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A Comparative Study on the Effects of Millisecond- and Microsecond-Pulsed Electric Field Treatments on the Permeabilization and Extraction of Pigments from *Chlorella vulgaris*

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Abstract The interdependencies of the two main processing parameters affecting "electroporation" (electric field strength and pulse duration) while using pulse duration in the range of milliseconds and microseconds on the permeabilization, inactivation, and extraction of pigments from *Chlorella vulgaris* was compared. While irreversible "electroporation" was observed above 4 kV/cm in the millisecond range, electric field strengths of ≥10 kV/cm were required in the microseconds range. However, to cause the electroporation of most of the 90 % of the population of *C. vulgaris* in the millisecond (5 kV/cm, 20 pulses) or microsecond (15 kV/cm, 25 pulses) range, the specific energy that was delivered was lower for microsecond treatments (16.87 kJ/L) than in millisecond treatments (150 kJ/L). In terms of the specific energy required to cause microalgae inactivation, treatments in the microsecond range also resulted in greater energy efficiency. The comparison of extraction yields in the range of milliseconds (5 kV, 20 ms) and microseconds (20, 25 pulses) under the conditions in which the maximum extraction was observed revealed that the improvement in the carotenoid extraction was similar and chlorophyll *a* and *b* extraction was slightly higher for treatments in the microsecond range. The specific energy that was required for the treatment in the millisecond range (150 kJ/L) was much higher than those required in the microsecond range (30 kJ/L). The comparison of the efficacy of both types of pulses on the extraction enhancement just after the treatment and after a post-pulse incubation period seemed to indicate that PEF in the millisecond range created irreversible alterations while, in the microsecond range, the defects were a dynamic structure along the post-pulse time that caused a subsequent increment in the extraction yield.

Keywords Microalgae · Extraction · PEF · Pulse duration

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Introduction

Pulsed electric field (PEF) technology represents a sustainable innovative solution for optimizing the extraction of intracellular compounds of interest from walled microorganisms while simultaneously reducing energetic inputs and processing costs (Toepfl et al. 2006a, b). PEF processing involves the application of repetitive short pulses (less than a few ms) of a high electric field to a biological material placed between two electrodes. If the applied external electric field is applied with sufficient intensity, an increment in the permeability of the cytoplasmic membrane of cells to ions and macromolecules is observed as a consequence of the formation of local defects or pores (Ivorra 2010) This phenomenon of electropermeabilization, which is also called membrane "electroporation" has been observed in individual microbial prokaryotic and eukaryotic cells in suspension, as well as in intact plant or animal tissues (Mahnic-Kalamiza et al. 2014). Membrane "electroporation" may be reversible or irreversible, depending on the processing parameters that

are applied and which cause the membrane to be either transiently or permanently permeable.

The direct effect of the external field on a vesicle is to modulate the transmembrane potential. It is generally accepted that, in order to induce "electroporation" the applied external electric field must induce an increment of the transmembrane voltage of the cells above a critical threshold. The external electric field strength that is required to reach the transmembrane voltage threshold is called the critical electric field strength (Teissie et al. 2005) The occurrence of the critical electric field strength has been shown to be dependent on the size and geometry of the cell (Kotnik et al. 2012). While electroporating microbial cells with characteristic diameters shorter than 10 μm, an external electric field in the range of 10–15 kV/cm is necessary. Further, the "electroporation" of plant and animal cells with characteristic diameters that may be ten times higher requires a lower external field (i.e., in the range of 0.5–2 kV/cm) (Toepfl et al. 2006a, b). In addition to electric field strength, the main processing parameter that determines cell electroporation is the treatment time during which the cells are subjected to the electric field, which depends on the number of repetitive pulses and the duration of the applied pulse. The pulse lengths that were used in different studies and which sought to release intracellular compounds from bacteria, yeast or plant cells are in the millisecond to microsecond range (Ganeva et al. 2001; Flisar et al. 2014; Coustets et al. 2015; De Vito et al. 2008). Generally, a reduction of the pulse duration from milliseconds to microseconds has to be compensated by an increase in the electric field intensity (Weaver et al. 2012).

Chlorella vulgaris is a freshwater unicellular microalgae. It contains the green photosynthetic pigments chlorophyll *a* and *b*, lutein and other primary carotenoids, such as α and β-carotenes in its chloroplast (Gouveia et al. 1996). The food, cosmetic, and pharmaceutical industries are able to take advantage of the wide range of biological activities of the bioproducts produced by *C. vulgaris*. However, the development of industrial-scale production systems of bioproducts from microalgae is still affected by economic and technical constrains. In particular, one of the main bottlenecks of the process is attributed to the estimated high cost and low yields of the operating phases in which bioproducts are extracted from microalgae.

Recently, several studies have demonstrated that PEF shows a promising application for improving extraction of lipids, proteins and pigments from microalgae (electroextraction) (Barba et al. 2014; Coustets et al. 2013; Goettel et al. 2013; Eing et al. 2013; Grimi et al. 2013; Luengo et al. 2014; Sheng et al. 2011; Zbinden et al. 2013; Parniakov et al. 2015). These studies have demonstrated that electroextraction from microalgae depends on different processing parameters, including electric field strength, number of repetitive pulses, pulse duration, pulse frequency, pulse polarity, and treatment temperature. However, the different microalgae investigated the extracted compounds, experimental conditions, and pulse generator used in published papers made it difficult to define the most optimized PEF processing conditions that are required for obtaining the maximum extraction yield of metabolites with lower energetic consumption.

