Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric fields from *Chlorella vulgaris*

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26 **Abstract**

27 Influence of the of temperature of biomass (10-40 ºC) treated by pulsed electric fields
28 (PEF) at different intensities (10-25 kV/cm) on electroporation of the microalgae
29 *Chlorella vulgaris* and on the extraction enhancement of lutein was investigated.
30 The occurrence of reversible and irreversible electroporation increased with electric
31 field strength and medium treatment temperature. On the other hand, increasing the
32 treatment medium temperature increased the sensitivity of *C. vulgaris* cells to
33 irreversible electroporation.
34 Response surface methodology was used to identify optimal PEF treatment conditions
35 for enhancing lutein extraction yield (LEY) from fresh *C. vulgaris* biomass.
36 Considering the cultivation temperature of *C. vulgaris* (25-30ºC) and the low increase in
37 the LEY when the PEF treatments were applied at temperatures above 30ºC, a treatment
38 of 25 kV/cm-100 μs at 25-30ºC that increased the LEY around 3.5-4.2-fold in
39 comparison with the control, resulting in the most suitable treatment conditions for
40 maximizing the lutein extraction at the lowest energy cost.

41 **Industrial relevance**

42 In recent years, industrial interest in microalgae as source of bioproducts such as natural
43 additives or active ingredients for food and cosmetic formulations has arisen. However,
44 there are still several obstacles to fully take advantage, such as the ability to
45 successfully extract these compounds from the cell biomass. Electroporation of
46 microalgae by PEF-technology could be an alternative to conventional cell disruption
47 techniques. Therefore, identifying critical factors affecting the enhancement of
48 bioproducts extraction from microalgae is necessary to establish PEF as a true option.

49 **Keywords:** microalgae, lutein, extraction, PEF, permeabilization, temperature.

50

1. **Introduction**.

In recent years, industrial interest in microalgae as source of bioproducts such as natural additives or active ingredients for food and cosmetic formulations has arisen (Becker, 2007; Schwenzfeier, Wierenga & Gruppen, 2011). *Chlorella vulgaris* is a unicellular microalgae that contains not only a high amount of green photosynthetic pigments such as chlorophyll a and b but also contains lutein and other primary carotenoids such as α and ß-carotene (Gonzalez & Bashan, 2000; Kitada et al., 2009).

Lutein is a xanthophyllic compound used as food colorant by the European Union (E-161 b).. This xanthophyll has also a potential role in preventing retinal degeneration, some types of cancer, and cardiovascular diseases due to its antioxidant capabilities (Arnal et al., 2009; Carpentier, Knaus & Suh, 2009). Lutein is located in the microalgae chloroplast and thermal degradation of lutein starts to be significant at temperatures above 60 °C . Currently, the commercial source of lutein is marigold flowers (*Tagetes erecta* L.) (Hojnik, Skerget & Knez, 2008; Sowbhagya, Sampathu & Krishnamurthy, 2004). Recently, microalgae have been proposed as a potential source of this compound because some microalgae species have a higher lutein content than marigold flowers, and they have shown yield productivities hundreds of times higher than marigold crops on a per-square-meter basis (Del Campo, Garcia-Gonzalez & Guerrero, 2007; Fernandez-Sevilla, Acien Fernandez & Molina Grima, 2010). In addition, compared to higher plants, microalgae can be cultivated in bioreactors on a large scale and thus they are a continuous and reliable source of the product without depending on environmental conditions, once it can be cultivated indoors.

Obtaining high-value bioactive extracts from microalgae requires culturing the microalgae, recovering the biomass, and purifying the metabolite from the biomass. The ability to successfully and efficiently extract the compounds from the cell biomass

76 without causing significant degradation is one of the main goals to taking full advantage
77 of microalgae as a valuable source of bioproducts. Although some products of interest
78 are excreted by microalgae to the growth medium, generally products produced by
79 microalgae are localized in the intracellular space either accumulated in vesicles or in
80 cytoplasm. The presence in most microalgae species of a cell wall surrounding the cells
81 but especially of an intact cytoplasmic membrane which acts as a semipermeable barrier
82 greatly influences extraction of these compounds. Traditionally, extraction of
83 microalgae products is conducted from dry biomass with organic or aqueous solvents,
84 depending on the polarity of the compound to be extracted (Ambrozova et al., 2014;
85 Ceron, Campos, Sanchez, Acien, Molina & Fernandez-Sevilla, 2008). Drying
86 microalgal biomass requires a significant amount of energy and may cause loss of
87 valuable food compounds through oxidation. It is preferable to use moist biomass in the
88 product recovery scheme to reduce energy costs and preventing degradation of
89 compounds.

90 The Pulsed Electric Fields (PEF), is a technology causing cell membrane
91 permeabilization. PEF is based on the fact that when a cell membrane is exposed to a
92 sufficiently intense electric field of short duration (milliseconds to microseconds), it
93 undergoes electrical breakdown which renders it permeable to molecules otherwise
94 unable to cross it. This external electric field needs to be above a critical value to induce
95 electroporation. Depending on the processing parameters applied, the membrane can
96 either become transiently or permanently permeable, making electroporation either
97 reversible or irreversible. While in reversible electroporation, pores created by the
98 electric field are able to reseal after the treatment application, in irreversible
99 electroporation the pores in the cytoplasmic membrane stay permanent. Application of
100 PEF for improving extraction of compounds of interest from microalgae requires

