameters of PEF-treated and control samples ($D_{4,3} \approx 70$
265 μm). These results are consistent with previous findings on different microalgal strains (Carullo et
266 al., 2018; 't Lam et al., 2017b), which demonstrated that PEF is a relatively mild cell disruption
267 method, preserving the initial structure of the algae cells.
268 Conversely, HSH treatments drastically affected the *A. platensis* trichomes, with the PSD
269 characterized by a single wide peak within the range of 3 – 90 μm (Figure 1). As a consequence, the

270 $D_{4,3}$ value was significantly ($p \leq 0.05$) reduced up to about 20 μm, likely due to the breakage of
271 microalgal trichomes under the high-shearing forces created by the equipment. Remarkably, Park et
272 al. (2015) did not observe any significant alteration in the mean cell size of *C. vulgaris* microalgae
273 (0.1 – 2 % DW) when subjected to a shear homogenization treatment at 7000 rpm, even for very long
274 processing times (t = 6 – 24 h). However, the higher intensity of the shear treatment used in this work
275 (20000 rpm) and the different morphological characteristics of the microalgal strains could explain
276 the observed differences.
277 It is worth noting that, when cascade treatments (HSH + PEF) were applied, no significant ($p > 0.05$)
278 variations neither in the PSD curves nor in the $D_{4,3}$ values were detected in comparison with the
279 individual HSH treatment, further confirming the almost negligible effect of PEF on *A. platensis*
280 microstructure.
281 The interpretation of the data of Figure 1 can be supported by the micrographs of untreated, and
282 treated (individual HSH or PEF, combined HSH + PEF) microalgae (Figure 2), obtained by either
283 optical (a) and SEM (b) microscopy. Figure 3a shows that the fluid-mechanical stresses generated
284 during HSH treatments lead to the partial disruption of microalgal trichomes, with an evident
285 reduction of the length of the algal filaments, in agreement with the reduction in $D_{4,3}$ shown in Figure
286 1. However, Figures 1 and 2 also suggest that HSH treatment had only a limited effect on the
287 disruption of the individual cells constituting the trichomes, in agreement with the results already
288 observed in previous works on different microalgae, where it is suggested that more intense
289 treatments (e.g., HPH) are needed (Carullo et al., 2018, 2020).
290 Interestingly, PEF treatment did not affect the trichomes structure, with the only visible effect of the
291 treatment being the release of intracellular matters towards the aqueous phase (colored areas around
292 the trichomes in Figure 2a) due to membrane permeabilization of algae cells. Similar conclusions
293 were drawn by both Carullo et al. (2020) and Käferböck et al. (2020) when studying the extractability
294 of valuable compounds upon PEF processing of microalgae *Arthrospira platensis* and *Arthrospira*
295 *maxima,* respectively. The authors noticed the presence of filamentous structures and colored spots

296 in the surrounding media of PEF treated microalgae, which were attributed to the leakage of proteins
297 and pigments upon the formation of pores at the membrane level.
298 In accordance with the results of Figure 1, the application of PEF treatments to HSH-treated samples
299 did not cause any additional visible fragmentation of trichomes, even though an intensification in the
300 colored areas around microalgal trichomes could be observed. This behavior might be likely
301 explained by considering the different mechanisms of cell disintegration occurring when HSH or PEF
302 treatments are applied. It can be hypothesized that the effect of trichome clusters breakage exerted by
303 HSH positively impacted on the efficiency of the subsequent electroporation step, with a clear
304 increase in the leakage of intracellular matter. These statements are supported by the findings of Donsì
305 et al. (2007), who reported that the inactivation of *S. cerevisiae* achieved upon PEF processing strictly
306 depended on the initial cell concentration in the treated medium. Specifically, it has been found that,
307 at cell concentration greater than 10^5 CFU/mL, yeasts showed a tendency to form clusters that
308 protected the inner cells from the action of the electric field, thus reducing the lethality of PEF
309 treatments.
310 Additional information on the effect of individual or combined treatments on the morphology of *A.*
311 *platensis* trichomes were provided by SEM analysis (Figure 2b). The surface of intact cells appeared
312 to be regular and smooth, which is consistent with the findings of Choi & Lee (2018). However, the
313 HSH treatment caused the partial fragmentation of the trichomes, with evidence of some disrupted
314 cells (yellow arrows in Figure 2b). In contrast, PEF processing only led to an increase in surface
315 roughness and the formation of cracks and depressions on the surface of the cells, which could be
316 ascribed to the mentioned electroporation phenomena and subsequent leakage of intracellular
317 compounds. Similar findings were previously reported by Han et al. (2019), who observed an increase
318 in surface roughness of *C. pyrenoidosa* cells upon the electroporation effect induced by the
319 application of PEF treatment at 20 kV/cm. The increase in surface roughness upon
320 electropermeabilization was previously reported for different types of microalgal strains, such as
321 *Chlorella vulgaris* (Carullo et al., 2018), *Chlorella pyrenoidosa* (Han et al., 2019), and *A. platensis*

322 (Carullo et al., 2020). Interestingly, when HSH and PEF were coupled, an enhanced effect on cell
323 morphology was observed, resulting from the contribution of the individual treatments, causing a
324 greater extent of cell permeabilization. However, none of the utilized technologies (HSH, PEF, HSH
325 + PEF) lead to the formation of finely sized cell debris, obtained in the case of full cell disintegration
326 by high-pressure homogenization treatments (Carullo et al., 2020).

327

328 *3.2.Effect of combined HSH – PEF treatment on the extractability of water-soluble compounds*

329 The quantification of the release of intracellular compounds in the aqueous phase enabled to further
330 highlight the differences among the investigated treatments (HSH, PEF, HSH + PEF) in terms of the
331 cell disruption efficiency.
332 Figure 3 reports the content of WSP (a), CH (b), C-PC (c), and EP (d) of supernatants from untreated
333 (control) samples and for individual or combined treatments. For the sake of comparison, also the
334 extraction yields and extract purity obtained after complete cell disruption by HPH treatments (P =
335 150 MPa, n_P = 3) are reported.
336 Results show that the intact cellular structure of *A. platensis* trichomes enabled only a marginal
337 leakage of water-soluble compounds during the extraction step, yielding final concentrations of WSP,
338 CH, and C-PC in the aqueous supernatant of 12.3 mg_{BSA}/g_{DW}, 4.2 $mg_{D\text{-}Glu}/g_{DW}$, and 0.2 mg/g_{DW},
339 respectively. In agreement with previous findings (Carullo et al., 2018; Jaeschke et al., 2019), this
340 release results from the concentration gradient between cells and the external medium, eventually in
341 combination with spontaneous cell lysis. Instead, when single HSH or PEF treatments were applied,
342 significant ($p \leq 0.05$) increases in the concentrations of WSP (Figure 3a), CH (Figure 3b), and C-PC
343 (Figure 3c) in the supernatant were detected, in a manner dependent on the targeted compound. In
344 particular, assuming a 100% extraction yield upon HPH treatment, HSH enabled the extraction of
345 about 25.8 % (w/w) of total proteins, 24.2 % (w/w) of total carbohydrates, and 27.9 % (w/w) of total
346 C-PC. The similar extraction yields of target compounds detected after HSH processing could be