The purpose of the present investigation was to compare the interdependencies of the two main processing parameters that affect "electroporation" (electric field strength and pulse duration) using a pulse duration in the range of milliseconds and microseconds on the permeabilization, inactivation, and extraction of pigments from *C. vulgaris*.

Materials and Methods

Cell Culture

Chlorella vulgaris (BNA 10-007, National Bank of Algae, Canary Islands, Spain), were grown in a BG-11 medium that contained the following components: 15 g/L $NaNO_3$; 4.0 g/L K_2HPO_4; 7.5 g/L $MgSO_4{\cdot}7H_2O$; 3.6 g/L $CaCl_2{\cdot}2H_2O$; 0.6 g/L citric acid; 6 g/L ammonium ferric citrate green; 0.1 g/L EDTA·Na_2; 2.0 g/L Na_2CO_3; trace metal solution (H_3BO_3 2.86 g/L; $MnCl_2{\cdot}4H_2O$ 1.81 g/L; $ZnSO_4{\cdot}7H_2O$ 0.22 g/L; $Na_2MoO_4{\cdot}2H_2O$ 0.39 g/L; $CuSO_4{\cdot}5H_2O$ 0.08 g/L; $Co(NO_3)_2{\cdot}6H_2O$ 0.05 g/L). For a solid medium, 1.5 g of technical agar was added to 100 mL of the medium. Medium BG 11 (liquid and solid) was autoclaved at 121 °C for 20 min.

The cells were photoautotrophically cultured in a 5 L Erlenmeyer flask that was bubbled with air (6 mL/s) at 30 °C in light:dark cycles (12:12) with white fluorescent lamps (2 klux). The cultures were initially inoculated with 1×10^6 cells/mL. The cell density was determined by microscope (microscope L-Kc, Nikon, Tokyo, Japan) in a Thoma cell chamber (ServiQuimia, Constantí, Spain). Experiments were conducted with cells in the stationary phase of growth (between the 10th and 15th day after inoculation).

PEF Equipment

PEF Equipment for Millisecond-Pulse Delivery

The PEF equipment used in this investigation (Deex Bio) was supplied by Betatech (Saint-Orens-de-Gameville, France). The electric system was made of two S20u generators, each able to deliver square voltage pulses up to

2 kV under 6A with an adjustable pulse duration up to a few milliseconds. The delays between pulses were down to 15 ms. An analog switch was used between the two generators and the pulsing chamber to invert the polarity between each pulse in order to prevent gas bubble formation and other electrochemical reactions at the surface of the stainless steel electrodes (Fig. 1a). For *C. vulgaris* treatment, a batch parallel-electrode treatment chamber with a distance of 0.3 cm between electrodes and an area of 0.78 cm^2 was used.

PEF Equipment for Microsecond-Pulse Delivery

The PEF equipment used in this investigation was supplied by ScandiNova (Modulator PG, ScandiNova, Uppsala, Sweden). The apparatus generates square waveform pulses of a width of 3 µs (Fig. 1b) with a frequency of up to 300 Hz. The maximum output voltage and current were 30 kV and 200 A, respectively. The equipment consists of a direct-current power supply which converts the 3-phase line voltage to a regulated DC voltage. It charges up six IGBT switching modules (high-power, solid-state switches) to a primary voltage of around 1000 V. An external trigger pulse gates all of the modules and controls its discharge to a primary pulsed signal of approximately 1000 V. Finally, a pulse transformer converts this primary 1000 V pulse to a high-voltage pulse of the desired high voltage. For *C. vulgaris*, treatment with a batch parallel-electrode treatment chamber with a distance between electrodes of 0.25 cm and an area of 1.76 cm^2 was used.

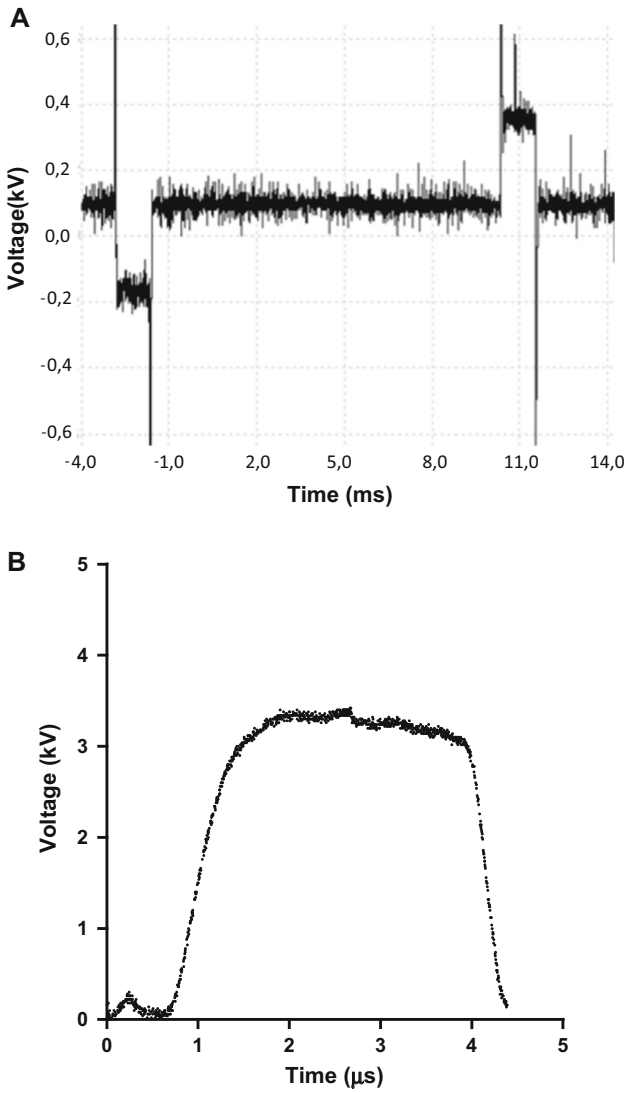


Fig. 1 Diagrams of the pulse shape of millisecond (**a**) and microsecond (**b**) duration pulses