101 irreversible rather than reversible electroporation. Several studies have demonstrated the
102 potential of PEF to enhance extraction of compounds such as lipids and carotenoids
103 from fresh microalgae biomass (Flisar, Meglic, Morelj, Golob & Miklavcic, 2014;
104 Goettel, Eing, Gusbeth, Straessner & Frey, 2013; Grimi, Dubois, Marchal, Jubeau,
105 Lebovka & Vorobiev, 2014; Luengo, Condon-Abanto, Alvarez & Raso, 2014; Zbinden
106 et al., 2013). However, practical application of technology requires conducting further
107 research to understand the influence of main processing parameters to optimize
108 processing conditions for obtaining maximum microalgae electroporation with lower
109 energy requirements. Besides electric field strength and treatment time that are the
110 characteristic processing parameters of PEF technology, processing temperature has
111 been demonstrated to be a key parameter affecting cell membrane electroporation.
112 When PEF has been investigated as a nonthermal method for microbial inactivation,
113 several studies have demonstrated that application of PEF at higher temperatures
114 decreases the critical electric field required to kill microorganisms and causes a greater
115 level of microbial inactivation at temperatures that are not lethal (Saldana, Alvarez,
116 Condon & Raso, 2014; Timmermans, Groot, Nederhoff, van Boekel, Matser &
117 Mastwijk, 2014). On the other hand, it has been also reported that higher temperatures
118 enhance electroporation of plant cell tissues (Lebovka, Praporscic, Ghnimi & Vorobiev,
119 2005). However, the effect of the temperature on the PEF-induced electroporation of
120 microalgae and on the subsequent improvement in the extraction of compounds of
121 interest from microalgae has not been investigated.
122 The aim of this investigation was to assess the influence of temperature of applied PEF
123 on reversible or irreversible electroporation of the microalgae *C. vulgaris* and on the
124 enhancement extraction efficient of lutein.
125

126 **2. Material and Methods**

127 **2.1. Cell culture.**

128 *C. vulgaris* (BNA 10-007, National Bank of Algae, Canary Islands, Spain), were grown
129 in BG-11 medium contained the following components: 15 g L^{-1} $NaNO_3$; 4.0 g L^{-1}
130 K_2HPO_4; 7.5 g L^{-1} $MgSO_4.7H_2O$; 3.6 g L^{-1} $CaCl_2.2H_2O$; 0.6 g L^{-1} Citric acid; 6 g L^{-1}
131 ammonium ferric citrate green; 0.1 g L^{-1} $EDTA.Na_2$; 2.0 g L^{-1} Na_2CO_3; trace metal
132 solution (H_3BO_3 2.86 g L^{-1}; $MnCl_2.4H_2O$ 1.81 g L^{-1} ; $ZnSO_4.7H_2O$ 0.22 g L^{-1};
133 $Na_2MoO_4.2H_2O$ 0.39 g L^{-1}; $CuSO_4.5H_2O$ 0.08 g L^{-1}; $Co(NO_3)_2.6H_2O$ 0.05 g L^{-1}). For a
134 solid medium, 15 g of technical agar were added to 1l of the medium. Medium BG 11
135 (liquid and solid) was autoclaved at 121 ^{0}C for 20 min.

136 Cells were cultured photoautotrophycally in 1 l Roux flask bubbled with air (6 ml/s), at
137 30 °C, in light:dark cycles (12:12) with white fluorescent lamps (15 µmol m^{-2} s^{-1}).
138 Cultures were initially inoculated with 1 × 10^6 cells/ml. Cell density was determined by
139 microscope (microscope L-Kc, Nikkon, Tokyo, Japan) in a cell chamber Thoma
140 (ServiQuimia, Constantí, Spain). Cells between the 10th and the 15th day (stationary
141 phase) were subjected to experiments.

142 For dry weight determination, 1 ml of culture was dried until achieving constant weight
143 (GeneVac Ltd, UK).

144 **2.2. PEF treatments.**

145 PEF equipment used in this investigation was previously introduced by Saldana et al.,
146 2010. Microorganisms were treated in a tempered batch parallel-electrode treatment
147 chamber at different temperatures (10.0, 25.0, 40.0 ± 1.0 °C) with a distance between
148 electrodes of 0.25 cm and an area of 1.76 cm^2. The temperature of the treatment

149 medium was measured with a thermocouple before and after the PEF treatment and the
150 temperature variations were always lower than 2 °C. The energy per pulse (W) was
151 calculated using the following equation:

152 $$W = \int_0^i \sigma \cdot E(t)^2 dt \qquad (1)$$

153 where k (S/m) is the electrical conductivity of the treatment medium; E (V/m) is the
154 electric field strength; and t(s) is the duration of the pulse. The total energy (kJ) applied
155 (W) was calculated by multiplying the energy per pulse (W′) by the number of pulses.
156 The total specific energy (kJ/kg) applied (W) was determined by dividing the total
157 energy by the mass of treated medium.
158 Before treatment, microorganisms were centrifuged at 3000 ×g for 10 min at 25 °C and
159 resuspended in a citrate–phosphate McIlvaine buffer (1 mS/cm; pH 7). The microbial
160 suspension (0.44 ml) at a concentration of 10^9 CFU/ml was placed into the treatment
161 chamber by means of a 1 ml sterile syringe (TERUMO, Leuven, Belgium). *C. vulgaris*
162 cells were subjected up to 50 square 3 µs waveform pulses at 0.5 Hz of 2.5, 3.75, 5 and
163 6.25 kV applied between the electrodes separated a gap of 0.25 cm. These voltages
164 resulted in electric field strengths of 10, 15, 20, 25 kV/cm respectively that
165 corresponded with specific energies per pulse of 0.30, 0.66, 1.2, 1.86 kJ/l of culture
166 (concentration 10^9 cells/ml). Total specific energy ranged from 1.5 to 93 kJ/l of culture.
167 The current intensities were 19, 33, 43 and 55 A when the voltages applied were 2.5,
168 3.75, 5 and 6.25 kV respectively.
169

170 **2.3. Counting of viable cells**

171 PEF-treated and control cell suspensions were serially diluted in McIlvaine buffer (1
172 mS/cm; pH 7) sterile solution. From selected dilutions, 20 µl were streak plated into