347 potentially attributed to the undifferentiated release of intracellular matter deriving from the
348 mechanical disruption of a fraction of the *A. platensis* microalgal cells, whereas a large fraction of
349 undamaged cells (and undamaged cell walls/membranes) in smaller-sized fragmented trichomes still
350 limited the release process. The electroporation of cell membranes caused by PEF greatly increased
351 the extractability of WSP (174 mg_{BSA}/g_{DW}), CH (101 $mg_{D\text{-}Glu}/g_{DW}$), and C- PH (26 mg/g_{DW}), in
352 comparison with untreated samples. In agreement with previous findings (Carullo et al., 2018, 2020;
353 Goettel et al., 2013; Pataro et al., 2017; Postma et al., 2016), Figure 3 shows the capability of PEF to
354 selectively release low molecular weight proteins and carbohydrates, whereas larger macromolecular
355 compounds, such as C-PC, remained trapped in the intracellular structure. The larger release of WSP
356 and CH detected after PEF processing of *A. platensis* (Figures 3a - 3b), in comparison with those
357 reported in other works for green microalgae (Carullo et al., 2018; Goettel et al., 2013; Pataro et al.,
358 2017; Postma et al., 2016; 't Lam et al., 2017b), could be partially explained by considering the
359 differences in cell wall composition. The algae tested in this work are characterized by a relatively
360 weak cell wall, mainly composed of murein (peptidoglycan) without any cellulose (Lu et al., 2006;
361 Safi et al., 2014), whereas green microalgae (e.g. *Chlorella vulgaris*, *Auxenochlorella protothecoides,*
362 *Neochloris oleoabundans, Chlamydomonas reinhardtii*) are protected by a hard bilayer of cellulose
363 and hemicellulose (Safi et al., 2015), thus offering greater resistance to disruption processes and,
364 hence, to the further diffusion step.
365 The use of a cascade approach markedly promoted the extractability of water-soluble compounds
366 towards the external medium, in comparison with individual HSH or PEF treatments (Figure 3). In
367 particular, a clear synergistic effect was observed for the release of both WSP (Figure 4a) and C-PC
368 (Figure 3c), which were increased up to 460 mg_{BSA}/g_{DW}, and 53 mg/g_{DW}, respectively, while an
369 additive effect was observed in the case of CH (139 $mg_{D\text{-}Glu}/g_{DW}$).
370 Interestingly, because of the synergy between shear forces and electric field, the extractability of
371 target compounds was comparable to that achieved by HPH processing, with an estimated recovery
372 of 67.2 % (w/w) of total WSP, 87.7 % (w/w) of total carbohydrates, and 88.6 % (w/w) of total C-PC.

373 Coherently with the results of Figures 1 and 2, it can be concluded that the fragmentation of trichomes
374 induced by HSH likely increased the free microalgal surface, thus improving the efficiency of the
375 electropermeabilization treatment of *A. platensis* cells.
376 Despite the scarce data available on the combined use of PEF and mechanical disruption techniques,
377 except for HPH (Grimi et al., 2014), the present results are consistent with the findings previously
378 reported for the combination of PEF treatments with other physical or chemical stress factors,
379 including temperature, alkaline extraction, enzymes or supercritical fluids (Carullo et al., 2020;
380 Luengo et al., 2015; Martinez et al., 2017; Parniakov et al. 2015; Pataro et al., 2019; 't Lam et al.,
381 2017a). For example, Pataro et al. (2019) investigated the effect of PEF pre-treatment on the
382 extractability of pigments from *Nannochloropsis oceanica* microalgae by supercritical CO_2. It was
383 observed that the mild cell permeabilization induced by the electrical pre-treatment (10 kV/cm; 100
384 $kJ/kg_{SUSP.}$) facilitated the penetration of CO_2 into microalgal cells, with consequent increases in the
385 recovery of both carotenoids and chlorophyll *a*. Martinez et al. (2017) reported, instead, the enhanced
386 susceptibility of *A. platensis* cell membranes to PEF treatments (20 kV/cm, 150 μs), upon application
387 of mild heating to 40 °C, which significantly improved the recovery of C-PC during extraction, in
388 comparison with electropermeabilization at 25 °C.
389 In general, the application of highly effective cell disruption treatments, such as HPH, is required to
390 achieve full microalgal cell disintegration and, hence, maximum extraction yields (Carullo et al.,
391 2018; Safi et al., 2014, 2015). However, due to the undifferentiated release of intracellular matters,
392 the extracts obtained by HPH are generally characterized by low purity (Carullo et al., 2020). This is
393 confirmed by the results of Figure 3d, showing that PEF ensured a higher purity of C-PC extract from
394 *A. platensis* cells suspensions (0.97) than HSH or HPH (0.51 – 0.53). However, the purity of C-PC
395 extracts from combined treated microalgae was not significantly ($p > 0.05$) different from that granted
396 by PEF alone. This means that in the cascade treatment, PEF still exerts a positive impact on the
397 selective extraction of C-PC, while the main effect of HSH pre-treatment is to quantitatively improve
398 the recovery yields of intracellular compounds upon fragmentation of *A. platensis* trichomes.

399 Remarkably, the C-PC extract from combined HSH + PEF treatments could be used without further
400 refining stages, because amply exceeding the minimum purity ratio (0.7) required for food-grade
401 products (Rito-Palomares et al., 2001).
402 The results of Figure 3d are also reinforced by the UV-Vis absorption spectra of supernatants from
403 untreated (control) and treated (HSH, PEF, HSH + PEF) microalgae, shown in Figure 4. The HSH
404 treatment alone leads to the release of all the intracellular matter from disrupted cells, including
405 different pigments, such as carotenoids and chlorophylls, which remain suspended in the water phase,
406 thus imparting a green coloration to the extract. Instead, the application of PEF treatment, with a
407 milder impact on the cellular structure, enabled to selectively recover the proteins and especially C-
408 PC, as testified by the light-blue coloration of supernatant, with the absence of peaks associated with
409 yellow-red (420 nm) or green (662 nm) pigments. These results are in good agreement with the
410 findings of Grimi et al. (2014), who showed that PEF was unable to cause the release of water-
411 insoluble pigments from *Nannochloropsis sp*. cells in the aqueous phase.
412 The spectrum of the extract from the combined treatment reflects the synergistic action of HSH and
413 PEF, with the significant increase in the peak height at 280 nm (WSP) and 615 nm (C-PC) above the
414 peaks for individual treatments. However, it is worth noting that in the combined treatment the
415 extractability of water-insoluble pigments was not enhanced, thus preserving the selective behavior
416 of PEF technology when applied alone.
417 These observations are also confirmed by SDS-PAGE analysis, reported in the Supplementary
418 Material. The electrophoretograms of extracts from individual HSH and PEF treatments and
419 combined HSH + PEF (Figure S1 of Supplementary Material) show that, regardless of the applied
420 cell disruption method, a wide range of protein subunits were released, mostly within 17 – 130 kDa
421 of molecular weight. However, the extracts from the PEF-treated sample exhibited two more distinct
422 bands between 17 kDa and 20 kDa, which are compatible with α and β subunits of C-PC (Kumar et
423 al., 2014). The higher intensity of the bands detected at 17 – 20 kDa for PEF and HSH + PEF than
424 HSH alone is representative of the higher selectivity of PEF processing towards C-PC. Similar to our

425 observations, Jaesckhe et al. (2019) demonstrated that, in comparison with BM processing, extracts
426 from PEF-treated *A. platensis* microalgae showed a greater separation of C-PC characteristic bands,
427 which was utilized as an indicator of purity.
428
429 *3.3. Energy efficiency analysis*

430 The different cell disruption techniques investigated in this work (HSH, PEF, HSH + PEF, and HPH)
431 have been compared in Table 1 in terms of consumed energy per unit mass of target compound
432 extracted, in the perspective of application in the *A. platensis* biorefinery. The individual HSH or PEF
433 treatments were more energy-efficient than HPH for all the targeted compounds (WSP, CH, and C-
434 PC) because full cell disruption by HPH required high pressure (150 MPa) and 3 homogenization
435 passes. Moreover, the PEF treatment was characterized by a remarkably higher energy efficiency than
436 HSH in the case of CH and C-PC, whereas a slightly lower efficiency for WSP recovery was
437 determined. These results could be likely associated with the limited capability of PEF alone to unlock
438 high-molecular-weight proteins, rather than CH and C-PC, in comparison with the mechanical cell
439 disruption induced by HSH. Interestingly, the application of a cascade treatment required
440 significantly lower energy consumption per unit mass of WSP and C-PC than individual HSH and
441 PEF treatments, in agreement with the synergistic effect observed in Figures 4a and 4c, while a slight
442 increase in energy consumption in comparison with the individual PEF treatment was determined for
443 CH. Table 1 also shows that the combined approach reduced the energy consumption required for
444 full cell disruption (HPH) on average by more than 50%, with the additional benefit of facilitating
445 the separation/purification steps in downstream processing, due to either the selective release of target
446 molecules and the absence of cell debris.
447 However, it can be expected that the energy efficiency of the different cell disruption methods
448 investigated in this work is greatly affected by the concentration in the biosuspension and the structure

