Algal Research 31 (2018) 60–69

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from the microalgae *Chlorella vulgaris*

Daniele Carullo[a], Biresaw Demelash Abera[a], Alessandro Alberto Casazza[b], Francesco Donsì[a], Patrizia Perego[b], Giovanna Ferrari[a,c], Gianpiero Pataro[a,*]

[a] Department of Industrial Engineering, University of Salerno, via Giovanni Paolo II, 132, 84084 Fisciano, SA, Italy
[b] Department of Civil, Chemical and Environmental Engineering, University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy
[c] ProdAl Scarl, University of Salerno, via Ponte don Melillo, 84084 Fisciano, SA, Italy

ARTICLE INFO

Keywords:
Microalgae
Pulsed electric fields
High pressure homogenization
Cell disruption
Proteins
Carbohydrates

ABSTRACT

Pulsed Electric Fields (PEF) and High Pressure Homogenization (HPH) are promising and scalable cell disruption technologies of microalgae cells. In this work, the permeabilization degree, morphological properties, and extractability of intracellular compounds from microalgae *Chlorella vulgaris* suspensions (1.2%, w/w) were investigated as a function of PEF treatment at different electric field strengths (10–30 kV/cm) and total specific energy input (20–100 kJ/kg), in comparison with the more disruptive HPH treatment (150 MPa) at different number of passes (n_P = 1–10). The conductivity and the particle size analyses, as well as the SEM images, clearly showed that PEF induces the permeabilization of the cell membranes in an intensity-dependent manner, without producing any cell debris, whereas HPH treatment causes the total disruption of the algae cells into small fragments. Coherently with the lower permeabilization capability, PEF promoted the selective extraction of carbohydrates (36%, w/w, of total carbohydrates), and low molecular weight proteins (5.2%, w/w, of total proteins). On the other hand, HPH induced the undifferentiated release of all the intracellular content, resulting in a 1.1 and 10.3 fold higher yields than PEF, respectively of carbohydrates and protein.

These results suggest that, in a multi-stage biorefinery, PEF could represent a suitable cell disruption method for the selective recovery of small-sized cytoplasmic compounds, while HPH should be placed at the end the cascade of operations allowing the recovery of high molecular weight intracellular components.

1. Introduction

Chlorella vulgaris is a freshwater eukaryotic microalga with a mean diameter ranging from 2.5 to 5 μm [1] belonging to the division of Chlorophyta. It has drawn large attention over the last decades because of its capability to accumulate large amounts of valuable components, especially proteins (51–58%), but also polyunsaturated fatty acids (14–22%), carbohydrates (12–17%), nucleic acids (4–5%), vitamins and minerals [2,3]. Moreover, it accumulates also chlorophyll (1–2%) that imparts the characteristic green color, masking the other less concentrated pigments, such as lutein and other carotenoids [4]. The extraction of all these intracellular compounds, which can be used as natural additives or active ingredients for food, cosmetic, pharmaceutical and animal feed products, as well as in the production of biofuels [5,6], is crucial for achieving an economically feasible microalgae biorefinery [7].

However, these compounds are located in different parts of the cells, protected by the rigid cell wall and membranes surrounding the cytoplasm and the internal organelles (e.g., chloroplast), which greatly limit their rate of mass transfer during extraction. Conventional extraction processes of these intracellular compounds are often conducted from dry biomass with organic or aqueous solvents, depending on the polarity of the target compounds [8,9]. However, these methods suffer from several limitations, namely the long extraction times and the use of relatively large amounts of solvent, and may lead to the co-extraction of undesirable components, with increased downstream processing costs [7,10]. In addition, the drying of microalgal biomass is reported to be one of the major energy-consuming steps within the overall process and is responsible for significant losses of valuable compounds [5,7].

For these reasons, the application of conventional or innovative cell disruption methods to wet biomass may considerably promote the implementation of the biorefinery concept on microalgae, enabling a

* Corresponding author.
E-mail address: gpataro@unisa.it (G. Pataro).

https://doi.org/10.1016/j.algal.2018.01.017
Received 12 October 2017; Received in revised form 26 January 2018; Accepted 26 January 2018

faster and more efficient release of intracellular compounds at low temperature. This also contributes to limit the degradation of the extracts and promotes the reduction of energy costs, of solvent consumption, as well as of the extraction time [7,10].

Among the cell disruption methods, the Pulsed Electric Fields (PEF) and the High Pressure Homogenization (HPH) treatments have emerged as promising methods for the mild and complete disruption of biological cells, respectively [9–14]. Moreover, both PEF and HPH can be easily scaled up to process large volumes of wet biomass in a wide range of solids concentration, thus avoiding the need for energy-intensive drying and possibly allowing to reduce the energy demand per unit biomass [5,15–18].

In PEF processing, the biomaterial is placed between two electrodes of a treatment chamber and exposed to high intensity electric fields (10–50 kV/cm), applied in the form of repetitive pulses of very short duration (from several nanoseconds to few milliseconds), which induce the permeabilization of cell membranes by electroporation, facilitating the subsequent release of intracellular matter [19]. Several studies highlighted the effectiveness of PEF to enhance the selective recovery of intracellular compounds from wet microalgal biomass, including lipids [20,21], pigments [8,10,14,22–23], carbohydrates, and water-soluble proteins of small molecular weight [6,9,14,18,23].

However, the extraction of molecules of higher molecular weight, or more bounded to the intracellular structure (e.g., proteins), requires the application of more effective cell disruption techniques, such as HPH [10].