PEF Treatments

Before treatments, microalgal cells were centrifuged at 3000×*g* for 10 min at 25 °C. They were re-suspended in a citrate–phosphate McIlvaine buffer (pH 7) of 0.150 ms/cm for the treatments in the millisecond range and 1 ms/cm for the treatments in the microsecond range. The microbial suspension at a concentration of 2×10^8 CFU/mL was placed into the treatment chamber by means of a 1-mL sterile syringe (TERUMO, Leuven, Belgium) and the PEF treatment was applied.

Chlorella vulgaris cells treated in the millisecond range were subjected up to 30 square, 1 ms waveform bipolar pulses at 0.25 Hz, of 3.5, 4, 4.5, and 5 kV/cm that corresponded with specific energies per pulse of 1.83, 2.4, 3.0, and 3.75 kJ/L of culture. The *C. vulgaris* cells treated in the microsecond range were subjected up to 50 square 3 µs waveform pulses at 0.5 Hz, of 10, 15, 20, and 25 kV/cm, thus corresponding with specific energies per pulse of 0.30, 0.67, 1.2, 1.87 kJ/L of culture.

The energy per pulse (W) was calculated using the following equation

$$W = \int \sigma E(t)^2 \mathrm{d}t, \quad (1)$$

where σ (s/m) is the electrical conductivity of the treatment medium, E (V/m) is the electric field strength, and t (s) is the duration of the pulse. The total energy (kJ) applied (W) was calculated by multiplying the energy per pulse (W') by the number of pulses. The total specific energy (kJ/L) applied (W) was determined by dividing the total energy by the volume of the treated medium.

Dielectric oil tempered at 25 °C was re-circulated through the inner part of electrodes of both treatment chambers to avoid the increase of the temperature of the treatment medium during the PEF treatment. In both protocols, the temperature increase of the treatment medium was measured with a thermocouple before and after the PEF treatment and the temperature variation was always less than 2 °C.

Enumeration of Viable Cells

PEF treated and untreated (control) cell suspensions were serially diluted in a sterile solution of peptone water. From the selected dilutions, 20 μL were plated into solid media. The plates were incubated at 30 °C for 7 days with the same light regime used for the liquid culture, and the number of CFU/mL were counted to determine the inactivation rate after the treatment. Longer incubation times did not increase the colony counts.

Staining Cells with Propidium Iodide

Two alternative staining protocols were followed under the same experimental conditions. Cells were either stained with PI (Sigma-Aldrich), before PEF treatment or once it had finished (5 min after the PEF treatment) according to the protocol described by Luengo et al. (2014). PI was added at a final concentration of 0.8 mM and the cells were incubated for 10 min at room temperature (longer times did not influence the fluorescence measurements). After the incubation time, the cells were washed twice and fluorescence was measured with a spectrofluorophotometer (mod. Genios, Tecan, Austria) using a 535-nm excitation filter (523–547 nm) and a 625-nm emission filter (608–642 nm).

The fluorescence data obtained after a given PEF treatment were expressed as a percentage of permeabilized cells in comparison to the fluorescence measurement obtained for cells after a PEF treatment (150 μs at 25 kV/cm) that permeabilized more than 99 % of the cells. Under these last conditions, the permeabilization of the cell population was checked using a fluorescence microscope (Nikon, Mod. L-Kc, Nippon Kogaku KK, Japan).

Pigment Extraction

Hundred microliters of non-treated or PEF-treated suspension were mixed with 1 mL of 96 % ethanol and vortexed. The mixture was macerated in the dark at room temperature for 20 min and centrifugated at $6000 \times g$ for 90 s. The absorbance of the supernatant was measured at 470, 649, and 664 nm against a 96 % ethanol blank. The concentration of chlorophyll *a* and *b* and the total carotenoids were calculated according to the equations below (Lichtenthaler 1987)

$$\text{Chlorophyll } a\,(C_a) = (13.36 \times A_{664}) - (5.19 \times A_{649}) \quad (2)$$

$$\text{Chlorophyll } b\,(C_b) = (27.43 \times A_{649}) - (8.12 \times A_{664}) \quad (3)$$

$$\text{Total carotenoids} = (1000 \times A_{470} - 2.13 \times C_a - 97.64 \times C_b)/209 \quad (4)$$

The concentrations are given in mg/mL.

Extraction in ethanol was conducted just after the application of the PEF treatment or after 1 h of incubation at room temperature of the *C. vulgaris* cells in the treatment medium.