449 and composition of the microalgae strain. Therefore, additional research is required to elucidate these
450 aspects and support the design of higher scale operations.
451

452 ## 4. Conclusions

453 The application of a HSH treatment, followed by electropermeabilization, to *A. platensis* suspensions
454 significantly improved the recovery of target compounds in comparison with single HSH or PEF
455 treatments, showing a synergistic effect. Moreover, for the combined treatment, extraction yields
456 comparable to those achieved by HPH were observed, with the additional advantage of a higher
457 extract purity and significantly lower energy consumption.
458 These promising results highlighted the potential of HSH + PEF treatments in the frame of microalgal
459 biorefinery to efficiently recover target compounds during water extraction, thus reducing the overall
460 processing costs and facilitating separation/purification steps in downstream processing.

461 **Conflict of Interest**

462 The authors declare that the research was conducted in the absence of any commercial or financial
463 relationships that could be construed as a potential conflict of interest.

464 **Author Contributions**

465 GP and GF contributed to conception and design of the study; DC was in charge of performing
466 chemical and statistical analysis; GP, FD, and DC performed the experiments; GF supervised the
467 study; DC wrote the first draft of the manuscript; All authors contributed to manuscript revision, read
468 and approval of the submitted version.

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472 **References**

473 Abelde, J., Betancourt, L., Torres, E., Cid, A., Barwell, C., 1998. Purification and characterization of
474 phycocyanin from the marine cyanobacterium Synechococcus sp. IO9201. Plant Sci. 136, 109–120.
475 doi: 10.1016/S0168-9452(98)00113-7.

476 Bennett, A., Bogorad, L., 1973. Complementary chromatic adaptation in a filamentous blue-green
477 alga. J. Cell Biol. 58, 419 – 435. doi: 10.1083/jcb.58.2.419.

478 Carullo, D., Abera, B.D., Casazza, A.A., Donsì, F., Perego, P., Ferrari, G., Pataro, G., 2018. Effect
479 of Pulsed Electric Fields and High-Pressure Homogenization on the aqueous extraction of
480 intracellular compounds from the microalgae Chlorella vulgaris. Algal Res. 31, 60 – 69. doi:
481 10.1016/j.algal.2018.01.017.

482 Carullo, D., Pataro, G., Donsì, F., Ferrari, G., 2020. Pulsed Electric Fields-Assisted Extraction of
483 Valuable Compounds From Arthrospira Platensis: Effect of Pulse Polarity and Mild Heating. Front.
484 Bioeng. Biotechnol. 8, 551272. doi: 10.3389/fbioe.2020.551272.

485 Chittapun, S., Jonjaroen, V., Khumrangsee, K., Charoenrat, T., 2020. C-phycocyanin extraction from
486 two freshwater cyanobacteria by freeze thaw and pulsed electric field techniques to improve
487 extraction efficiency and purity. Algal Res. 46, 101789. doi: 10.1016/j.algal.2020.101789.

488 Choi, W.Y., Lee, H.Y., 2018. Enhancement of Neuroprotective Effects of Spirulina platensis Extract
489 from a High-Pressure Homogenization Process. Appl. Sci. 8, 634. doi: 10.3390/app8040634.

490 Donsì, G., Ferrari, G., Pataro, G., 2007. Inactivation kinetics of Saccharomyces cerevisiae by pulsed
491 electric fields in a batch treatment chamber: The effect of electric field unevenness and initial cell
492 concentration. J. Food Eng. 78, 784 – 792. doi: 10.1016/j.jfoodeng.2005.11.027.

493 Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1957. Colorimetric Method for
494 Determination of Sugars and Related Substances. Anal. Chem. 28, 350-356. doi:
495 10.1021/ac60111a017.

496 Folin, O., Ciocalteau, V., 1927. On tyrosine and tryptophane determinations in proteins. J. Biol.
497 Chem. 73, 627–650.

498 Garcia, E.S., van Leeuwen, J., Safi, C., Sijtsma, L., Eppink, M.H.M., Wijffels, R.H., van den Berg,
499 C., 2018. Selective and energy efficient extraction of functional proteins from microalgae for food
500 applications. Bioresour. Technol. 268, 197–203. doi: 10.1016/j.biortech.2018.07.131.

501 Goettel, M., Eing, C., Gusbeth, C., Straessner, R., Frey, W., 2013. Pulsed electric field assisted
502 extraction of intracellular valuables from microalgae. Algal Res. 2, 401 – 408. doi:
503 10.1016/j.algal.2013.07.004.

504 Golberg, A., Sack, M., Teissie, J., Pataro, G., Pliquett, U., Saulis, G., Töpfl, S., Miklavcic, D.,
505 Vorobiev, E., Frey, W., 2016. Energy-efficient biomass processing with pulsed electric fields for
506 bioeconomy and sustainable development. Biotechnol. Biofuels 9, 1 – 22. doi: 10.1186/s13068-016-
507 0508-z.

508 Grimi, N., Dubois, A., Marchal, L., Jubeau, S., Lebovka, N.I., Vorobiev, E., 2014. Selective
509 extraction from microalgae Nannochloropsis sp. using different methods of cell disruption. Bioresour.
510 Technol. 153, 254–259. doi: 10.1016/j.biortech.2013.12.011.

511 Günerken, E., D'Hondt, E., Eppink, M.H.M., Garcia-Gonzales, L., Elst, K., Wijffels, R.H., 2015. Cell
512 disruption for microalgae biorefineries. Biotechnol. Adv. 33, 243 – 260. doi:
513 10.1016/j.biotechadv.2015.01.008.

514 Han, S.-F., Jin, W., Yang, Q., Abomohra, A.E.-F., Zhou, X., Tu, R., Chen, C., Xie, G.-J., Wang, Q.,
515 2019. Application of pulsed electric field pretreatment for enhancing lipid extraction from Chlorella
516 pyrenoidosa grown in wastewater. Ren. En. 133, 233 – 239. doi: 10.1016/j.renene.2018.10.034.

517 Jaeschke, D.P., Domeneghini Mercali, G., Ferreira Marczak, L.D., Müller, G., Frey, W., Gusbeth, C.,
518 2019. Extraction of valuable compounds from Arthrospira platensis using pulsed electric field
519 treatment. Bioresour. Technol. 283, 207 – 212. doi: 10.1016/j.biortech.2019.03.035.

520 Käferböck, A., Smetana, S., de Vos, R., Schwarz, C., Toepfl, S., Parniakov, O., 2020. Sustainable
521 extraction of valuable components from Spirulina assisted by pulsed electric fields technology. Algal
522 Res. 48, 101914. doi: 10.1016/j.algal.2020.101914.

523 Kumar, D., Dhar, D.W., Pabbi, S., Kumar, N., Walia, S., 2014. Extraction and purification of C-
524 phycocyanin from Spirulina platensis (CCC540). Ind J Plant Physiol. 19, 184–188. doi:
525 10.1007/s40502-014-0094-7.

526 Leonhardt, L., Käferböck, A., Smetana, S., Vos, R.d., Toepfl, S., Parniakov, O., 2020. Biorefinery of
527 Chlorella sorokiniana with Pulsed electric field pre-treatment. Bioresour. Technol. 301, 122743. doi:
528 https://doi.org/10.1016/j.biortech.2020.122743.

529 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin
530 phenol reagent. J. Biol. Chem. 193, 265 - 275.

531 Lu, H.K., Hsieh, C.C., Hsu, J.J., Yang, Y.K., Chou, H.N., 2006. Preventive effects of Spirulina
532 platensis on skeletal muscle damage under exercise-induced oxidative stress. Eur. J. Appl. Physiol.,
533 98, 220 – 226. doi: 10.1007/s00421-006-0263-0.