HPH is a purely mechanical process, during which a liquid dispersion of plant material or a cell biosuspension is forced by high pressure (50–300 MPa) through a micrometric disruption chamber, where the velocity increases rapidly and the pressure decreases to atmospheric conditions as the suspension exit the unit [15]. As a result, the biological cell suspension is subjected to extremely intense fluid-mechanical stresses (shear, elongation, turbulence, and cavitation), which cause the physical disruption of the cell wall and membranes [16,24,25].

Due to its high cell disruption efficiency [7], HPH is reported to markedly increase the extraction yield of several intracellular compounds from microalgae [7,14,26–28]. However, the HPH treatment causes the non-selective release of intracellular compounds, with the concurrent dispersion of cell debris, complicating the downstream separation processes [14]. Moreover, because of the intense interfacial shear stresses and inherent heating occurring in the homogenization valve, which might induce the degradation of compounds, such as proteins [29–30], HPH treatments always require an efficient heat dissipation at the homogenization valve.

Although several studies have already highlighted the potential of PEF and HPH pre-treatments in the microalgae biorefinery, to date, only the study of Safi et al. [28] has addressed the comparison of their efficiency in terms of cell disintegration, energy input and release of soluble proteins from microalgae *Nannochloropsis gaditana*. However, suspensions of this microalgae were prepared from a frozen paste and at different biomass concentration for PEF (15–60 g_{DW}/L) and HPH (100 g_{DW}/L) treatments.

Moreover, a deeper knowledge regarding the impact of these novel technologies at micro and macro scale is required, which is thoroughly necessary in view of their use in a cascade biorefinery approach of microalgae, where the control of the degree of cell breakage could be exploited to enable the fine tuning of the recovery process of intracellular components [6,7,31].

Therefore, the aim of this study is to investigate comparatively the effects of the main process parameters of both PEF and HPH treatments on the cell disintegration degree, the energy consumption, and the release of intracellular compounds (ionic substances, proteins, and carbohydrates) from fresh *C. vulgaris*, in order to select, for each investigated technology, the best treatment conditions in the perspective of their implementation in a biorefinery scheme.

2. Materials and methods

2.1. Microalgae and cultivation

The microalgal strain used in this study was *Chlorella vulgaris* (CCAP 211), purchased from the Culture Collection of Algae and Protozoa (Argyll, UK). It was cultivated in modified Bold's basal medium [32] at pH 7.0 ± 0.5, in a 5 L horizontal tubular photobioreactor illuminated by four 40 W fluorescent lamps from one side [33]. The composition (per liter of distilled water) of the modified medium was as follows: 1.5 g $NaNO_3$, 0.45 g $MgSO_4{\cdot}7H_2O$, 0.15 g NaCl, 0.45 g $K_2HPO_4{\cdot}3H_2O$, 1.05 g KH_2PO_4, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.003 g vitamin B_1, 7.5 10^{-6} g vitamin B_8, 7.5 10^{-6} g vitamin B_{12} and 6 mL of P-IV solution (Sigma Aldrich, Milan, Italy). The culture was aerated at a rate of 1000 cm^3/min with an air flow containing 2% (*v*/v) carbon dioxide. Growth conditions were monitored by optical density (OD) measurements at 625 nm using a UV–Vis spectrophotometer (Lambda 25 model, Perkin Elmer, Milan, Italy). The pH of the culture medium was monitored during the experiments using a pH meter (pH 211, HANNA Instruments, Woonsocket, RI). Microalgae were harvested during the end of the exponential phase at a biomass concentration of about 3 g_{DW}/L of suspension and then concentrated by centrifugation (centrifuge model 42426, ALC, Milan, Italy) at 4000 ×*g* for 10 min at 20 °C up to a final concentration of 12 g_{DW}/L. The concentrated biomass was pre-packed in high-density polyethylene bottles (Nalgene) cooled at 4 °C, and sent to the laboratories of ProdAl Scarl (University of Salerno, Italy). Samples were transported in an EPS box under refrigerated conditions and delivered within 24 h. PEF and HPH treatments were performed on the delivery day. The initial electrical conductivity of algae suspension was about 1.78 ± 0.03 mS/cm at 25 °C (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy).

2.2. PEF treatment

PEF treatments were conducted in a bench-scale continuous flow PEF unit, described in detail in a previous work [6]. Briefly, the unit consisted of a peristaltic pump to control the flow rate of the algae suspension through the system. The inlet temperature of the algae suspension was controlled using a stainless steel coil immersed in a water heating bath. The PEF treatment zone consisted of two modules, each made of two co-linear cylindrical treatment chambers, hydraulically connected in series, with an inner radius of 1.5 mm and a gap distance of 4 mm. The treatment chambers were connected to the output of a high voltage pulsed power (20 kV–100 A) generator (Diversified Technology Inc., Bedford, WA, USA) able to deliver monopolar square pulses (1–10 μs, 1–1000 Hz). The maximum electric field intensity (E, in kV/cm) and total specific energy input (W_T, in kJ/kg_{susp}) were measured and calculated as reported in Postma et al. [6]. T-thermocouples were used to measure the product temperature at the inlet and outlet of each module of the PEF chamber.

During PEF treatment, the algae suspension (12 g_{DW}/L) was pumped, from a feeding tank under stirring, through the treatment chamber at a constant flow rate of 33.3 mL/min. The pulse length was fixed at 5 μs, while the electric field strength (E) of 10, 20 and 30 kV/cm and total specific energy input (W_T) of 20, 60, and 100 kJ/kg_{susp} were set by varying the applied voltage and the pulse repetition frequency, respectively. All the experiments were carried out at an inlet temperature of each module of PEF chamber of 25 °C, while the maximum temperature increase at the exit of each module due to Joule effect never exceeded 10 °C.