Results

The Influence of the Pulse Duration (Milliseconds and Microseconds) at Different Electric Field Strengths on the Membrane Electroporation of *C. vulgaris*

The uptake of the fluorescent dye, PI, that was only permitted entry into permeabilized cells was the procedure used to evaluate the membrane permeabilization. Reversible and irreversible "electroporation" were assayed by comparing the fluorescent intensity of a PEF-treated microbial suspension when PI was added before or after the PEF treatment (Garcia et al. 2007). Reversible electroporation is shown when the fluorescent intensity of the microalgae suspension that is in contact with PI during the PEF treatment is higher than the fluorescent intensity of the suspension that was stained with PI after the PEF treatment. Figure 2 shows the influence of the electric field strength and treatment time on the PI uptake by *C. vulgaris* cells when PI was added before (white bars) or after (striped bars) the PEF treatment with pulses of a duration in the range of milliseconds (a) and microseconds (b). The uptake of PI increased with the electric field strength and treatment time for both types of pulses used depending on whether PI was added before or after the PEF treatment. In the millisecond range, reversible "electroporation" was detected in the range from 3.5 to 5 kV/cm and irreversible "electroporation" was observed above 4–5 kV/cm. However, in the microsecond range, the permeability of *C. vulgaris* to PI was unaffected at these electric field strengths (data not shown). A significant increment in the electric field strength was required to increase the permeability of the *C. vulgaris* membrane when pulses in the range of microseconds were applied. Electric field strengths of 10 kV/cm or higher were required to detect significant reversible and irreversible "electroporation" when pulses in the microsecond range were applied. Independent of the electric field strength and the duration of pulses, the existence of reversible electroporation was

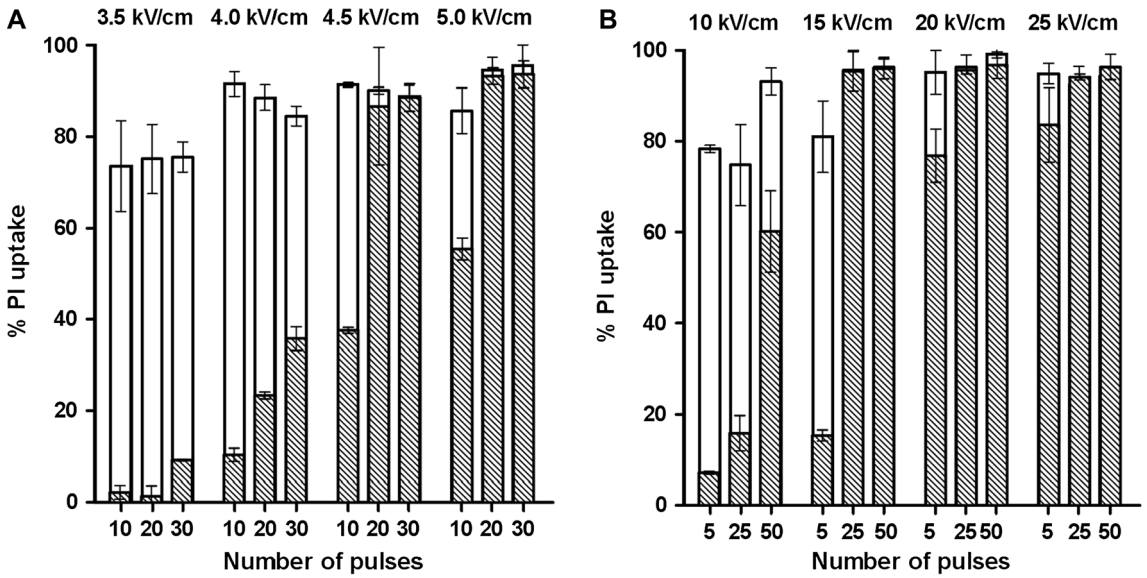


Fig. 2 Influence of the electric field strength and treatment time on the PI uptake when PI was added before (*white bars*) or after (*striped bars*) the PEF treatment for treatments in the range of **a** milliseconds (bipolar pulses of 1 ms) and **b** microseconds (monopolar pulses of 3 μs)

observed at the lowest treatment times delivered. According to the results obtained in this investigation, the intensity of the electric field strength that caused the complete irreversible electroporation of most (90 %) of the population of *C. vulgaris* was dependent on the duration and the number of the pulses. When millisecond pulses were used, this irreversibility was observed with 20 pulses at electric field strengths of 5 kV/cm. However, in the microsecond range, shorter treatment times (≥75 μs, 25 pulses) but higher electric field strength (≥15 kV/cm) were required to prevent resealing after the PEF treatment in more than 90 % of the population. Therefore, in order to obtain a similar degree of irreversible electroporation, the reduction of the pulse duration from milliseconds to microseconds must be compensated by a threefold increase in the electric field intensity.

As a given degree of cell permeabilization can be obtained by applying treatments of different electric field strengths and duration, it is important to know the combination of both parameters that require the minimum amount of total specific energy consumption from a process design point of view. When comparing the lowest treatment intensity required to cause the electroporation of most of the 90 % of the population of *C. vulgaris* in the millisecond (5 kV/cm, 20 pulses) or microsecond (15 kV/cm, 25 pulses) range, the specific energy delivered was lower for a shorter treatment duration at higher electric field strengths (16.87 kJ/L) than for a longer treatment duration at a lower electric field strength (150 kJ/L). Pulses in the microsecond range required lower specific energy even when the conductivity of the treatment medium was higher (1 ms/cm) than in the case of millisecond pulses (0.15 ms/cm). It has been reported that the electroporation in a medium of low conductivity requires less specific energy compared to a medium at higher conductivity (Frey et al. 2013) Therefore, extraction using pulses in the microsecond range could have been even more energy efficient in a medium of lower conductivity.

Our results on influence of pulse duration on electroporation degree disagree with previous results. Such prior studies reported the effect of pulse duration in the range of 10 μs to 1 ms on the efficiency of the disintegration of apple tissue cells by PEF which was estimated by means of the electrical conductivity (De Vito et al. 2008). In this investigation on plant tissues, it was reported that longer pulses were more effective and for obtaining the same disintegration degree at a given electric field more energy was required for shorter pulses. The effect of pulses of different durations for the disintegration of apple cells in tissue was compared in the same range of electric field strengths (100–400 V/cm).