534 Luengo, E., Martınez, J.M., Bordetas, A., Alvarez, I., Raso, J., 2015. Influence of the treatment
535 medium temperature on lutein extraction assisted by pulsed electric fields from Chlorella vulgaris.
536 Innov. Food Sci. Emerg. Technol. 29, 15–22. doi: 10.1016/j.ifset.2015.02.012.

537 Lupatini, A. L., Colla, L. M., Canan, C., Colla, E., 2016. Potential application of microalgae Spirulina
538 platensis as a protein source. J. Sci. Food Agric. 97, 724-732. doi: 10.1002/jsfa.7987.

539 Martinez, J. M., Luengo, E., Saldana, G., Alvarez, I., Raso, J., 2017. C-phycocyanin extraction
540 assisted by pulsed electric field from Artrosphira platensis. Food Res. Int. 99, 10421047. doi:
541 10.1016/j.foodres.2016.09.029.

542 Martinez, J. M., Delso, C., Alvarez, I., Raso, J., 2020. Pulsed electric field-assisted extraction of
543 valuable compounds from microorganisms. Compr. Rev. Food Sci. Food Saf. 19, 530 – 552. doi:
544 10.1111/1541-4337.12512.

545 Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for Biodiesel Production and Other
546 Applications: A Review. Ren. Sust. Energy Rev. 14, 217-232. doi: 10.1016/j.rser.2009.07.020.

547 Park, J.-Y., Lee, K., Choi, S.-A., Jeong, M.-J., Kim, B., Jin-Suk Lee, Oh, Y.-K., 2015. Sonication-
548 assisted homogenization system for improved lipid extraction from Chlorella vulgaris. Renew.
549 Energy 79, 3-8. doi: 10.1016/j.renene.2014.10.001.

550 Parniakov, O., Barba, F.J., Grimi, N., Marchal, L., Jubeau, S., Lebovka, N., Vorobiev, E., 2015.
551 Pulsed electric field assisted extraction of nutritionally valuable compounds from microalgae
552 Nannochloropsis spp. using the binary mixture of organic solvents and water. Innov. Food Sci.
553 Emerg. Technol. 27, 79 – 85. doi: 10.1016/j.ifset.2014.11.002.

554 Pataro, G., Goettel, M., Straessner, R., Gusbeth, C., Ferrari, G., Frey, W., 2017. Effect of PEF
555 Treatment on Extraction of Valuable Compounds from Microalgae C. vulgaris. Chem. Eng. Trans.
556 57, 67 – 72. doi: 10.3303/CET1757012.

557 Pataro, G., Carullo, D., Siddique, M.A.B., Falcone, M., Donsì, F., Ferrari, G., 2018. Improved
558 extractability of carotenoids from tomato peels as side benefits of PEF treatment of tomato fruit for

559 more energy-efficient steam-assisted peeling. J. Food Eng. 233, 65 – 73. doi:
560 10.1016/j.jfoodeng.2018.03.029.

561 Pataro, G., Carullo, D., Ferrari, G., 2019. PEF-assisted Supercritical CO2 Extraction of Pigments,
562 from Microalgae Nannochloropsis oceanica in a Continuous Flow System. Chem. Eng. Trans. 74, 97
563 – 102. doi: 10.3303/CET1974017.

564 Poojary, M.M., Barba, F.J., Aliakbarian, B., Donsì, F., Pataro, G., Dias, D.A., Juliano, P., 2016.
565 Innovative Alternative Technologies to Extract Carotenoids From Microalgae and Seaweeds. Mar.
566 Drugs 14, 214. doi: 10.3390/md14110214.

567 Postma, P. R., Pataro, G., Capitoli, M., Barbosa, M.J., Wijffels, R.H., Eppink, M.H.M., Olivieri, G.,
568 Ferrari, G., 2016. Selective extraction of intracellular components from the microalga Chlorella
569 Vulgaris by combined pulsed electric field – temperature treatments. Bioresour. Technol. 203, 80 –
570 88. doi: 10.1016/j.biortech.2015.12.012.

571 Postma, P.R., Suarez-Garcia, E., Safi, C., Yonathan, K., Olivieri, G., Barbosa, M.J., Wijffels, R.H.,
572 Eppink, M.H.M., 2017. Energy efficient bead milling of microalgae: Effect of bead size on
573 disintegration and release of proteins and carbohydrates. Bioresour. Technol. 224, 670–679. doi:
574 10.1016/j.biortech.2016.11.071.

575 Rito-Palomares, M., Nunez, M., Amador, D., 2001. Practical application of aqueous two-phase
576 systems for the development of a prototype process for c-phycocyanin recovery from Spirulina
577 maxima. J. Chem. Technol. Biotechnol. 76, 1273 – 1280. doi: 10.1002/jctb.507.

578 Safi, C., Ursu, A.V., Laroche, C., Zebib, B., Merah, O., Pontalier, P.-Y., Vaca-Garcia, C., 2014.
579 Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. Algal
580 Res. 3, 61–65. doi: 10.1016/j.algal.2013.12.004.

581 Safi, C., Frances, C., Ursu, A.V., Laroche, C., Pouzet, C., Vaca-Garcia, C., Pontalier, P.-Y., 2015.
582 Understanding the effect of cell disruption methods on the diffusion of Chlorella vulgaris proteins
583 and pigments in the aqueous phase. Algal Res. 8, 61–68. doi: 10.1016/j.algal.2015.01.002.

584 Safi, C., Cabas Rodriguez, L., Mulder, W.J., Engelen-Smit, N., Spekking, W., van den Broek, L.A.M.,
585 Olivieri, G., Sijtsma, L., 2017. Energy consumption and water-soluble protein release by cell wall
586 disruption of Nannochloropsis gaditana. Bioresour. Technol. 239, 204 – 210. doi:
587 10.1016/j.biortech.2017.05.012.

588 't Lam, G.P., van der Kolk, J.A., Chordia, A., Vermue, M.H., Olivieri, G., Eppink, M.H.M., Wijffels,
589 R.H., 2017a. Mild and Selective Protein Release of Cell Wall Deficient Microalgae with Pulsed
590 Electric Field. ACS Sustainable Chem. Eng. 5, 6046−6053. doi: 10.1021/acssuschemeng.7b00892.

591 't Lam, G.P., Postma, P.R., Fernandes, D.A., Timmermans, R.A.H., Vermue, M.H., Barbosa, M.J.,
592 Eppink, M.H.M., Wijffels, R.H., Olivieri, G., 2017b. Pulsed Electric Field for protein release of the
593 microalgae Chlorella vulgaris and Neochloris oleoabundans. Algal Res. 24, 181–187. doi:
594 10.1016/j.algal.2017.03.024.

595 Vanthoor-Koopmans, M., Wijffels, R.H., Barbosa, M.J., Eppink, M.H.M., 2013. Biorefinery of
596 Microalgae for Food and Fuel. Bioresour. Technol. 135, 142 – 149. doi:
597 10.1016/j.biortech.2012.10.135.

598 **Figure captions**

599 **Figure 1.** Particle size distribution (PSD) of untreated (control), treated by HSH (96 kJ/$kg_{SUSP.}$), PEF
600 (E = 20 kV/cm; W_T = 100 kJ/$kg_{SUSP.}$), and combined (HSH + PEF) processed *A. platensis*
601 suspensions. Insert shows the volume-weighted mean diameter ($D_{4,3}$) of the samples. Different letters
602 above the bars indicate significant differences among the mean values ($p \leq 0.05$).

603 **Figure 2.** Optical microscopy at 20 × (a) and **s**canning electron microscopy (SEM) at 5000 × (b) of
604 *A. platensis* cells, before (control) and after the applications of HSH and PEF applied individually
605 and in combination (HSH + PEF). Yellow arrows indicate the fragmentation of microalgal trichomes.