At the exit of the treatment chamber, treated and untreated (without applying PEF treatment) algae suspensions were collected in plastic tubes and placed in an ice water bath to be rapidly cooled up to a final temperature of 25 °C before undergoing the aqueous extraction process.

2.3. HPH treatment

HPH treatments were carried out by using an in-house developed laboratory scale high-pressure homogenizer [34]. The *C. vulgaris* suspensions, at the same concentration as for PEF treatment tests (12 g_{DW}/L), were forced to pass through a 100 μm diameter orifice valve (model WS1973, Maximator JET GmbH, Schweinfurt, Germany) upon pressurization by means of an air driven Haskel pump (model DXHF-683, EGAR S.r.l., Milan, Italy). The pressure drop across the orifice and the volumetric flow rate of the suspension were 150 MPa and 155 mL/min, respectively. In this work, the algae suspensions were treated with a different number of passes (n_P = 1–10). In order to prevent excessive heating, after each pass, the suspensions were cooled at 25 °C by passing through a tube-in-tube heat exchanger, located downstream of the orifice valve.

2.4. Water extraction

After processing, untreated and treated (PEF, HPH) samples were allowed to stand for 1 h at 25 °C under shaking at 160 rpm to allow intracellular components to diffuse out of the cells. After this resting time, the cell suspensions were centrifuged (10 min, 5700 × *g*) (PK121R model, ALC International, Cologno Monzese, IT) and the supernatants were transferred to fresh tubes and stored at −20 °C until further analysis.

2.4.1. Electrical conductivity measurement

Changing of the electrical conductivity (σ) of untreated and treated (PEF, HPH) algae suspensions was monitored periodically (Conductivity meter HI 9033, Hanna Instrument, Milan, Italy) over time for up to 24 h by maintaining the samples in a water bath set at a constant temperature of 25 °C.

The collected data were used also to evaluate (Eq. (1)) the cell disintegration index (Z_p), which has been successfully used as a reliable macroscopic indicator of the degree of cell membrane permeabilization induced by PEF [35,36]:

$$Z_P = \frac{\sigma_{PEF,t} - \sigma_0}{\sigma_{MAX} - \sigma_0} \quad (1)$$

where $\sigma_{PEF,t}$ is the electrical conductivity of PEF treated biosuspensions measured at time t, σ_0 is the conductivity of untreated algae suspension at time 0, and σ_{MAX} is the conductivity of biosuspension with completely disrupted algae cells (HPH treatment: p = 150 MPa, n_P = 5). The Eq. (1) gives Z_p = 0 for intact algae cells and Z_p = 1 for fully disrupted cells.

2.4.2. Particle size distribution (PSD) analysis

PSD of untreated and treated (PEF or HPH) algae suspensions were analyzed by laser diffraction at 25 °C, using a MasterSizer 2000 particle size analyzer (Malvern, United Kingdom). Using the Fraunhofer approximation, which does not require the knowledge of the optical properties of the sample, the size distribution of the algal suspension was determined, from which the mean particle size expressed as volume moment mean diameter ($D_{4,3}$) was evaluated for each processing condition. The parameters used in the determination of the PSD were the properties of water at 25 °C (refraction index = 1.33), which was used as dispersant medium.

2.4.3. Scanning electron microscopy (SEM) analysis

The morphological features and cellular details of algae cells were analyzed by using a Scanning Electron Microscopy (SEM). Pellets derived from the centrifugation of untreated and treated (PEF or HPH) algae suspensions were prepared as described by Kunrunmi et al. [37] with some modifications. At first, samples were fixed by immersion in a 2% (v/v) glutaraldehyde phosphate buffer solution. The buffer was then removed and the pellets were osmotically dehydrated with ethanol solutions of increasing concentration (25%, 50%, 75%, and 100% (v/v)). Afterwards, ethanol was removed from the pellet with supercritical CO_2 in a Quorum K850 critical point dryer (Quorum Technologies Ltd., London, UK) and the latter was then metallized by means of the Agar Auto Sputter Coater 103A (Agar Scientific Ltd., Stansted, UK), before being analyzed in a high-resolution ZEISS HD15 Scanning Electron Microscope (Zeiss, Oberkochen, Germany).

2.4.4. Dry matter (DM) content analysis

Approximately 40 mL of the supernatants collected from the centrifugation of untreated and treated (PEF or HPH) algae suspension were placed in aluminum cups and dried in an oven (Heraeus, Germany) at 80 °C until constant mass was achieved. DM was gravimetrically determined by weighing the samples before and after drying on an analytical balance (Gibertini, Italy). The dry mass content was expressed as g of dry matter/kg of supernatant ($g_{DW}/kg_{SUP.}$).

2.4.5. Proteins analysis

The water-soluble protein concentration in the supernatants was evaluated using the Lowry method [38], with some modifications. The Folin-Ciocalteau reactive [39], purchased from Sigma Aldrich (Milan, Italy), was initially diluted in two volumes of ultra-pure water (1:2, v/v); then 0.5 mL of the diluted reactive were added to 1 mL of supernatant, previously mixed with 5 mL of the reactive "C" [50 volumes of reactive "A" [(2% (w/v) Na_2CO_3 + 0,1 N NaOH) + 1 volume of reactive "B" (1/2 volume of 0.5% (w/v) $CuSO_4{\cdot}5H_2O$ + 1/2 volume of 1% $KNaC_4H_4O_6{\cdot}4H_2O$)] (Sigma Aldrich, Milan, Italy). Absorbance was measured at 750 nm against a blank (5 mL reactive 'C' + 1 mL deionized water + 0.5 mL Folin-Ciocalteau reactants) 35 min after the start of the chemical reaction by using a V-650 Spectrophotometer (Jasco Inc. Easton, MD, USA). Bovine serum albumin (BSA) (A7030, Sigma Aldrich, Milan, Italy) was used as standard and the results were expressed as mg equivalent of BSA per g of dry biomass.