Inactivation of *C. vulgaris* by PEF Treatment of Duration in the Range of Milliseconds and Microseconds at Different Electric Field Strengths

The loss of the membrane integrity created by the application of an external electric field that caused uncontrolled molecular transport across membranes may abolish the microalgae's capacity to maintain the microbial homeostasis, thus causing microalgae inactivation (Qin et al.

2014). The effect of the PEF on the loss of microalgae viability for treatments whose duration is in the range of milliseconds and microseconds was assayed. Figure 3 shows survival curves that correspond to the inactivation of *C. vulgaris* by PEF treatments at different electric field strengths and durations in the range of milliseconds (a) and microseconds (b). Figure 3 shows that, independent of the type of pulses applied, the inactivation of *C. vulgaris* increased by increasing the electric field strength and the treatment time. However, for both pulse durations used during the treatments, the inactivation kinetics of *C. vulgaris* was non-linear when the $\log_{10}$ of the survival fraction was plotted against the number of pulses. Upwardly concave survival curves similar to those obtained in this investigation are generally observed when other microbial cells, such as bacteria or yeast, are inactivated by PEF at durations in the microsecond range (Ohshima et al. 2002; Raso et al. 2000; Rodrigo et al. 2003). Therefore, our results indicate that the modification of the pulse duration from microseconds to milliseconds did not modify the shape of the inactivation survival curves. Similar to the membrane "electroporation", lower electric field strengths were required to obtain a significant loss of viability in the cells of *C. vulgaris* when pulses in the range of milliseconds where applied. While in the millisecond range, significant inactivation was detected in the range between 4 and 5 kV/cm; in the microsecond range, the application of electric field strengths at 10 kV/cm or higher was required. However, treatments at higher electric fields applied in the range of microseconds are more lethal than treatments in the millisecond range applied in a lower electric field. For example, while the most intense treatment applied in the range of milliseconds (5 kV/cm for 60 ms) inactivated the population of *C. vulgaris* around $1.7\log_{10}$ cycles, an inactivation of more than $3.5\log_{10}$ cycles was obtained in the most intense treatment applied in the microsecond range (25 kV/cm for 150 μs). On the other hand, in terms of the specific energy required to cause microalgae inactivation, treatments in the microsecond range also resulted in more energy efficiency. The specific energy (225 kJ/L) required to obtain the highest inactivation ($1.7\log_{10}$) for pulses with durations in the milliseconds was 2.4 times higher than the specific energy (93.7 kJ/L) required to obtain the highest inactivation ($3.8\log_{10}$) when pulses were applied in the microsecond range.

The microbial inactivation by PEF has been generally related to the permanent permeabilization of the microbial membrane. The relationships between the percentage of irreversible electropermeabilized cells and the percentage of dead cells estimated by plate counting for treatments applied in the millisecond (closed circles) and microsecond (open circles) ranges are shown in Fig. 4. The results show that an agreement between cell death and PI uptake that is represented by the theoretical straight line with slope 1 and intercept 0 shown in the figures was only observed when the percent of PI uptake was higher than 80 for both treatments in the millisecond and microsecond ranges. For the percent of PI uptakes that were lower than 80, independent of the pulse duration, the percentage of irreversible permeabilized cells was lower than the percentage of death cells. These results seems to indicate that a fraction of cells were inactivated during the treatment but was able to

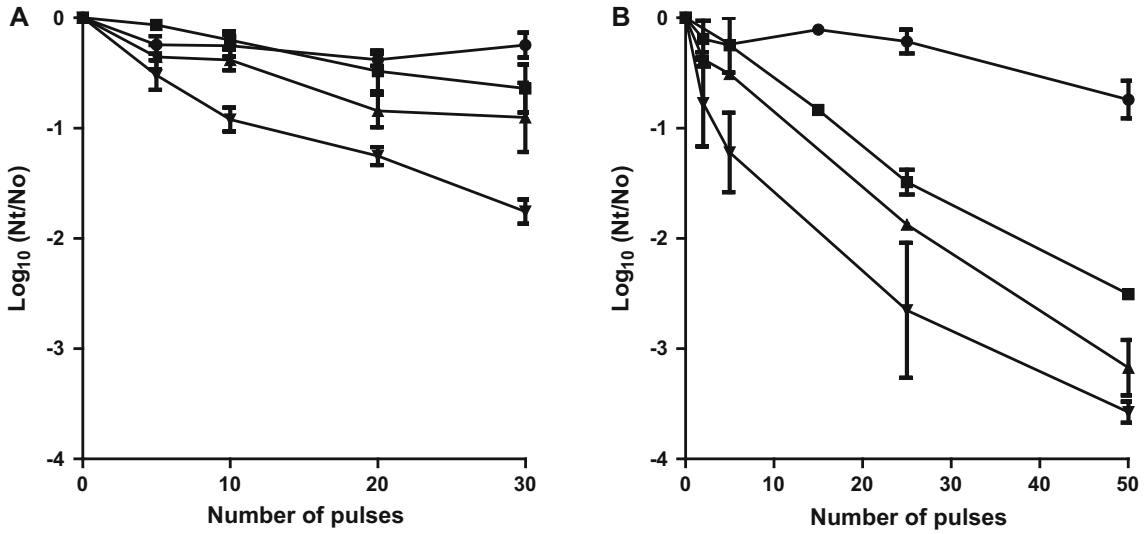


Fig. 3 Influence of the electric field strength and treatment time on the death of *C. vulgaris* cells treated by PEF in the range of **a** milliseconds (bipolar pulses of 1 ms) and **b** microseconds (monopolar pulses of 3 μs). Electric field strengths of 3.5 (*filled circle*), 4 (*filled square*), 4.5 (*filled triangle*), 5 (*filled inverted triangle*) kV/cm, and 10 (*filled circle*), 15 (*filled square*), 20 (*filled triangle*), 25 (*filled inverted triangle*) kV/cm