606 **Figure 3.** Concentration of water-soluble proteins (a), carbohydrates (b), C-phycocyanin (c), and
607 protein extract purity (d) of the supernatant from untreated (Control), and treated by HSH alone, PEF
608 alone, and the combination HSH + PEF *A. platensis* suspensions. Different letters above the bars
609 indicate significant differences among the mean values ($p \leq 0.05$).

610 **Figure 4.** UV-Vis absorption spectra of supernatants from untreated (Control) and treated by HSH
611 alone, PEF alone, and the combination HSH + PEF *A. platensis* suspensions. Insert shows the pictures
612 of the supernatant obtained after centrifugation of microalgae untreated (1), and treated by HSH (2),
613 PEF (3), and HSH + PEF (4).

Figure 1

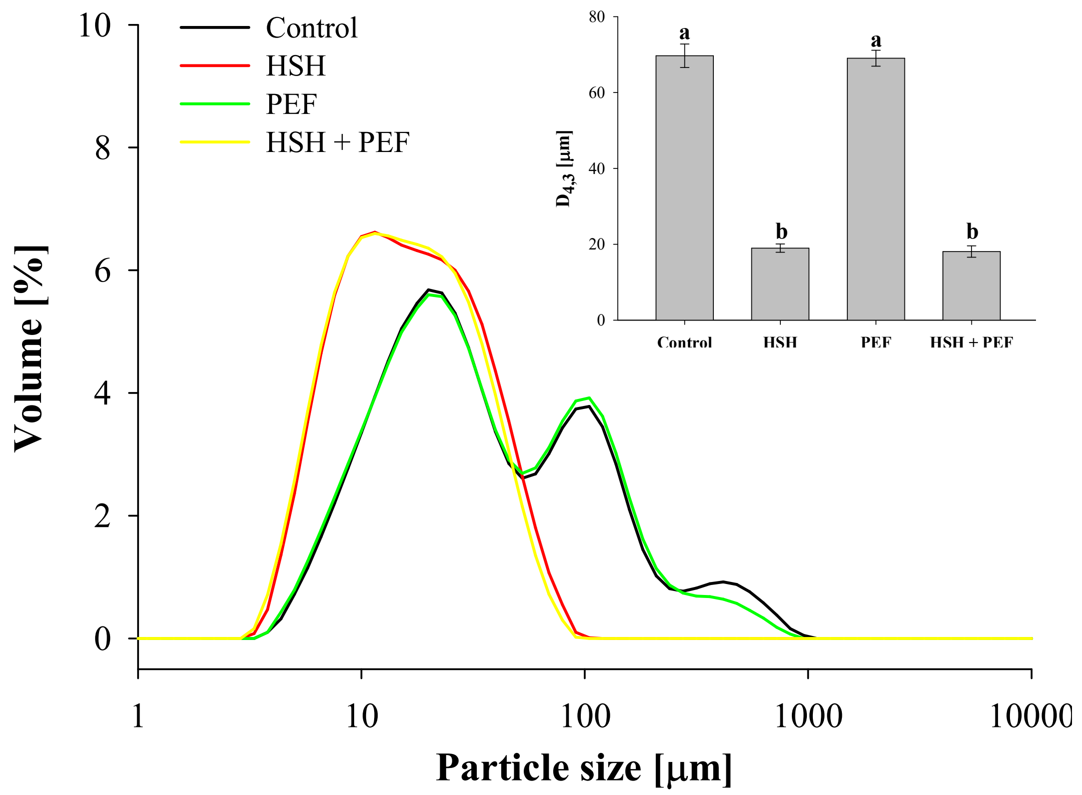
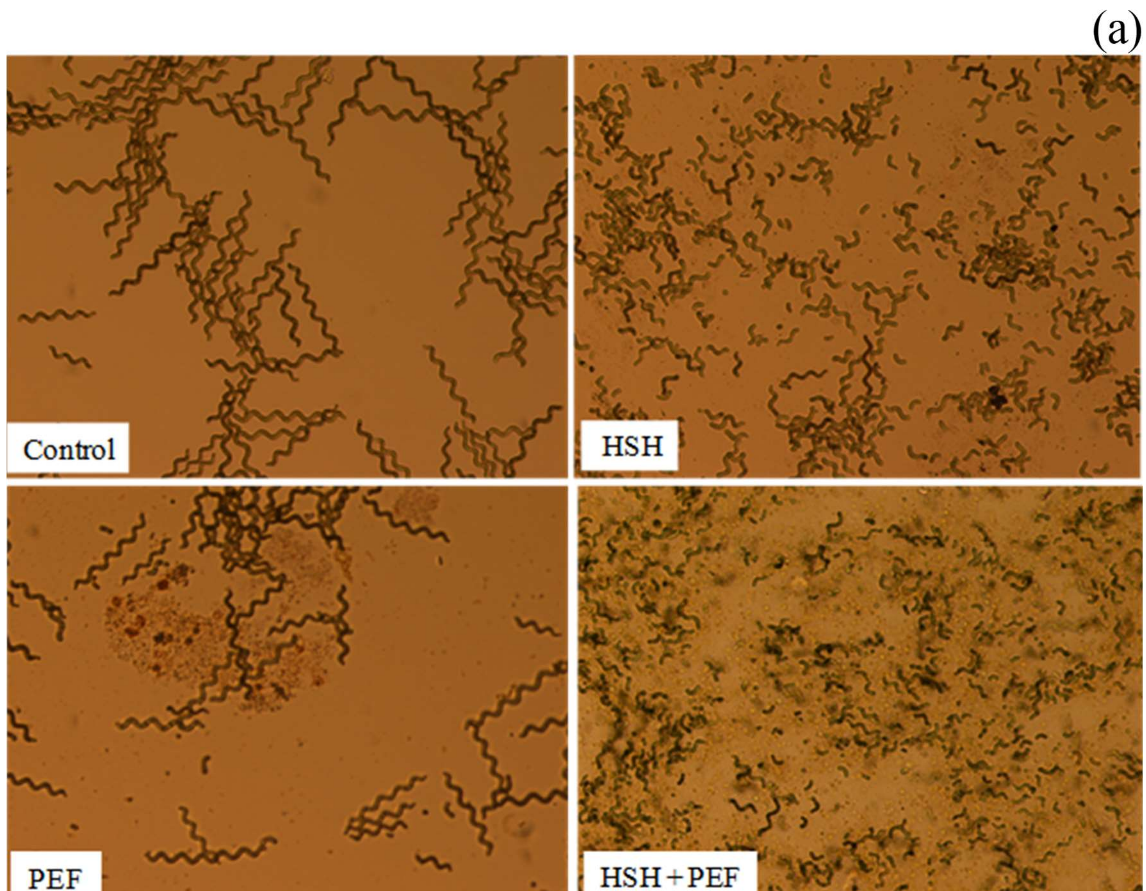