2.4.6. Carbohydrates analysis

The total carbohydrates concentrations of the supernatants were analyzed according to the method of DuBois et al. [40]. 0.2 mL of 5% (w/w) phenol and 1 mL of concentrated sulfuric acid (Sigma Aldrich, St. Louis, USA) was added to 0.2 mL of diluted supernatant (Dilution Factor = 5). Samples were incubated at 35 °C for 30 min before reading the absorbance at 490 nm against a blank of 0.2 mL 5% (w/w) phenol, 1 mL concentrated sulfuric acid and 0.2 mL of deionized water. D-Glucose (G8270, Sigma-Aldrich, Milan, Italy) was used as a standard and the results were expressed as equivalent mg of D-glucose per g of dry biomass.

2.5. Statistical analysis

All treatments and analyses were performed in triplicate and the results were reported as mean values with their respective standard deviations (SD). Statistically significant differences ($p \leq 0.05$) between the means were evaluated using one-way analysis of variance (ANOVA), performed with SPSS 20 (SPSS Inc., Chicago, USA) statistical package, and the Tukey's test.

3. Results and discussion

3.1. Impact of PEF and HPH treatments on the release of ionic intracellular components

The results of the measurements of the electrical conductivity of microalgae suspension have been successfully used as a valuable indicator to assess and quantify the amount of ionic intracellular components released from algae upon the application of the different cell disruption methods [9,14,18,41].

Fig. 1 shows the effect of PEF treatment intensity (E, W_T), as well as

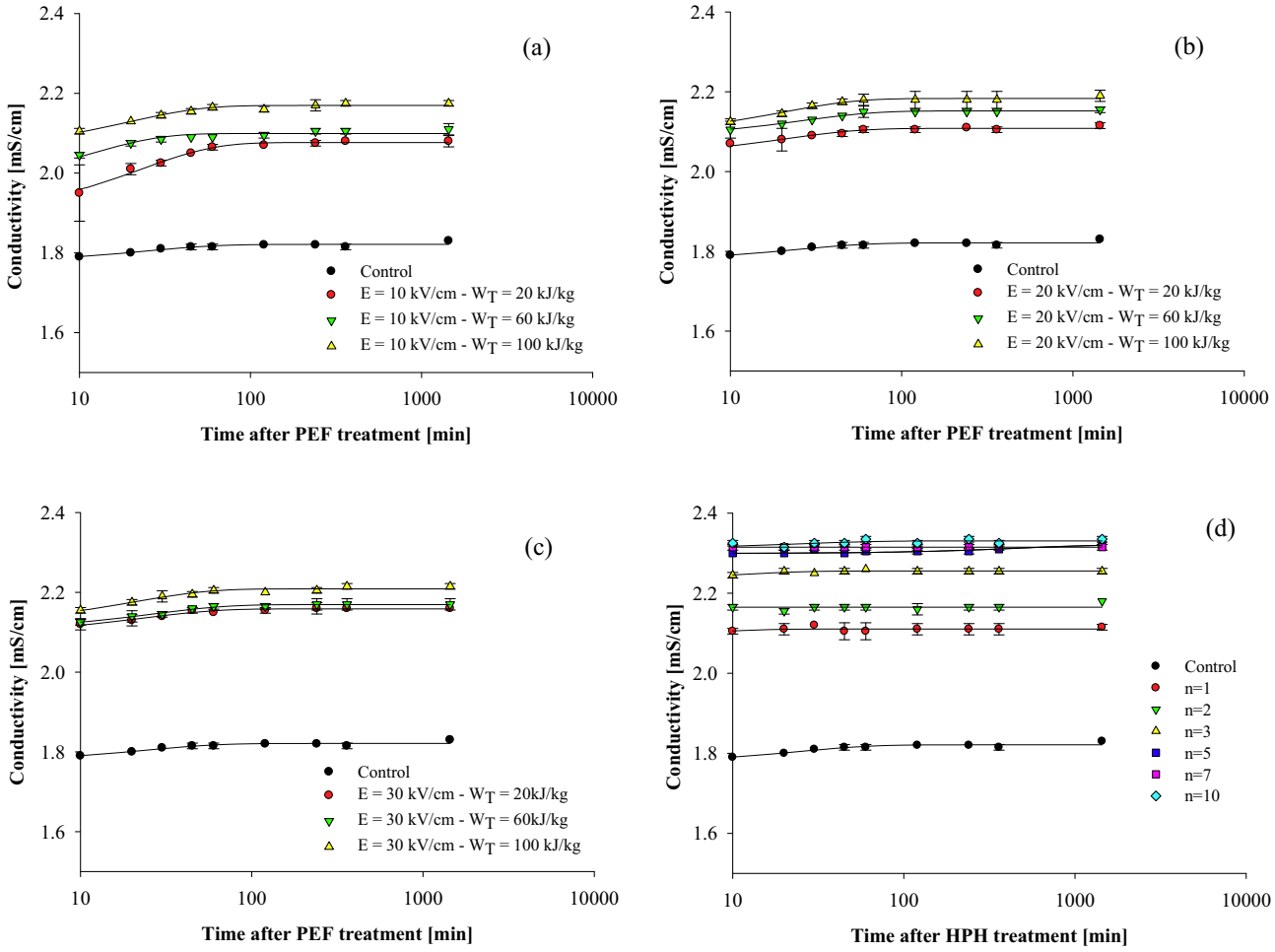

Fig. 1. Effect of incubation time after PEF and HPH treatment on electrical conductivity at 25 °C of (a–c) PEF (E = 10–30 kV/cm; W_T = 20–100 kJ/kg) and (d) HPH (150 MPa; np = 1–10) treated *C. vulgaris* suspension at a different number of passes. Control means untreated suspension. Data shown is the mean ± SD, n = 9.

the number of HPH passes (n_P) on the conductivity profiles of *C. vulgaris* suspensions over time at 25 °C.