173 solid media. Plates were incubated at 30 °C for 7 days with the same light regime used
174 for the liquid culture, and the number of CFU per milliliter was counted to determine
175 the % death cells after treatment. Longer incubation times did not increase the
176 microalgae counts.
177
178 **2.4. Staining cells with propidium iodide**.
179 Two alternative staining protocols were followed under the same experimental
180 conditions. Cells were either stained with popidium iodine (PI) (Sigma-Aldrich,) before
181 PEF-treatment or once the treatment had finished. PI was used to investigate
182 electroporation of microalgae because its molecular weight (668.4) is similar to
183 molecular weight of lutein (568.8).
184
185 **2.4.1. Staining cells before PEF treatments**
186 Before PEF treatments, microorganisms were centrifuged at 3000 ×g for 10 min at 25
187 °C and resuspended in a citrate–phosphate McIlvaine buffer (1 mS/cm; pH 7) to a final
188 concentration of approximately 10^9 cells/ml. Next, PI was added to cell suspensions to a
189 final concentration of 0.8 mM. Once the PEF treatment was finished, cell suspensions
190 were incubated for 10 min. Previous experiments had shown that longer incubation
191 times did not influence fluorescence measurements. Next, cell suspensions were
192 centrifuged and washed two times until no extracellular PI remained in the buffer, and
193 the dye trapped inside the cells was measured. Fluorescence was measured with a
194 spectrofluorophotometer (mod. Genios, Tecan, Austria) using a 535 nm excitation filter
195 (523–547 nm) and a 625 nm emission filter (608–642 nm).
196 Fluorescence data for cell suspensions were expressed as a percentage of permeabilized
197 cells based on the fluorescence value obtained for cells permeabilized after a PEF

198 treatment (150 µs at 25 kV/cm) in a citrate–phosphate buffer of pH 7.0, sufficient to
199 inactivate more than 99 % of cells. Under these conditions, permeabilization of the cell
200 population was checked using a fluorescence microscope (Nikon, Mod. L-Kc, Nippon
201 Kogaku KK, Japan).
202 The degree of permeabilization evaluated following this protocol corresponds to the
203 sum of the irreversible and reversible cell permeabilization.
204
205 **2.4.2. Staining cells after PEF treatments**
206 PI was added to a final concentration of 0.8 mM. With the purpose of standardize the
207 staining protocol PI was added 5 minutes after the PEF-treatment. No differences in the
208 PI uptake were observed when PI was added within 1 hour after the treatment. Cell
209 suspensions were incubated for 10 min, centrifuged, washed two times until no
210 extracellular PI remained in the buffer, and fluorescence was measured. In this case, the
211 degree of permeabilization corresponds only to irreversible permeabilization.
212 Fluorescence measurements were based on mean values obtained two different
213 microalgae suspensions.
214
215 **2.5. Pigment extraction.**
216 100 µl of non-treated or PEF-treated suspension were added to 1ml of 96 % ethanol and
217 vortexed. The mixture was macerated in the dark at room temperature for 20 minutes
218 and centrifuged at 6000 x g for 90 s.
219
220 **2.6. HPLC analysis of carotenoids**
221 HPLC/DAD analyses were performed on a Varian ProStar high-performance liquid
222 chromatograph (Varian Inc., Walnut Creek, CA, USA) equipped with a ProStar 240

223 ternary pump, a ProStar 410 autosampler, and a ProStar 335 photodiode array detector.
224 The system was controlled with a Star chromatography workstation v.6.41 (Varian). A
225 reversed-phase column Microsorb-MV 100-5 C18 (25 x 0.46 cm; 5 µm particle size)
226 with a precolumn (5 x 0.46 cm; 5 µm particle size) of the same material was used. The
227 temperature of the column and precolumn was maintained at 30ºC.
228 Pigments were eluted isocratically using acetonitrile:water:methanol (65 % : 2 % :23) as
229 a mobile phase for 25 minutes. Flow rate through the column was 1.5 ml/min, sample
230 injection 30 µl, and absorbance detection wavelength 443 nm. Prior to injection, all
231 samples were filtered through a 0.2 µm sterile syringe filter of cellulose acetate (VWR,
232 West Chester, PA, USA).
233 Lutein was identified by comparing its retention time and visible absorption spectra
234 with this of its standard. A calibration curve of lutein was injected to determine its
235 concentration in the extract.
236

237 **2.7. Experimental design**

238 Response surface methodology (RSM) was used to evaluate the effect of the treatment
239 parameters, electric field strength, treatment time, and temperature on the lutein
240 extraction yield (LEY) from *C. vulgaris*.
241 The obtained data after treated the cells with the conditions described in section 2.2
242 were modeled with the following second-order polynomial equation:

243 $$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i>j}^{k} \beta_{ij} X_i X_j \quad (1)$$

244 where Y is the response variable to be modeled, Xi and Xj are independent factors, β0 is
245 the intercept, βi is the linear coefficients, βij is the quadratic coefficients, βij is the
246 cross-product coefficients, and k is the total number of independent factors. A backward

247 regression procedure was used to determine the parameters of the models. This
248 procedure systematically removed the effects that were not significantly associated ($p > 0.05$)
249 with the response until a model with a significant effect was obtained.
250 The CCD and the corresponding analysis of the data were carried out by using the
251 software package Design-Expert 6.0.6 (Stat-Ease Inc., Minneapolis, MN, USA).
252

253 **2.8. Statistical analysis**

254 Experiments were performed in triplicate and the presented results are means ± standard
255 deviation. One-way analysis of variance (ANOVA) using Tukey's test was performed
256 to evaluate the significance of differences between the means values. The differences
257 were considered significant at $p < 0.05$. GraphPad PRISM (GraphPad Software, San
258 Diego, California, USA) was used to perform the statistical analysis.
259