Fig. 5 Influence of electric field strength in treatments of 20 pulses (bipolar pulses of 1, 40 ms) and 25 pulses (monopolar pulses of 3, 75 μs) on the extraction yield of carotenoids (**a**) chlorophyll *a* (**b**) and chlorophyll *b* (**c**) from *C. vulgaris* just after the PEF treatment (*white bars*) and after 1 h of incubation in the treatment medium after the PEF treatment (*gray bars*)

recover the integrity of the membrane by becoming impermeable to the PI that was added after the PEF treatment and before dying. This behavior, which was previously observed for *C. vulgaris* treated by PEF in the microsecond range, was also confirmed in the present work for PEF treatments applied in the millisecond range and on CHO cells treated with pulses in the millisecond duration (Luengo et al. 2014; Rols et al. 1998). An effective extraction of intracellular compounds required irreversible cell membrane permeabilization in order to facilitate solvent access into the cell and the release of intracellular material. Quantification of the number of inactivated cells with pulses in the microsecond or millisecond range could be a suitable indicator of the efficacy of electropulsation for improving the extraction of compounds from *C. vulgaris* for those more intense treatments that cause an irreversible electroporation of more than the 80 % of the cell population.

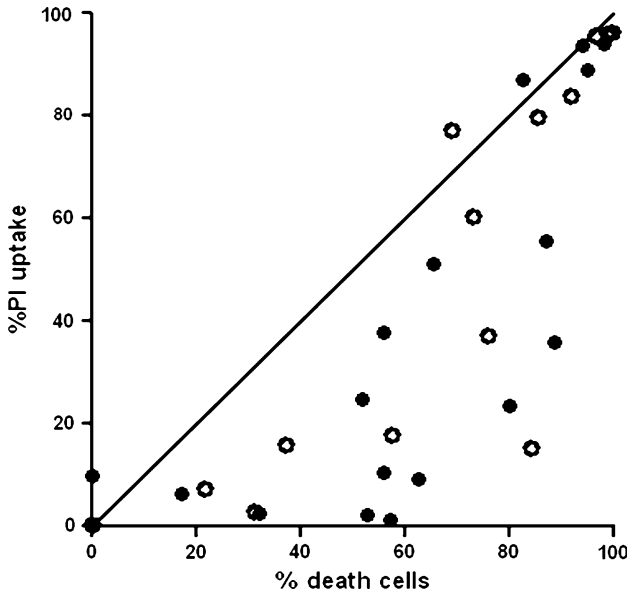
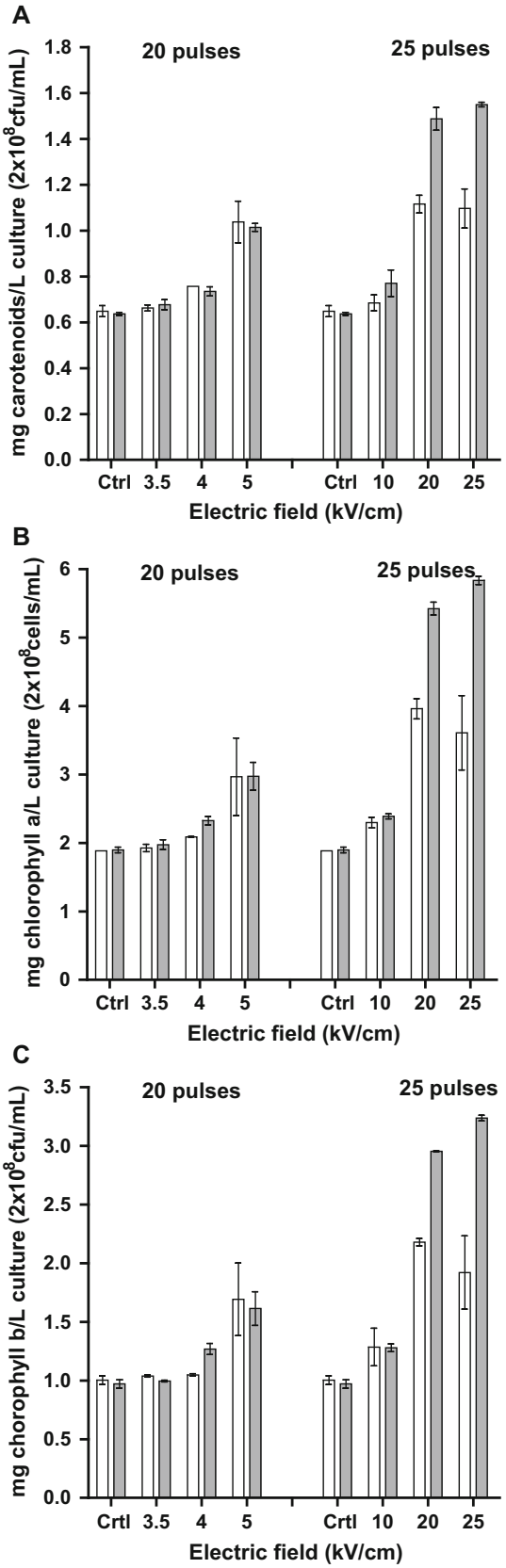