For the sake of comparison, in the same graphs, also the time-conductivity profile of the untreated algae suspension is shown. Results demonstrate that the initial conductivity (1.78 mS/cm) of untreated suspension increased only slightly with the incubation time, likely due to a spontaneous release of a small fraction of intracellular ionic compounds, reaching a saturation value (1.82 mS/cm) already after 30 min of incubation.

The electroporation effect induced by the application of PEF treatment at different field strength (10–30 kV/cm) and energy input (20–100 kJ/kg) promoted a rapid release of the ionic intracellular compounds, which resulted in a substantial increase in the electrical conductivity, with respect to the untreated suspension (Fig. 1a–c). After PEF treatment, the saturation value, reached after 1 h of incubation, increased with the increase of the field strength and energy input, due to a faster diffusion of the ionic intracellular substances into the aqueous phase, which is in agreement with the electroporation theory. A further increase of the incubation time did not cause any significant increase in the conductivity value, which leveled off to a final value in the range between 2.08–2.21 mS/cm, depending on the PEF treatment intensity applied.

A progressive increase of the content of ionic compounds in the extracellular medium when increasing the intensity of the PEF treatment was also observed by Goettel et al. [18], which also reported that 79% of the total released ions from *Auxenochlorella protothecoides* already occurred in the first hour after treatment. Similarly, Postma et al. [6] and Pataro et al. [9] reported that increasingly intense PEF treatments promoted the progressive permeabilization of the *C. vulgaris* cells, and that an incubation time of 1 h was sufficient to allow small ions to diffuse out of the cells, in agreement with the results reported in Figs. 1a–c.

The data of Figs. 1a–c suggest the achievement of an irreversible electroporation after PEF treatment [18], by markedly improving the mass transfer rate of ionic compounds through the cell structure, which is partially damaged by the electrical treatment.

Coherently with this assumption, when compared to PEF treatments, the HPH treatments resulted in a significant increase in the conductivity of *C. vulgaris* suspension, whose extent was greater when increasing the number of HPH passes, as shown in Fig. 1d. More specifically, the mechanical disruption of the algae cells appeared to be extremely fast, leading to an almost instantaneous diffusion of the intracellular compounds into the aqueous phase, as observed also by Safi et al. [26].

Considering that HPH is a purely mechanical on-off disruption process, it is likely that after each pass a certain fraction of algae cells are completely broken, while the residual cells remain intact, in agreement with the observation of the significant extraction yield of ionic compounds after the multi-pass HPH treatment, as reported in Fig. 1d.

Coherently, the results of Fig. 1d also show that above 5 passes, the conductivity did not change significantly, and tended to an asymptotic value of 2.3 mS/cm, because the residual fraction of intact cells has become extremely small. However, such asymptotic value was

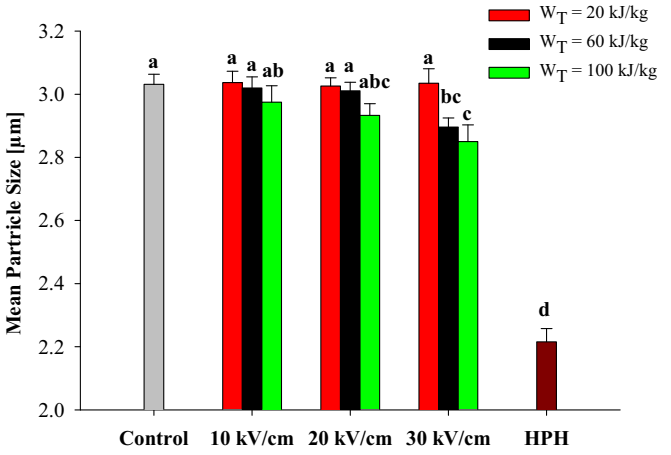

Fig. 2. Mean particle size of untreated (control), PEF treated (E = 10–30 kV/cm; W_T = 20–100 kJ/kg) and HPH treated (p = 150 MPa; n_P = 5) *C. vulgaris* suspension. Different letters above the bars indicate significant differences among the mean values of the samples ($p \leq 0.05$). Data shown is the mean ± SD, n = 9.

significantly higher than that measured after the application of the most intense PEF treatment, confirming that the release of ionic compounds by PEF is incomplete.

Thus, setting the conditions of Z_P = 1 in correspondence of 5 HPH passes, the cell disintegration efficiency of PEF varied in a range dependent on the treatment intensity applied: the lowest value of Z_P (0.47) was observed for a PEF treatment intensity of 10 kV/cm and 20 kJ/kg, whereas, increasing the electric field strength and energy input, a maximum Z_P value of 0.85 was recorded.

3.2. Effect of PEF and HPH treatment on C. vulgaris cell structure

In this work, particle size distribution (PSD) analyses and SEM observations were carried out in order to gain insight on the impact of PEF and HPH treatments on the size and structure of *C. vulgaris* cells.

Fig. 2 depicts the mean particle size $D_{4,3}$ for untreated (control), PEF treated at variable field strength and energy inputs, and HPH (n_P = 5) treated microalgae suspensions.

The PSD curves of untreated algae suspension revealed the presence of a single peak between 1 and 10 µm (data not shown), which was characterized by a mean cell size of 3.03 ± 0.03 µm (Fig. 2).