260 **3. RESULTS AND DISCUSSION.**

261 **3.1. Effect of PEF on the permeabilization of *C. vulgaris* cells.**

262 Figure 1 shows the influence of PEF treatments of 75 μs (25 pulses of 3 μs) at several
263 electric field strengths and different temperatures (10, 25 and 40 ºC) on the
264 electroporation of the cytoplasmatic membrane of *C. vulgaris* evaluated by the uptake
265 PI added before (1A) and after (1B) the PEF treatment.
266 Independently of the staining protocol, the uptake of PI increased with the electric field
267 strength and the treatment temperature. When PI was added before the application of the
268 PEF-treatment to detect the occurrence of reversible and irreversible permeabilization,
269 the maximum percentage of PI uptake was observed even at the lower electric field
270 investigated (10 kV/cm) when cells were treated at 25 and 40 ºC. However, at 10 ºC, 15
271 kV/cm or higher electric field strengths were required to obtain the same degree of

272 permeabilization. On the other hand, to obtain the maximum irreversible electroporation
273 (addition of PI after the treatment) (Fig 1B), it was necessary to apply treatments of at
274 least 15 kV/cm at 25 and 40 ºC. At the lower temperature investigated (10 ºC), the
275 maximum degree of electroporation obtained at 25 and 40 ºC was not achieved even at
276 the highest electric field applied (25 kV/cm).
277 The difference between the % of PI uptake when PI was added before or after the PEF
278 treatment reveals the existence of cells reversibly electroproated. At 25 and 40 ºC,
279 reversible electroporation was only observed at the lowest electric field assayed (10
280 kV/cm). While at 15 kV/cm or higher, the % of PI uptake with the two staining
281 protocols assayed was similar, at 10 ºC, the % of PI uptake was always lower when PI
282 was added after the PEF treatment. For example, at 10 kV/cm, 75% of PI uptake was
283 detected when the dye was added before the treatment, but no uptake was observed
284 when added after the treatment, meaning that the cells of *C. vulgaris* electroporated
285 during the treatment were able to reseal their membranes after the treatment. According
286 to these results, increasing the treatment temperature decreases the critical electric field
287 to induce irreversible electroporation in the cell membranes of *C. vulgaris*. While at 10
288 ºC permanent electroporation required electric field strengths higher than 10 kV/cm, at
289 25 and 40 ºC, treatments at 10 kV were sufficient to render cells irreversibly
290 electroporated.
291 Results shown in Figure 1 indicate that in the range of temperatures investigated, the
292 uptake of PI by the population of *C.vulgaris* cells was not influenced by the temperature
293 at electric field strengths above 15 kV/cm when PI was added before the treatment. In
294 this range of electric field strength, while the electroporation of the cells of *C. vulgaris*
295 was irreversible for most of the population at 25 and 40 ºC, at 10 ºC, a proportion of the
296 cells were reversibly electroporated during the treatment. So these cells were able to

297 reseal pores after the treatment, and its membrane became impermeable to PI when
298 added after the treatment. Theoretical models proposed to explain electroporation
299 indicate that the electric field induces a potential across the cytoplasmic membrane,
300 causing a structural reorganization of the lipid bilayer that leads to the formation of
301 aqueous pores (Joshi, Hu, Schoenbach & Hjalmarson, 2002; Saulis & Venslauskas,
302 1993). Depending on the parameters of the electric field pulses, electroproation can be
303 either reversible or irreversible. According to results shown in Figure 1 when PEF was
304 applied at 10 ºC, the increase of the permeability of the cytoplasmic membrane during
305 the treatment was similar to the increase observed at higher temperatures. However,
306 while at 25 and 40ºC the electroporation was irreversible, at 10 ºC, the membrane of a
307 proportion of the cells returned to its natural state after the treatment. Reversibility or
308 irreversibility of electroporation has been correlated with the size and the number of
309 pores in the lipid bilayer by electroporation. Phase transitions of the membrane
310 phospholipids from gel to liquid-crystalline phase are temperature-related, becoming the
311 phospholipid bilayer less ordered and packed at higher temperatures (Reigada, 2014;
312 Stanley, 1991). The higher organization of the lipid bilayer at lower temperatures could
313 cause external electric fields of a given intensity inducing a smaller number of pores or
314 small size pores than at higher temperatures, facilitating that the membranes return to its
315 natural state afterward.
316 Application of PEF for improving extraction of compounds of interest from microalgae
317 requires permanent electroporation, because the estimated lifetimes of the pores after
318 the field is removed when transient electroporation occurs is estimated to be in the range
319 from milliseconds up to few minutes(Saulis, 2010). These times result too short to
320 enhance the extraction of the compounds localized in the intracellular space of
321 microalgae, because generally they are located in vacuoles, chloroplasts, or vesicles.

322 Generally, in the literature it is assumed that only irreversible electroporation is directly
323 correlated with cell death (Unal, Yousef & Dunne, 2002; Wouters, Bos & Ueckert,
324 2001). The relationship between the percentage of PI uptake when the PI was added
325 after the PEF treatment and the percentage of dead cells is estimated by plate counting
326 after the treatment are shown in Figure 2. The figure shows all the inactivation data
327 obtained at different temperatures by applying the treatment conditions to the *C.*
328 *vulgaris* population described in the material and methods section. A theoretical straight
329 line with slope 1 and intercept 0 that would represent a perfect agreement between % of
330 PI uptake (permeabilization) and cell death has been included in Figure 2. Figure 2
331 shows that when the percentage of irreversible permeabilized cells was lower than 80%,
332 the percentage of death cells was higher than the number of irreversible permeabilized
333 cells independent of the temperature of PEF application. Therefore these results indicate
334 that an amount of cells inactivated during the treatment were able to return the
335 cytoplasmic membrane to its initial state after the treatment. As it is expected that the
336 cytoplasmic membrane will remain unpermebilized in these cells, determination of the
337 number of cells inactivated by PEF could not be a good indicator for quantification of
338 the level of cells *C. vulgaris* that have been irreversibly electroporated in a process that
339 aims to improve extraction of intracellular compounds by PEF.
340
341 **3.2. HPLC profile of pigments extracted from *C.vulgaris*.**
342 A suitable procedure for improving extraction of biocompounds from microalgae
343 should improve the extraction yield without causing significant degradation in the
344 compounds of interest**.** The extracts obtained from *C.vulgaris* using ethanol as a solvent
345 after the application of PEF treatments of different intensities at different temperatures
346 were analyzed by reverse-phase HPLC. Figure 3 compares chromatogram profiles