Figure 2



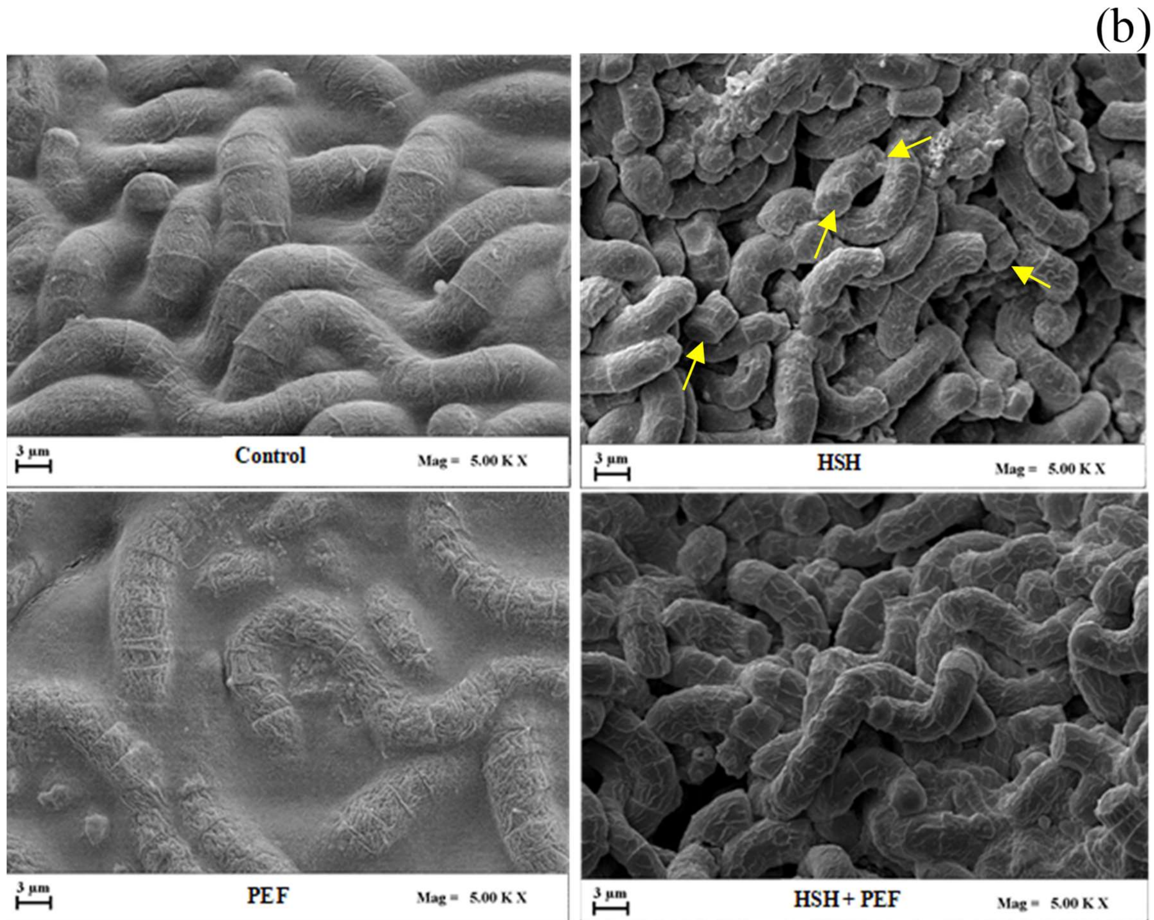
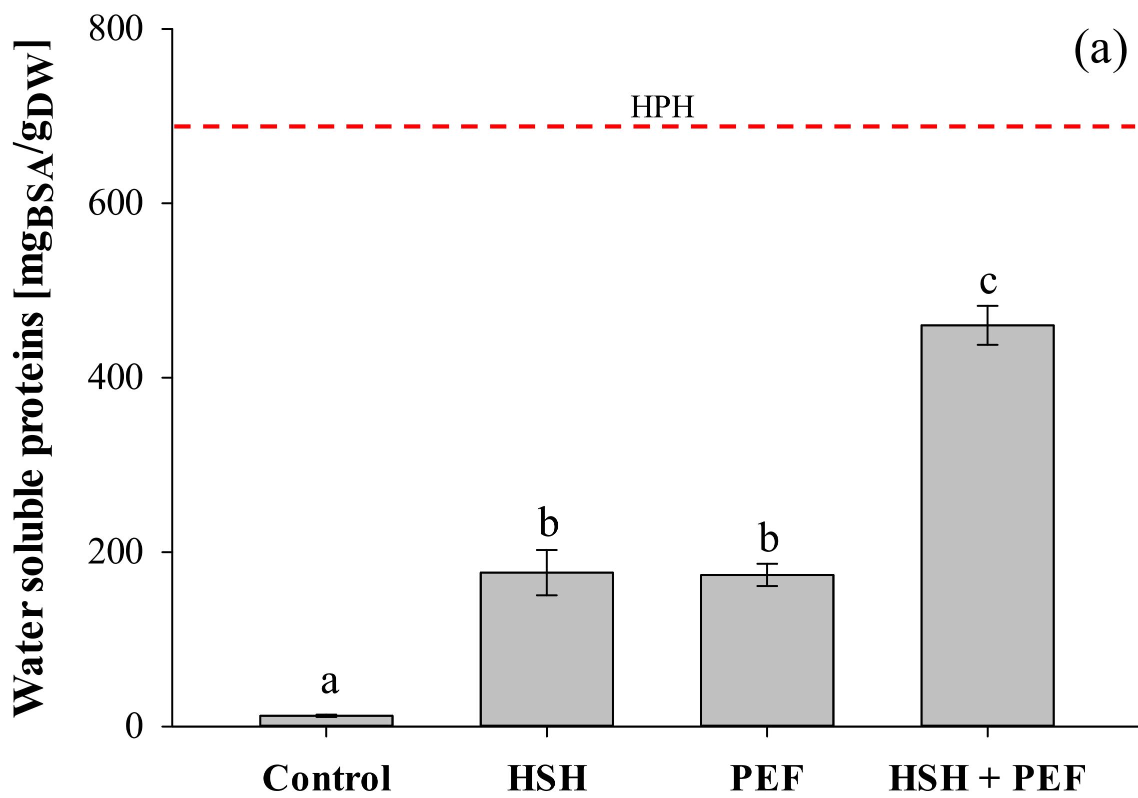
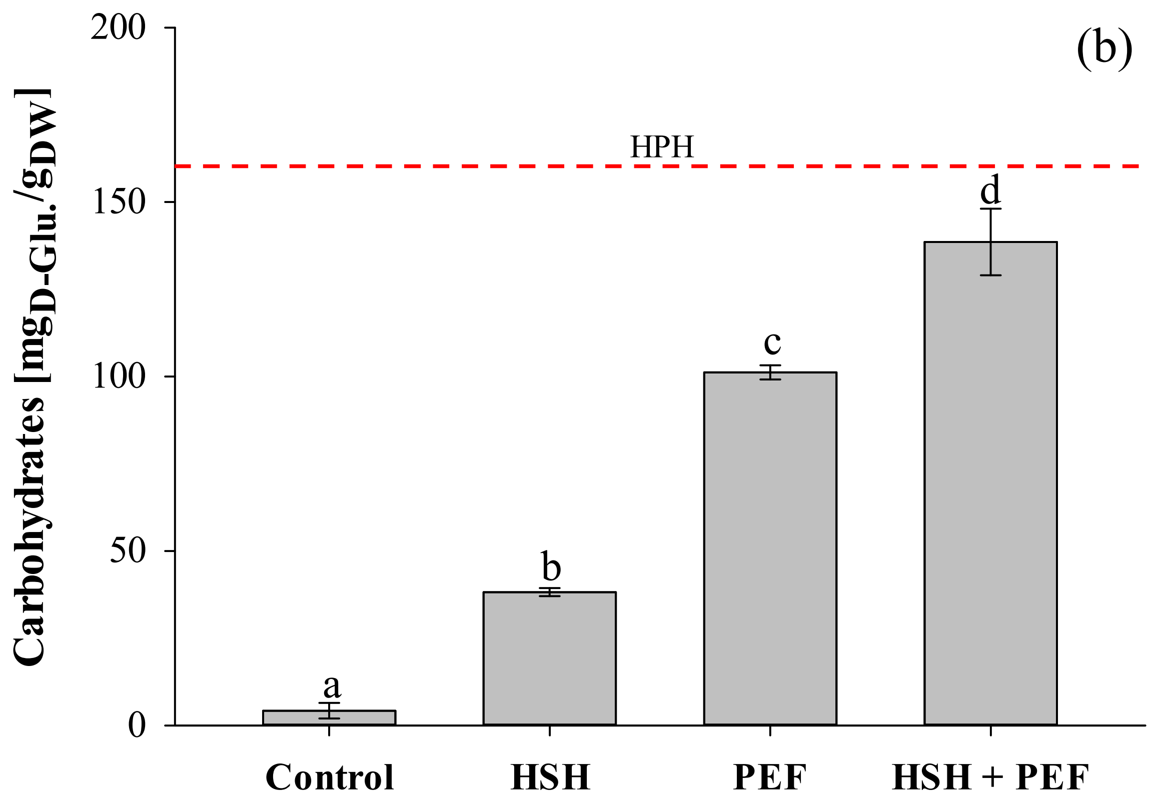
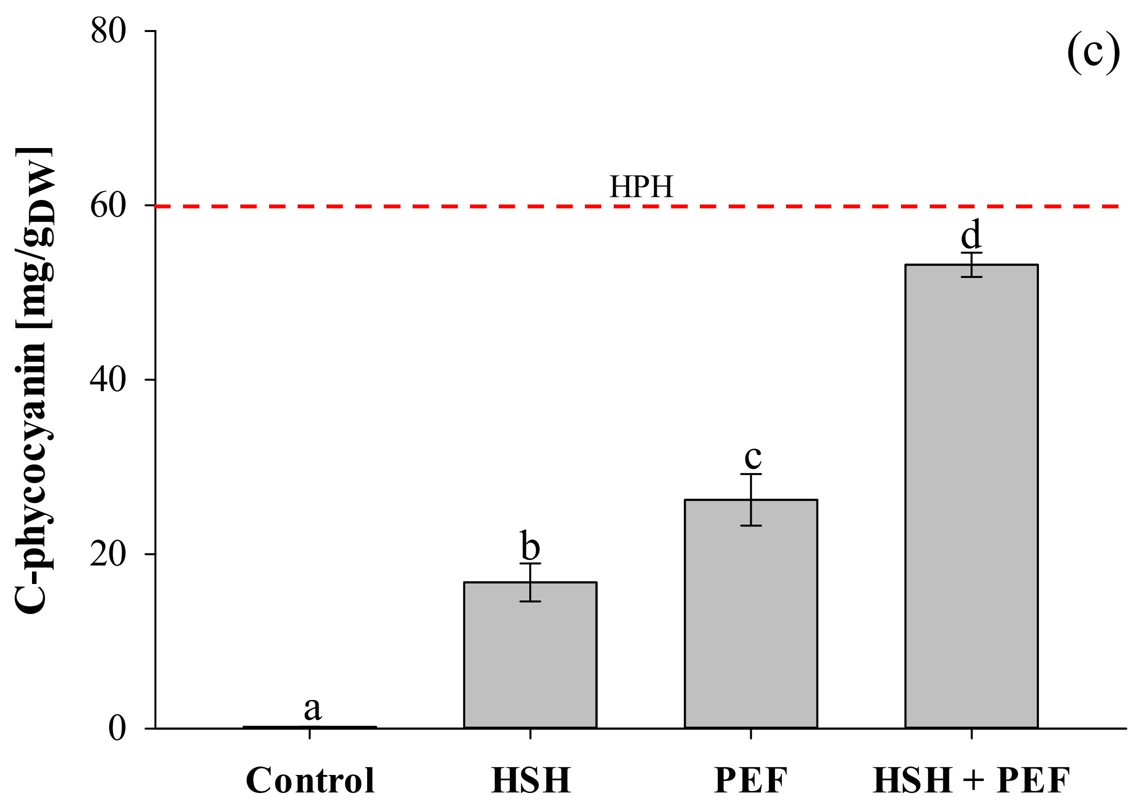