The size distribution curve of PEF-treated algae suspension was very similar to that of the untreated sample (data not shown), showing only a slight decrease of the mean cell size with increasing the treatment intensity (E and W_T). In fact, the value of the mean cell size significantly ($p \leq 0.05$) decreased by about 6% only upon the application of the most intense PEF treatment conditions (E = 30 kV/cm, $W_T \geq 60$ kJ/kg) (Fig. 2). These results seem to confirm that PEF is a relatively mild cell disruption method, preserving the initial structure of the algae cells.

The application of 5 HPH passes, instead, led to a significant change in the PSD curves of the microalgae suspension, highlighting a bimodal distribution, in which a second peak between 0.1 and 1 µm appeared (data not shown). As a result, a strong reduction in the mean cell size down to a value of 2.22 ± 0.04 µm was observed (Fig. 2), which is likely due to the complete cell disruption and the consequent formation of cell debris.

Partially in contrast with these results, Spiden et al. [42] found that the effect of an HPH treatment on *Chlorella* microalgae at different pressures (p = 30–107 MPa) only led to a slight decrease in the mean cell size, which was in agreement with the only partial fragmentation achieved. Eventually, in our case, the application of a higher pressure (p = 150 MPa) was capable of inducing the complete disruption of the cells, which is in agreement with the previous findings of Safi et al. [28]. Similarly, Shene et al. [27] and Samarasinghe et al. [17], studying the effect of HPH processing (p = 70–310 MPa, n_P = 1–6) on *Nannochloropsis oceanica* microalgae, reported that the cells were fully disrupted in fragments, with a corresponding decrease in mean particle size.

In order to better interpret the results of Figs. 1 and 2, also SEM analyses were carried out on untreated, PEF-treated (E = 20 kV/cm; W_T = 20–100 kJ/kg), and HPH-treated (n_P = 5) microalgae, as shown in Fig. 3.

Untreated *C. vulgaris* cells exhibited their characteristic near-spherical shape and a diameter ranging from 1.5 and 4.5 µm, which relate to the findings reported in the current literature [43].

The SEM images of Fig. 3 clearly show the different impact of PEF and HPH treatments on the microalgal cell structure. Interestingly, the results clearly show, for the first time, the occurrence of a shrinkage phenomenon in PEF-treated algae cells, which, gradually lose their initial near-spherical shape with increasing the applied energy input but were never disintegrated into cell debris. The observed shrinkage could be associated with the partial release of the intracellular compounds through the electroporated cell membranes (Fig. 1b), which led in some cases to cell collapse (Fig. 3). Similar results were observed at different electric field strengths (data not shown).

In contrast, a complete disruption of the cells and the formation of small fragments was observed after 5 passes HPH treatment, which was consistent with the results of Figs. 1 and 2.

Similarly, the formation of cell fragments was observed by other authors upon the application of HPH treatments to *Chlorella* [26,44] and *Neochloris abundans* [45] microalgae, highlighting the strong efficacy of HPH treatment as a method of complete cell disruption.

3.3. Influence of PEF and HPH treatments on the release of intracellular compounds

The cell disruption efficiency of PEF and HPH treatments were also compared by monitoring the extractability of intracellular compounds by dry matter analyses and by measuring the amount of water-soluble compounds (proteins and carbohydrates) released into the supernatants obtained from untreated and treated (PEF, HPH) algae suspension.

3.3.1. Dry matter of supernatants

The total amount of released intracellular compounds was evaluated by measuring the dry matter content in the supernatant of untreated, PEF-treated at different field strength and energy inputs, and HPH-treated (n_p = 5) microalgae suspensions.

The results showed in Fig. 4 are in agreement with the conductivity measurements of Fig. 1. The application of PEF treatment markedly increased the dry matter content of supernatants, when compared with the untreated sample. A higher field strength and energy inputs resulted in a higher degree of membrane permeabilization, leading to a significantly ($p \leq 0.05$) higher release of intracellular compounds into the aqueous phase. The maximum value of dry matter content was detected at the most intense PEF treatment conditions (E = 30 kV/cm; W_T = 100 kJ/kg), which was 2.4 times higher than that detected in the supernatant of the untreated microalgae suspension. However, among PEF treated samples, statistically significant differences ($p < 0.05$) were observed only between samples treated at 10 kV/cm and 20 kJ/kg with those treated either at 20 kV/cm and 100 kJ/kg or at 30 kV/cm for an energy input > 20 kJ/kg. Remarkably, the results of Fig. 4 are in agreement with the previous findings of Goettel et al. [18]. The authors observed a continuous increase of cell components in the medium surrounding *Auxenochlorella protothecoides* when the energy input was increased up to 200 kJ/kg at a constant field strength (34 kV/cm). Moreover, in our case, the release of intracellular soluble compounds by PEF varied in the range 13–18% of total cell dry weight, which is also in agreement with the results obtained by Goettel et al. [18], who found that a PEF treatment at 30.5 kV/cm and 155 kJ/kg caused the spontaneous release of intracellular matter up to 15% of the initial biomass