347 detected at 443 nm for extracts obtained in an extraction from untreated and pretreated
348 by PEF (40º C - 25kV/cm-50 pulses of 3µs) *C. vulgaris* cells. Similar chromatogram
349 profiles were obtained for the extracts obtained in different experimental conditions
350 investigated. According to Figure 3, the application of a PEF treatment in the most
351 intense conditions increased the amount of compounds extracted but did not affect the
352 extraction of a selected compound. As reported by other authors(Updike & Schwartz,
353 2003), PEF treatment did not cause pigment degradation. Similar results have been
354 observed when the application of PEF has been investigated to improve the extraction
355 of other compounds of interest from the cells of plant tissues (Lopez, Puertolas,
356 Hernandez-Orte, Alvarez & Raso, 2009; Puertolas, Cregenzan, Luengo, Alvarez &
357 Raso, 2013)
358

359 **3.3. Improvement of the extraction of lutein from *C. vulgaris* by PEF treatments at**
360 **different temperatures.**

361 The lutein extraction yield (LEY) resulting from the extraction with ethanol from the
362 control (untreated *C.vulgaris* cells) and the PEF-treated *C.vulgaris* cells at the
363 treatments conditions described in section 2.2 are shown in Table 1. Values of LEY
364 varied from 163 to 753 µg/g dw of *C. vulgaris* culture. These values are within the
365 range of the values reported in the literature by other authors that have investigated the
366 accumulation of this pigment in these microalgae (Jeon et al., 2014; Kitada et al., 2009).
367 Results shown in Table 1 indicate the potential of PEF for improving the extraction of
368 lutein from a fresh biomass of *C. vulgaris*. As compared with the untreated biomass, the
369 concentration of lutein was around 4.5-fold higher when the fresh biomass was
370 previously electroporated at 40ºC by a PEF of 25 kV/cm for 75 µs. Treatments of the
371 same intensity at 10 and 25 ºC increased the LEY 3.2- and 2.3-fold, respectively. The

372 high ethanol concentration used for extraction could cause the denaturation of the
373 cytoplasmatic membrane faciliting the lutein release. However, our results indicate that
374 this denaturation is not very effective in improving mass transfer though the
375 cytoplasmatic membrane because a previous electroporation increased significantly the
376 extraction of lutein.
377 The improvement in the extraction of lutein as a consequence of the prior
378 permeabilization of the cytoplasmic membrane of *C. vulgaris* by PEF depended on the
379 PEF treatment conditions. When cells were treated at 10 ºC, no statistically significant
380 increase in LEY were observed at the lowest electric field strengths applied (10 and 15
381 kV/cm) even after the application of the longest treatment duration (150 µs). At this
382 temperature, these results agree with the data obtained in the % of PI uptake when the
383 dye was added after the treatment to detect irreversible electroporation, since the % of
384 PI uptake was lower than 5 %. However, at 15 kV/cm, 40 % of PI uptake detected when
385 the cells were treated with 150 µs did not correspond with the lack of lutein extraction
386 improvement observed. These results could indicate that the number or the size of the
387 pores produced by these PEF treatments allows PI uptake, but they were not big enough
388 to improve lutein extraction. At 25 and 40 ºC, the LEY increased significantly at electric
389 fields of 15 kV/cm or higher even at the shortest treatment time applied (15 µs).
390 However, at these higher temperatures, although a PEF treatment of 20 kV/cm for 75 µs
391 was enough to obtain 100 % of PI uptake, to obtain the maximum LEY at each
392 temperature, it was necessary to apply more intense electric field strengths (25 kV/cm).
393 According to these results, although some positive correlation (R=0.8, data not shown)
394 was observed between the PI uptake and the lutein extraction in the cells of *C. vulgaris*
395 treated by PEF at different temperatures, data on irreversible electroporation evaluated

396 by the uptake of PI are not suitable to define the PEF treatment conditions to obtain the
397 highest LEY.
398

399 **3.4. Mathematical modeling**

400 To quantify the influence of PEF processing parameters (electric field strength,
401 treatment time and temperature) on the LEY from *C. vulgaris* fresh biomass, a multiple
402 regression analysis was conducted fitting the experimental data presented in Table 1 for
403 equation 1. Ethanol was used as solvent for untreated and PEF treated cells of *C.*
404 *vulgaris*. Backward regression procedure did not eliminate any equation term because
405 all the terms of equation 1 were statistically significant ($p < 0.05$). Coefficients of
406 equation 1 and the statistics used to test the adequacy of the model are shown in Table
407 2. The P-value of the models was less than 0.05, which indicates that the model is
408 significant and therefore the terms in the models have a significant effect on the
409 responses. The determination coefficient was 0.90, which means that only 10 % of the
410 total response variation remained unexplained by the model developed. The adjusted R^2
411 values that correct the R^2 according to the number of responses and terms in the model
412 were close to the R^2. The value of the *RMSE* parameter shows that model produced
413 predictions close to the observed data. A bias factor (B_f) of 1 means that the model was
414 a good predictor of LEY and the accuracy factors (A_f) show that, on average, the
415 predictions differ from the observations by 18 %. The F-values for the model
416 parameters are useful indicators of the significance of the effects of the variables and
417 their interactions. According to the F-values shown in Table 2, changes in electric field
418 strength and the temperature had the most significant effect on the LEY. However, since
419 the square terms of the electric fields strength and temperature were also significant, this
420 means that its effect on LEY is nonlinear. The negative effect of the square term of