Figure 3




(b)
HPH
Carbohydrates [mg_D-Glu./g_DW]
200
150
100
50
0
a
b
c
d
Control
HSH
PEF
HSH + PEF


(c)
HPH
C-phycocyanin [mg/g_DW]
80
60
40
20
0
a
b
c
d
Control
HSH
PEF
HSH + PEF

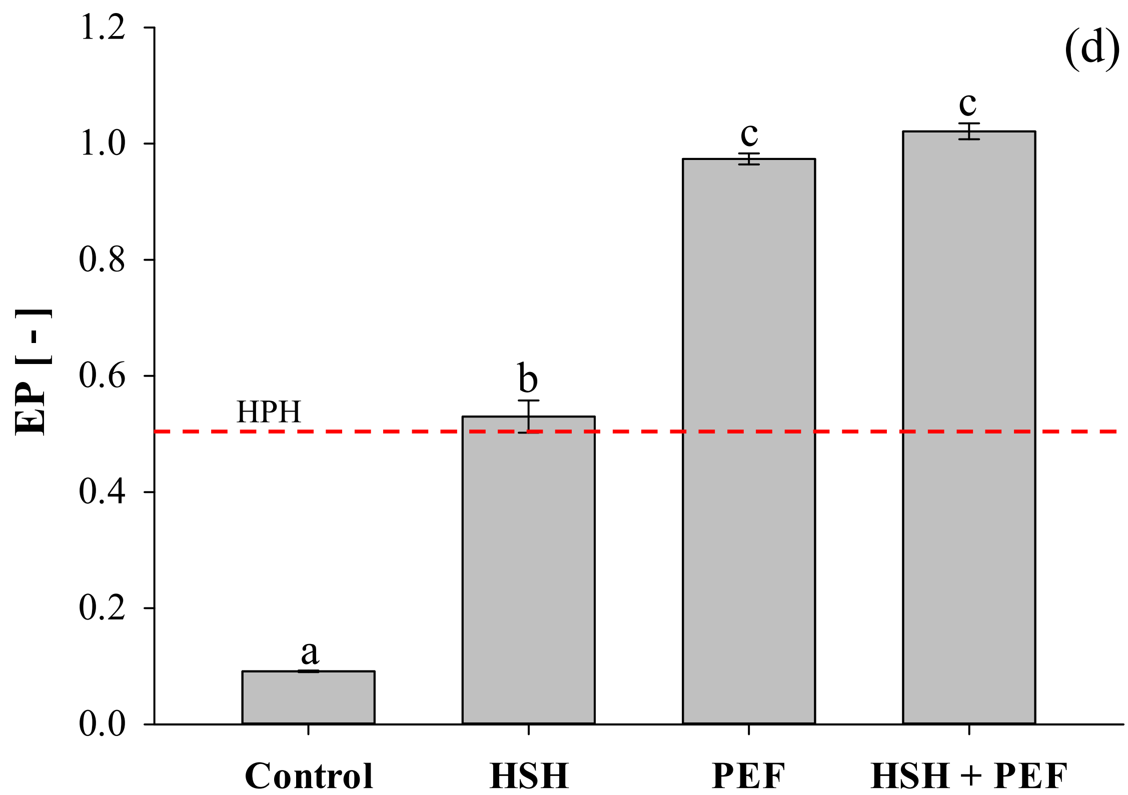


Figure 4

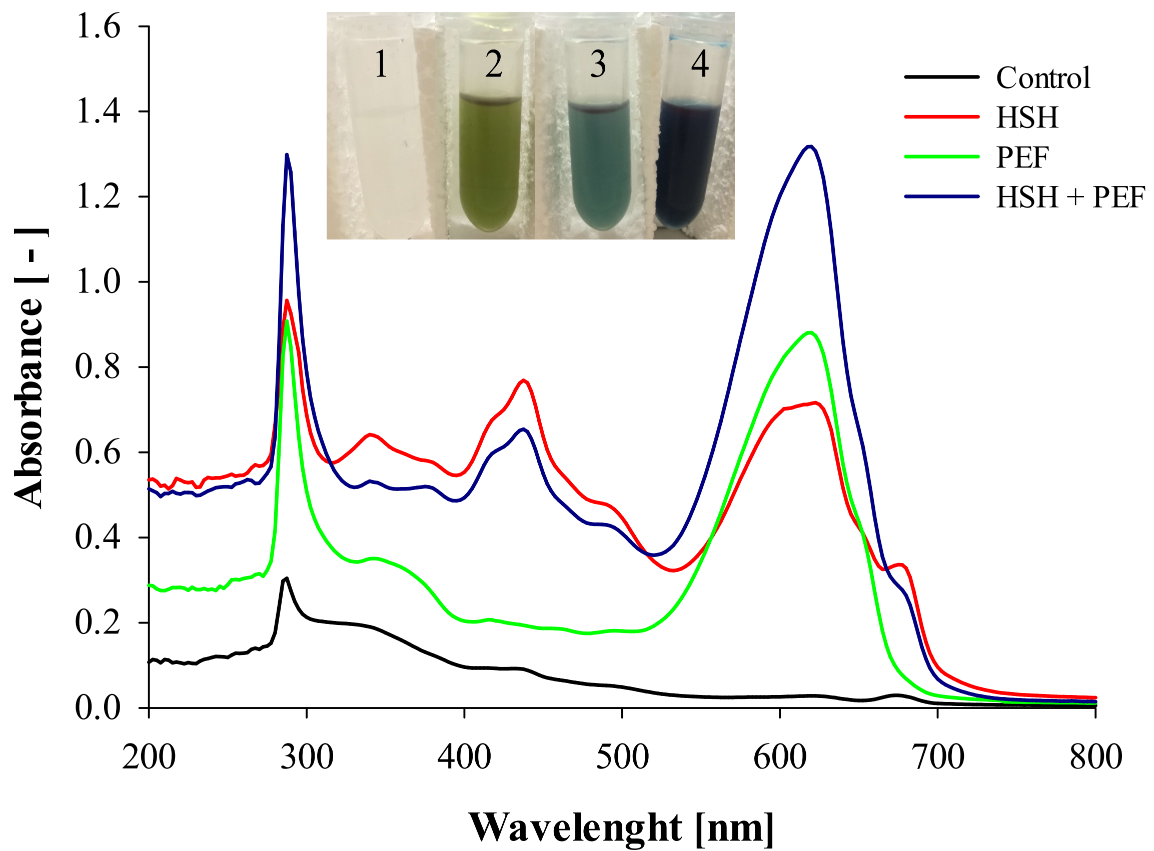


Table 1. Specific energy consumptions (EC, in kWh/kg_{DW}) of the different cell disruption techniques (HSH, 20000 rpm – 96 kJ/kg_{SUSP}; PEF, 20 kV/cm – 100 kJ/kg_{SUSP}; HSH + PEF; HPH, 150 MPa – 3 passes) for the recovery of a unit mass of target compounds (WSP: water-soluble proteins, C-PC: C-phycocyanin, CH: carbohydrates) from A. platensis microalgae suspensions. Different lowerscript letters within the same column indicate statistical differences among samples ($p < 0.05$).

Disruption method	Specific energy consumption (kWh/kg_{DW})		
	WSP	C-PC	CH
HSH	7.6 ± 1.1^{ab}	79.6 ± 10.5^{b}	34.9 ± 1.0^{c}
PEF	8.1 ± 0.6^{b}	52.9 ± 6.1^{a}	13.7 ± 0.3^{a}
HSH + PEF	5.9 ± 0.3^{a}	51.2 ± 1.3^{a}	19.7 ± 1.4^{b}
HPH	10.5 ± 0.7^{c}	110.5 ± 5.9^{c}	53.7 ± 2.2^{d}

SUPPLEMENTARY MATERIAL

Extraction improvement of water-soluble compounds from *Arthrospira platensis* through the combination of high-shear homogenization and pulsed electric fields

SDS-PAGE analysis

Experimental

The electrophoretic pattern of supernatants from single HSH, single PEF, and HSH + PEF treated microalgal biosuspensions were obtained by reducing SDS-PAGE analysis. Separation of proteins was performed in a TV100Y twin-plate mini-gel unit equipped with a power supply unit (APELEX-Massy, France). The acrylamide for the separating gel (12% w/v) was prepared in 1.5 M Tris-HCl buffer (pH = 8.8), with a stacking gel (6% w/v) prepared in 0.5 M Tris-HCl buffer (pH = 6.8). All supernatants and standards (25 μL) were diluted with the same amount of loading buffer (0.125 M, pH = 6.8) containing SDS (2% w/w), glycerol (10% w/w), bromophenol blue (0.02% w/w), and β-mercaptoethanol (5% w/w) as reducing agent. The mixture was boiled for 2 minutes, centrifuged, and loaded into the prepared gel, with the experiments run at constant current intensity (25 mA) for 1h. Gels were then stained with staining solution (0.2% Coomassie Brilliant Blue R 250, 10% acetic acid, 25% isopropanol) for overnight. Later, a de-staining solution (30% methanol, 10% acetic acid) was used until the background became clear.