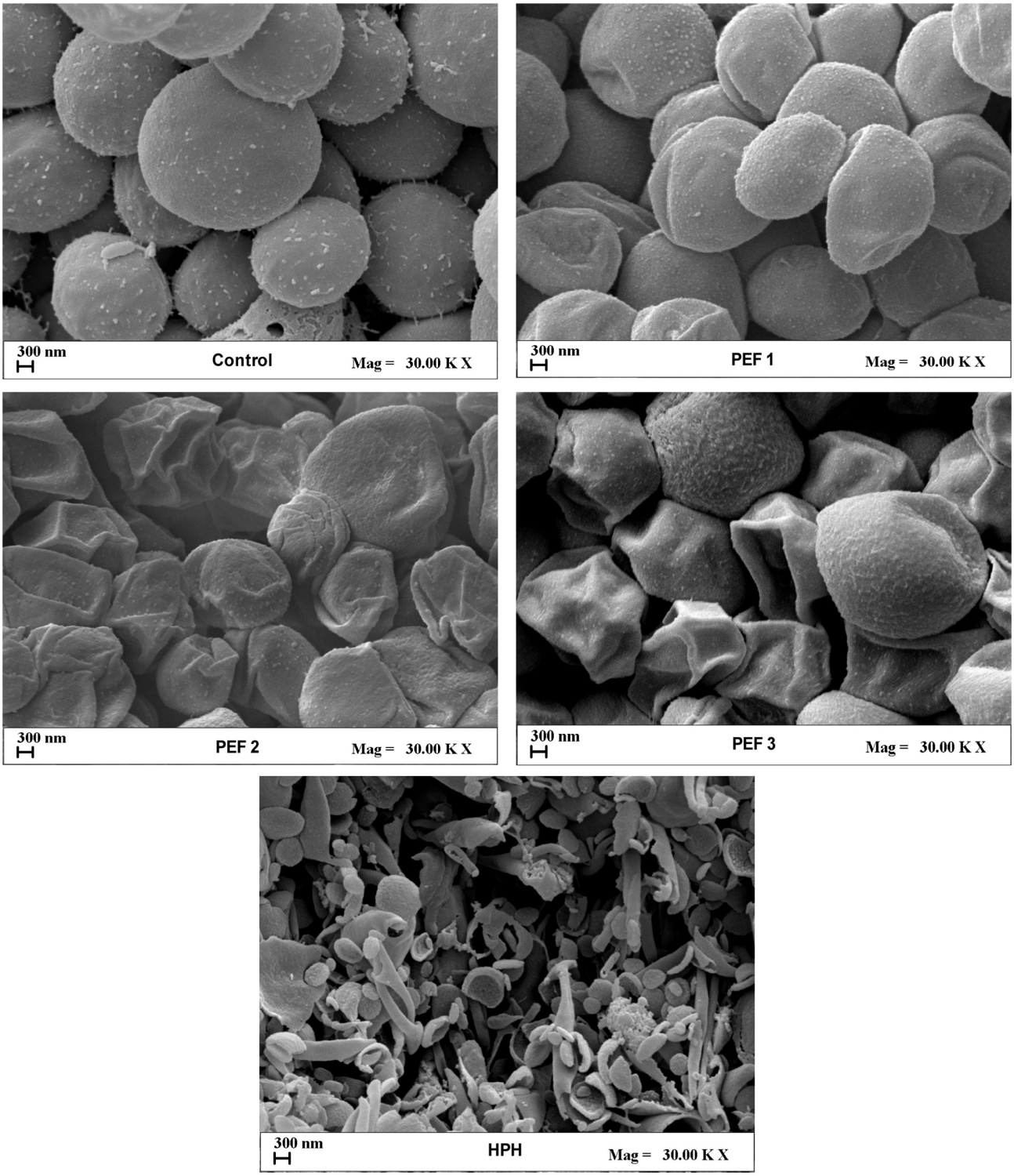

Fig. 3. Scanning electron microscopy (SEM) of *C. vulgaris* cells before (Control) and after PEF (20 kV/cm) at total specific energy input of 20 kJ/kg (PEF1), 60 kJ/kg (PEF2), 100 kJ/kg (PEF3), and HPH (p = 150 MPa; n_p = 5) treatment of the microalgal suspension.

dry weight (109 g/kg_{DW}). Pataro et al. [9] also observed a slightly higher leakage of intracellular matter from *C. vulgaris* cells with increasing the field strength (from 27 to 35 kV/cm) and energy input (from 50 to 150 kJ/kg).

The stronger cell disintegration effect, achieved after 5 passes HPH treatment (Figs. 1-3), led to a highly efficient extraction of intracellular matter (Fig. 4), whose extent reached up to 64% of the total cell dry weight.

The results of Fig. 4 were also confirmed by visual observation of the supernatants. In fact, while the supernatants obtained from centrifugation of fresh and PEF treated microalgal suspensions appeared colorless, those obtained from HPH treated samples were characterized by a green color (data not shown). This was likely due to the presence of cell debris (Fig. 3) containing green pigments, which, being extremely reduced in size, did not precipitate in the pellet after centrifugation [26]. With this assumption, it can be stated that part of the supernatant

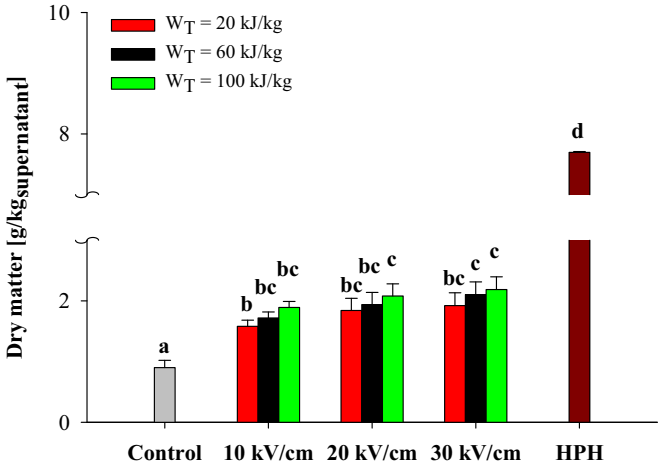

Fig. 4. Dry matter content in the supernatant of untreated (Control) and treated *C. vulgaris* suspension 1 h after PEF (E = 10–30 kV/cm; W_T = 20–100 kJ/kg) or after HPH (p = 150 MPa; n_P = 5) treatment. Different letters above the bars indicate significant differences among the mean values of the samples ($p \leq 0.05$). Data shown is the mean ± SD, n = 9.

dry matter content from the HPH treated cells could be due to the presence of submicrometric residues, which remained suspended in the aqueous phase, making the downstream separation processes more difficult.