421 temperature and time indicates an optimum value for temperature and time; above this
422 value, the temperature or time increase did not substantially increase the LEY.
423
424 **3.5. Influence of electric field strength, treatment time and temperature on the**
425 **improvement of the extraction of lutein from *C. Vulgaris*.**
426 Graphical representations were obtained using the regression model (Table 2)
427 considering the responses within the range of experimental conditions assayed (Figures
428 4A and 4B). The effect of treatment time (number of pulses multiplied by the pulse
429 width) and temperature of application of the PEF treatment at 25 kV/cm to a fresh
430 biomass of *C. vulgaris* on the subsequent extraction of lutein is shown in Figure 4A. At
431 any temperature, the LEY increased with the treatment time until around 100 µs.
432 Beyond this time, further increases of the treatment time did not appreciably increase
433 the lutein extraction. Increasing the treatment temperature led to a reduction of the
434 treatment time required to obtain a given LEY and, as a consequence, it resulted in a
435 decrease in the total specific energy required. For example, a lutein extraction of 400
436 µg/g dw of *C. vulgaris* culture required a treatment of 100 µs that corresponded to an
437 energy input of 71 kJ/l of culture when the treatment temperature was 10ºC. However,
438 the same LEY was obtained with a treatment of 15 µs at 25 ºC that corresponded to an
439 energy input of 31 kJ/l of culture.
440 These results support other studies in which it has been observed that the increase of the
441 temperature of application of PEF treatments causes a reduction of the specific energy
442 input required to obtaining a given reduction in the viability of a microbial population
443 with the aim of food pasteurization (Saldana, Puertolas, Monfort, Raso & Alvarez,
444 2011).

445 The influence of the PEF electric field strength to the fresh biomass of *C. vulgaris* at
446 different temperatures on the subsequent extraction of lutein is shown in Figure 4B.
447 Treatment time selected to plot figure 4B was 100 µs because according to Figure 4A, it
448 was the shortest treatment duration that permitted obtaining the highest LEY at the
449 temperatures investigated. It is observed that at any temperature, the LEY increased by
450 augmenting the intensity of the electric field strength applied to the fresh microalgae
451 biomass. However, increases in the range of 10 to 20 kV/cm were less effective than in
452 the range of 20 to 25 kV/cm. For example, at 20ºC, while an increase of 5 kV/cm units
453 of electric field from 10 to 15 kV/cm increased the extraction of lutein 39 µg/g dw of *C.*
454 *vulgaris*, from 20 to 25 kV/cm the observed increases was 112 µg/g dw of *C. vulgaris*.
455 This behavior could be due to the fact that lutein is located inside the chloroplast and
456 needs to cross not only the cytoplasmic membrane but also the chloroplast membrane to
457 be extracted from the fresh biomass of *C. vulgaris*. At higher electric field strengths,
458 addition of the cytoplasmic membrane to the PEF treatment could also electroporate the
459 membrane of the chloroplast, facilitating lutein extraction. Electric field strength
460 threshold is inversely related to cell size (Kotnik, Kramar, Pucihar, Miklavcic & Tarek,
461 2012). Since chloroplast size is smaller than cell size, a higher electric field strength
462 could be necessary to electroporate it. The electric fields (100 kV/cm) used for different
463 authors for permeabilization of cell organelles with pulses of a duration of nanoseconds
464 confirms that higher electric fields are required to affect the integrity of chloroplast.
465 (Esser, Smith, Gowrishankar, Vasilkoski & Weaver, 2010).
466 The electroporation of the chloroplast membrane could be also influenced by the
467 temperature of application of PEF, since the degree of permeabilization is larger at
468 higher temperatures. According to the results shown in Figure 1B, independently of the
469 temperature of application of the PEF treatment, the complete population of *C. vulgaris*

470 biomass was permeabilized after a treatment at 25 kV/cm for 100 µs. However, Figure
471 4B shows that an increase of the temperature of PEF treatment application at this
472 intensity from 10 to 40 ºC increased the LEY from 400 to 700 µg/g dw of *C. vulgaris*.
473 The higher electroporation of the chloroplast membrane at 40 than at 10ºC could
474 explain the higher extraction of lutein observed at higher temperatures. Since the
475 chloroplast membranes is a lipid bilayer like the cytoplasmic membrane, the higher
476 organization of the lipid bilayer at lower temperatures could also be the cause because
477 an external electric field of a given intensity induced a smaller degree of electroporation
478 at lower temperatures.. Independent of the reason because an increase of the
479 temperature of application of the PEF treatment causes an improvement in the amount
480 of extracted lutein, Figure 4B shows that enhancement in the extraction was dependent
481 on the range of temperatures in which the increase is performed. For example, at 25
482 kV/cm, increasing the temperature of 10ºC to 20 ºC increased the LEY by 35 %, but an
483 increase lower than 10% was observed by raising the treatment temperature from 30 to
484 40 ºC. From a practical point of view, the smaller increases in LEY were observed when
485 the treatment temperature is above 30 ºC and the energy cost required to increase the
486 temperature of the biomass advises against applying the treatment at temperatures
487 higher that 30ºC to improve the extraction of lutein from *C. vulgaris* biomass.