Results

Proteins separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, as well as the distribution of proteins among fractions. Figure S1 shows the electrophoretograms of extracts from single HSH/PEF (lanes 2 – 3)

and HSH + PEF (lane 4) treated *A. platensis* suspensions. The pattern of a protein marker (lane 1) was also reported to identify the molecular weight of released proteins.

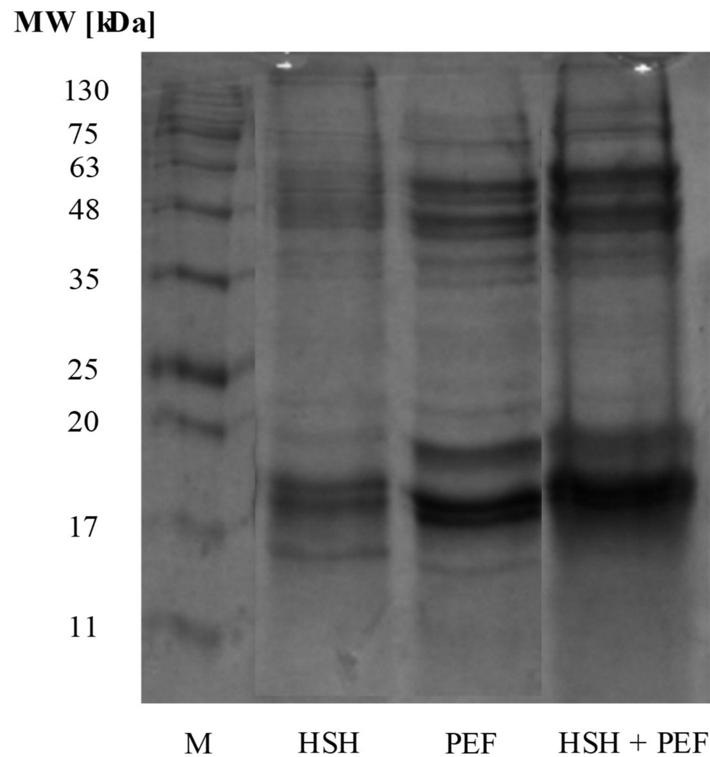


Figure S1. SDS-PAGE patterns of supernatant from single HSH, single PEF, and combined (HSH + PEF) treated *A. platensis* biosuspensions. The letter M in the first lane stands for "marker". Lane (HSH), (PEF), (HSH+PEF) refer to single HSH, single PEF, and combined HSH + PEF treated *A. platensis* suspensions, respectively.

Regardless of the applied single cell disruption method, it can be observed that a wide range of protein subunits could be released, mostly within 17 – 130 kDa of molecular weight (lanes 2 – 3). However, extracts from PEF treated sample exhibited two more distinct bands between 17 kDa and 20 kDa, which are compatible with α and β subunits of C-PC (Kumar et al., 2014). Based on the Lowry method, used to quantify the amount of water-soluble proteins in microalgal extracts, an equal amount of proteins was added per each lane. Therefore, the greater intensity of the bands detected in lane 3 at 17 – 20 kDa, with respect to those of lane 2, is representative of the higher selectivity of PEF

processing towards C-PC, in agreement with the results of Figures 4d and 5. Similarly to our observations, Jaesckhe et al. (2019) demonstrated that, in comparison with bead milling processing, extracts from PEF treated *A. platensis* microalgae showed a greater separation of C-PC characteristic bands, which was utilized as an indicator of purity.

In conclusion, as previously observed from the results of Figure 4c, the application of a sequential HSH-PEF treatment led to an increase in the intensity of C-PC subunit bands, thus again confirming its greater extractability with respect to single disruption techniques.