Fig. 4 Relationship between the percentages of cell permeabilization assessed by PI staining after PEF against the percentage of death cells treated by PEF in the range of milliseconds (*filled circle*) and microseconds (*open circle*). To show the degree to which each treatment causes membrane permeabilization, a theorical *straight line* with *slope* = 1 and *intercept* = 0, is included



The Effect of PEF Treatments in the Range of Millisecond and Microsecond Durations on the Extraction of Carotenoids and Chlorophylls *a* and *b* from *Chlorella vulgaris*

Figure 5 compares the influence of the electric field strength on the extraction of carotenoids (Fig. 5a), chlorophyll *a* (Fig. 5b) and chlorophyll *b* (Fig. 5c) from *C. vulgaris* cells treated by PEF for a cumulated duration of 20 (40 ms) and 25 pulses (75 μs). Although experiments were conducted at different processing times in the range of milliseconds (10, 20, 40, and 60 ms) and microseconds (6, 15, 75, and 150 μs), treatments shorter that 40 ms (20 pulses) and 75 μs (25 pulses) were ineffective in improving extraction. Further, longer treatments did not significantly increase extraction (data not shown). The extraction of the three compounds was assayed just after the application of the PEF treatment (white bars) and after pre-incubating the cells for 1 h in the treatment medium (striped bars). Extraction from the PEF-untreated *C. vulgaris* cells (control) is also shown in Fig. 5. When the extraction was conducted just after the application of the PEF treatment, the extraction yield of the three pigments, in comparison to the control, increased significantly for the treatments of 40 ms at 5 kV/cm and for the treatments of 75 μs at 20 and 25 kV/cm. However, in the last case, the use of field larger than 20 kV/cm did not increase the extraction of the three pigments. Goettel et al. (2013) investigated the extraction of intracellular compounds from the microalgae *Auxenochlorella protothecoides*; they also observed that increasing the electric field strength from 23 to 43 kV/cm did not have a great influence on the amount of intracellular compounds that were released. These authors considered that the lowest field strength applied (23 kV/cm) was already enough to achieve the maximum irreversible permeabilization.

The comparison of extraction yields in the millisecond and microsecond ranges under the conditions in which the maximum extraction was observed revealed that the improvement in the carotenoid extraction was similar and chlorophyll *a* and *b* extraction was slightly higher for treatments in the microsecond range. For example, after a PEF at 5 kV/cm, the extraction yields for carotenoids and chlorophylls *a* and *b* were 1.06, 2.90, and 1.69 mg/L of culture, respectively. After a PEF treatment at 20 kV/cm, the yields were 1.09, 3.95, and 2.17 mg/L of culture, respectively. However, the required specific energy for the treatment in the millisecond range (150 kJ/L) was much higher than that required in the microsecond range (30 kJ/L). Another important difference concerning the effects of millisecond and microsecond pulses was observed when the extraction was conducted after 1 h of pre-incubation. Significant increments in the subsequent extraction yields were observed after 1 h of pre-incubation in the treatment media for PEF treatment applied in the microsecond range, but not for those applied in the millisecond range or in the control. For example, after a PEF treatment at 20 kV/cm for 75 μs, the extraction yields for carotenoids and chlorophylls *a* and *b* were 2.3, 2.9, and 3 times higher, respectively, in comparison to the control.

The higher extraction yield of the three pigments after 1 h of incubation in the samples treated by PEF in the microsecond range could be related to the evolution of the pore population post-treatment stage. While the lower electric field applied with pulses in the millisecond range created stable pores, the higher electric field strength applied in the microsecond range caused the creation of local defects in the membrane that evolved (i.e., in terms of pore size enlargement and/or an increase in the number of pores during the incubation time). This increment in the permeabilization of the membrane could facilitate both the diffusion of the eluting solvent (ethanol) into the cytoplasm interacting with the pigments and the latter diffusion of the pigments toward the bulk solvent. On the other hand, the increment in the permeabilization of the *C. vulgaris* membrane during the incubation time in the aqueous media could also cause the chloroplast plasmolysis due to osmolytic disequilibrium in the cytoplasmatic space. Therefore, after 1 h of incubation the chloroplast membrane where the three pigments are located could become permeabilized, thus facilitating both the diffusion of the ethanol into the chloroplast and the diffusion of the pigment toward the cytoplasm.

In conclusion, our results show that the application of PEF treatments when using millisecond or microsecond pulses is effective on the permeabilization, inactivation, and extraction of pigments of *C. vulgaris*. In order to obtain similar effects using pulses of different durations, the reduction of the pulse duration from milliseconds to microseconds needed to be compensated for by an increment in the electric field intensity. However, in terms of specific energetic requirements, treatments at higher electric field strengths in the microsecond range were more effective than treatment at lower electric field strengths for the millisecond range in order to cause similar effects (permeabilization, inactivation, and extraction yield). The comparison of the efficacy of both types of pulses on the extraction enhancement just after the treatment and after a post-pulse incubation period seems to indicate that PEF in the millisecond range at a lower electric field strength creates irreversible alterations, while in the microsecond range, the defects were a dynamic structure along the post-pulse time that caused a subsequent increment in the extraction yield.

However, further studies are needed in order to determine the exact mechanism of action of the combination of pulse duration and electric field strength intensity on the electroporation of *C. vulgaris* by PEF.