488 **Conclusions**

489 In this investigation it has been demonstrated that in addition to the electric field
490 strength and treatment time, treatment temperature is a critical parameter that influences
491 electroporation of *C. vulgaris* and subsequent extraction of lutein from a fresh biomass
492 of this microalgae treated by PEF. Evaluation of the irreversible electroporation of cells
493 of *C. vulgaris* detected by the increase of the fluorescence of the population when the
494 day PI was added after the PEF treatment or quantification of cells of *C. vulgaris*

495 inactivated by the PEF treatment was not a suitable procedure for defining optimal PEF
496 treatment conditions at different temperatures to achieve the maximum extraction of
497 lutein from a fresh biomass of *C. vulgaris*.
498 Commercial viability of the products derived from microalgae is significantly
499 dependent on the cost of these extraction processes, and generally the downstream
500 recovery of the products can be substantially more expensive than the cultivation of the
501 microalgae. Indeed, to make production of extracts from microalgae economically
502 feasible, it is necessary to optimize the biomass processing for metabolite recovery.
503 Considering that the cultivation temperature of *C. vulgaris* is between 25 and 30 ºC, the
504 low increase in the LEY when the PEF treatment is applied at temperatures above 30ºC
505 and the energy cost derived of increasing the temperature of the culture to be treated by
506 PEF, from a practical point of view, the most appropriate approach for improving
507 extraction of lutein by PEF at the lowest energy cost would be to locate the PEF
508 treatment chamber in the outflow tube of the photobioreactors where the microalgae are
509 cultivated.
510
511 **Acknowledgements.**
512 This research was supported by the Government of Aragón (Grupo de Investigación
513 Consolidado A20), and the European Social Fund. E.L. gratefully acknowledges the
514 financial support for her doctoral studies from the Department of Science, Technology
515 and University of the Aragon Government.
516
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627 pretreatment for greener solvent lipid extraction from microalgae. *Biotechnology and*
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631

632 **Figure captions**.

633 Figure 1. Influence of electric field strength at different treatment temperatures on the PI
634 uptake when PI was added before (A) and after (B) the PEF treatment. Treatment time:
635 75µs (25 pulses of 3µs). 10 °C (●), 25 °C (○), 40 °C (■).

636 Figure 2.Relationship between the percentages of cell permeabilization assessed by PI
637 staining after PEF against the percentage of death cells. To show the degree to which
638 each treatment causes membrane permeabilization, a theoretical straight line with slope
639 =1 and intercept =0, is included. 10 °C (●), 25 °C (○), 40 °C (■).

640 Figure 3. HPLC profile at 443 nm for extracts obtained in a extraction from pretreated
641 by PEF (40º C - 25kV/cm-50 pulses of 3µs) (A) and untreated (B) *C. vulgaris* cells.

642 Figure 4. Influence of treatment time (A) and electric field strength (B) on the lutein
643 extraction yield obtained from *C.vulgaris* cells PEF-treated at several temperatures. 10
644 °C (—), 20 °C (- -), 30 °C (—), 40 °C (- -).

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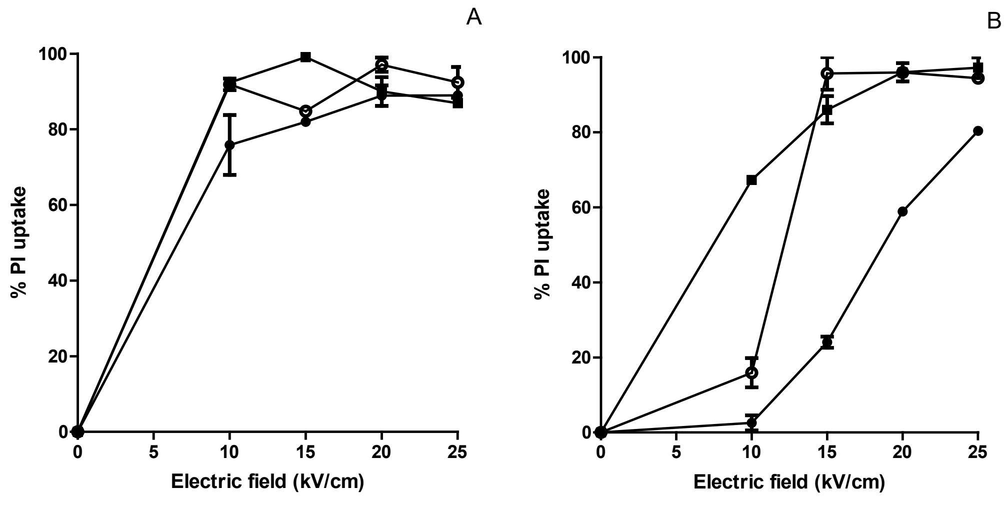
648 Figure 1.



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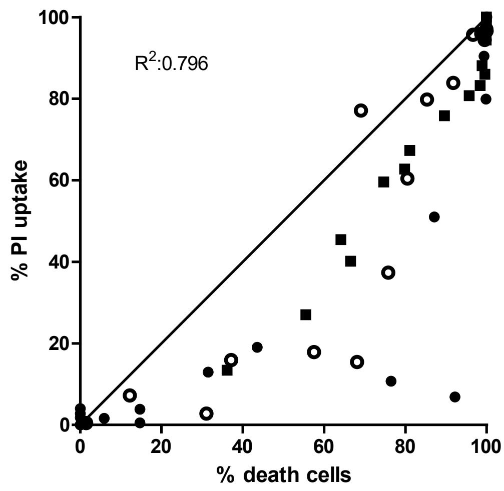
651 Figure 2.



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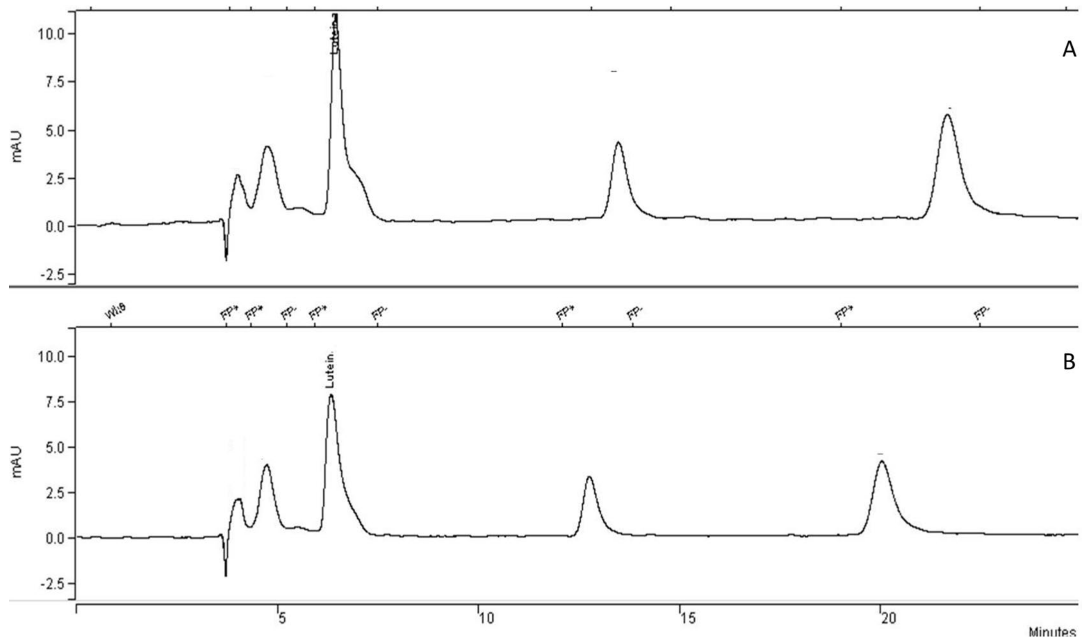
664 Figure 3.



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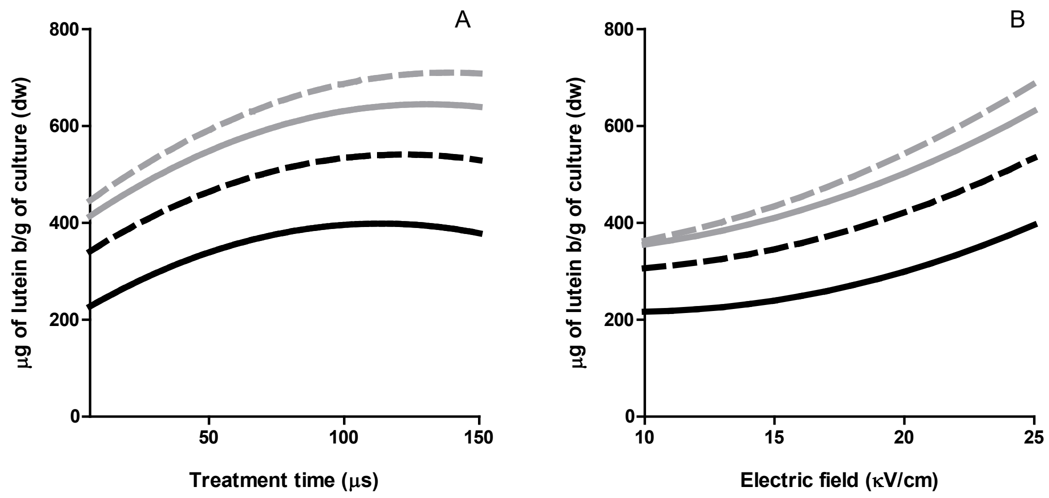
667 Figure 4



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678 Table 1 Lutein extraction yield (LEY) resulting from ethanol extraction from untreated and
679 PEF-treated *C.vulgaris* cells.

Treatment temperature (°C)	Electric field strenght (kV/cm)	Treatment time (µs)	LEY ± IC 95% (µg/g dw)
	0	0	163.10± 6.91
10	10	15	166.37± 20.39
		75	188.83± 36.67
		150	208.68± 26.02
	15	6	140.62± 34.55
		15	138.06± 35.97
		75	185.54± 29.90
		150	169.42± 81.12
	20	6	161.35± 25.25
		15	248.69± 2.00
		75	260.62± 48.61
		150	249.41± 75.44
	25	6	185.49± 44.61
		15	257.43± 50.51
		75	383.32± 11.30
		150	451.38± 16.13
25	10	15	292.75± 41.25
		75	307.92± 38.41
		150	324.27± 28.48
	15	6	249.45± 15.66
		15	249.00± 12.31
		75	290.86± 19.86
		150	371.45± 40.69
	20	6	296.98± 81.94
		15	376.09± 104.54
		75	396.27± 12.88
		150	441.40± 99.32
	25	6	342.96± 53.95
		15	455.99± 82.74
		75	524.82± 171.72
		150	513.67± 118.71
40	10	15	228.13± 9.60
		75	284.13± 7.88
		150	279.26± 7.53
	15	6	205.95± 20.24
		15	265.26± 14.25
		75	400.96± 11.80
		150	490.07± 18.96
	20	6	256.89± 41.13
		15	287.85± 25.67
		75	529.65± 36.05

	150	523.65±	29.47
25	6	446.08±	51.99
	15	492.96±	52.50
	75	712.64±	59.13
	150	753.09±	36.61

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685 Table 2 Coefficients, F-values and p- values of the ANOVA analysis for the quadratic model
686 developed to describe the influence of the temperature (T), treatment time (t) and electric field
687 strength (E) on the lutein extraction yield from *C. vulgaris* cells.

	Coefficient	**Fvalue**	**pvalue**
Intercept	+147.36		
Temperature (T)	+9.30	111.67	< 0.0001
Time (t)	+1.84	45.86	< 0.0001
Electric field (E)	-22.07	131.76	< 0.0001
T^2	-0.20	8.55	0.0056
t^2	-0.01	18.59	0.0001
E^2	+0.73	31.28	< 0.0001
T*t	+0.02	6.06	0.0185
T*E	+0.32	15.53	0.0003
t*E	+0.05	6.15	0.0177
Model		38.35	< 0.0001
R2	0.900		
R2-adj	0.877		
RMSE	46.77		
Af	1.18		
Bf	1.00		

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