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References

Barba FJ, Nabil G, Vorobiev E (2015) New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. Food Eng Rev 7:45–62

Coustets M, Al-Karablieh N, Thomsen C, Teissie J (2013) Flow process for electroextraction of total proteins from microalgae. J Membr Biol 246:751–760

Coustets M, Ganeva V, Galutzov B, Teissie J (2015) Millisecond duration pulses for flow-through electroinduced protein extraction from E. coli and associated eradication. Bioelectrochem 103:82–91

De Vito F, Ferrari G, Lebovka NI, Shynkaryk NV, Vorobiev E (2008) Pulse duration and efficiency of soft cellular tissue disintegration by pulsed electric fields. Food Bioprocess Technol 1:307–313

Eing C, Goettel M, Straessner M, Gusbeth C, Frey W (2013) Pulsed electric field treatment of microalgae—benefits for microalgae biomass processing. IEEE Trans Plasma Sci 41:2901–2907

Flisar K, Meglic SH, Morelj J, Golob J, Miklavcic D (2014) Testing a prototype pulse generator for a continuous flow system and its use for E. coli inactivation and microalgae lipid extraction. Bioelectrochem 100:44-51

Frey W, Gusbeth C, Schwartz T (2013) Inactivation of *Pseudomonas putida* by pulsed electric field treatment: a study on the correlation of treatment parameters and inactivation efficiency in the short-pulse range. J Membr Biol 246:769–781

Ganeva V, Galutzov B, Eynard N, Teissié J (2001) Electroinduced extraction of β-galactosidase from *Kluyveromyces lactis*. Appl Microbiol Biotechnol 56:441–413

Garcia D, Gomez N, Manas P, Raso J, Pagan R (2007) Pulsed electric fields cause bacterial envelopes permeabilization depending on the treatment intensity, the treatment medium pH and the microorganism investigated. Int J Food Microbiol 113:219–227

Goettel M, Eing C, Gusbeth C, Straessner R, Frey W (2013) Pulsed electric field assisted extraction of intracellular valuables from microalgae. Algal Res 2:401–408

Gouveia L, Veloso V, Reis A, Fernandes H, Novais J, Empis J (1996) Evolution of pigment composition in *Chlorella vulgaris*. Bioresour Technol 57:157–163

Grimi N, Dubois A, Marchal L, Jubeau S, Lebovka NI, Vorobiev E (2013) Selective extraction from microalgae *Nannochloropsis* sp. using different methods of cell disruption. Bioresour Technol 153:254–259

Ivorra A (2010) Tissue electroporation as a bioelectric phenomenon: basic concepts. In: Rubinsky B (ed) Irreversible electroporation. Springer, Heidelberg, pp 23–61

Kotnik T, Kramar P, Pucihar G, Miklavcic D, Tarek M (2012) Cell membrane electroporation—part 1: the phenomenon. Electr Insul Mag 28:14–23

Lichtenthaler H (1987) Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Luengo E, Condon-Abanto S, Alvarez I, Raso J (2014) Effect of pulsed electric field treatments on permeabilization and extraction of pigments from *Chlorella vulgaris*. J Membr Biol 247:1269–1277

Mahnic-Kalamiza S, Vorobiev E, Miklavcic D (2014) Electroporation in food processing and biorefinery. J Membr Biol 247:1279–1304

Ohshima T, Okuyama K, Sato M (2002) Effect of culture temperature on high-voltage pulse sterilization of *Escherichia coli*. J Electrostat 55:227–235

Parniakov O, Barba FJ, Grimi N, Marchal L, Jubeau S, Lebovka N, Vorobiev E (2015) Pulsed electric field assisted extraction of nutritionally valuable compounds from microalgae *Nannochloropsis* spp. using the binary mixture of organic solvents and water. Innov Food Sci Emerg Technol 27:79–85

Qin S, Timoshkin IV, Maclean M, Wilson MP, MacGregor SJ, Given MJ, Anderson JG, Wang T (2014) Pulsed electric field treatment of microalgae: inactivation tendencies and energy consumption. IEEE Trans Plasma Sci 42:3191–3196

Raso J, Alvarez I, Condón S, Sala Trepat FJ (2000) Predicting inactivation of *Salmonella senftenberg* by pulsed electric fields. Innov Food Sci Emerg Technol 1:21–29

Rodrigo D, Ruíz P, Barbosa-Cánovas GV, Martínez A, Rodrigo M (2003) Kinetic model for the inactivation of *Lactobacillus plantarum* by pulsed electric fields. Int J Food Microbiol 81:223–229

Rols MP, Delteil C, Golzio M, Teissie J (1998) Control by ATP and ADP of voltage-induced mammalian-cell-membrane permeabilization, gene transfer and resulting expression. Eur J Biochem 254:382–388

Sheng J, Vannela R, Rittmann BE (2011) Evaluation of cell disruption effects of pulsed-electric-field treatment of Synechocystis PCC 6803. Environ Sci Technol 45:3795–3802

Teissie J, Golzio M, Rols MP (2005) Mechanisms of cell membrane electropermeabilization: a minireview of our present (lack of ?) knowledge. Biochim Biophys Acta 1724:270–280

Toepfl S, Heinz V, Knorr D (2006a) Applications of pulsed electric fields technology for the food industry. In: Raso J, Heinz V (eds) Pulsed electric fields technology for the food industry. Springer, New York, pp 197–221

Toepfl S, Mathys A, Heinz V, Knorr D (2006b) Review: potential of high hydrostatic pressure and pulsed electric fields for energy efficient and environmentally friendly food processing. Food Rev Int 22:405–423

Weaver JC, Smith KC, Esser AT, Son RS, Gowrishankar TR (2012) A brief overview of electroporation pulse strength-duration space: a region where additional intracellular effects are expected. Bioelectrochemistry 87:236–243

Zbinden MDA, Sturm BSM, Nord RD, Carey WJ, Moore D, Shinogle H, Stagg-Williams SM (2013) Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae. Biotechnol Bioeng 110:1605–1615