3.3.2. Extractability of carbohydrates and proteins

Fig. 5 shows the concentration (on DW basis) of carbohydrates (Fig. 5a) and proteins (Fig. 5b) detected in the aqueous supernatant obtained 1 h after PEF treatment of *C. vulgaris* suspensions at different field strength and energy input.

When no PEF treatment was applied, only very low amounts of carbohydrates (7.06 mg/g_{DW}) and proteins (1.65 mg/g_{DW}) were released in the aqueous phase, which may be ascribed to either a concentration gradient across the intact cell membranes or to a spontaneous cell lysis.

The permeabilization effect of the cell membranes induced by the application of PEF treatment, instead, improved the mass transfer of intracellular compounds, leading to a significantly ($p \leq 0.05$) higher content of both carbohydrates and proteins, as compared to the untreated samples, being the extraction efficiency increased up to 20-fold for proteins and 8-fold for carbohydrates.

Among the PEF treated samples, the effect of the field strength applied (Fig. 5) appeared less important than that of the energy input within the investigated range, especially for the protein extraction, which is in agreement with previous findings [9,41]. In particular, a significant ($p \leq 0.05$) increase in the content of both intracellular compounds was detected only when the field strength was increased from 10 to 20 kV/cm and for a fixed energy input of 100 kJ/kg for proteins, and 20 kJ/kg for carbohydrates, respectively. In contrast, while significant differences ($p \leq 0.05$) in the protein content were detected when PEF treatments were carried out at different energy inputs (Fig. 5a), regardless of the field strength applied, only a slighter effect of the energy input was observed for the extraction of carbohydrates, which was significant ($p \leq 0.05$) only when the energy input was increased from 20 to 60 kJ/kg at 10 kV/cm and between 20 and 100 kJ/kg at 30 kV/cm (Fig. 5b).

A slightly increasing trend when increasing the energy input from 50 to 150 kJ/kg was previously observed by both Goettel et al. [18] with the microalgae *A. protothecoides* at a fixed field strength applied of 34 kV/cm, and Pataro et al. [9] with the microalgae *C. vulgaris* at a fixed field strength applied of 27 kV/cm. Postma et al. [6], instead, did not find any significant difference in the release of carbohydrates from *C. vulgaris* treated by PEF at 50 and 100 kJ/kg at 17.1 kV/cm.

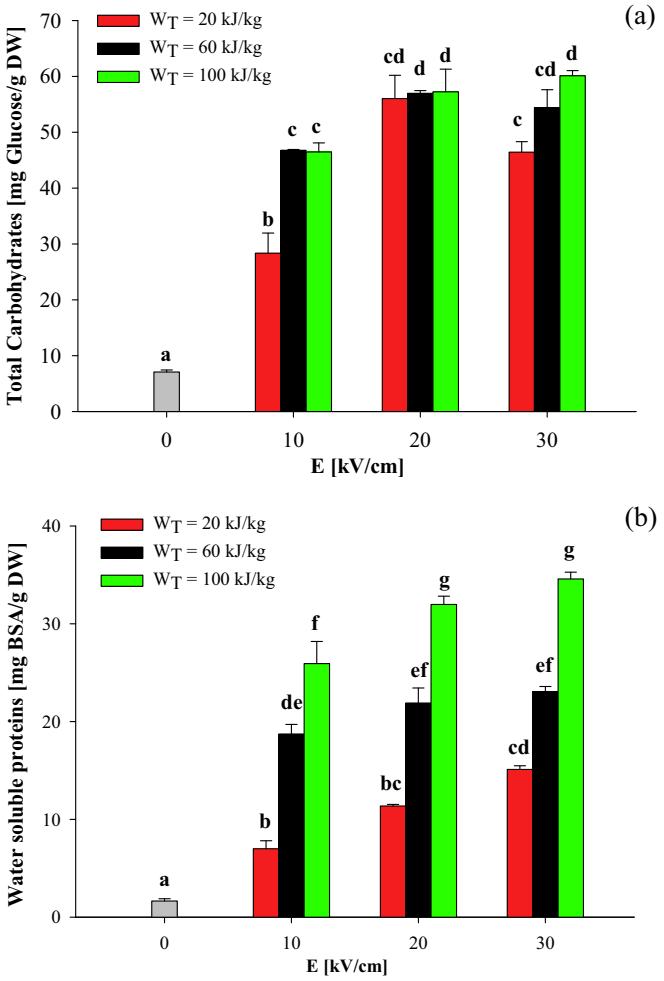

Fig. 5. Concentration of carbohydrates (a) and proteins (b) in the supernatant of untreated (0 kV/cm) and treated *C. vulgaris* suspension 1 h after PEF treatment as a function of the field strength and for different energy input. Different letters above the bars indicate significant differences among the mean values of the samples ($p \leq 0.05$). Data shown is the mean ± SD, n = 9.

From the results of Fig. 5 it can be concluded that a field strength of 20 kV/cm and an energy input of 100 kJ/kg could be sufficient to achieve efficient protein and carbohydrates extraction by PEF.

In particular, assuming a carbohydrates and proteins content of 16% and 61% on DW, respectively [6], the amount of these compounds released after PEF treatment (20 kV/cm, 100 kJ/kg) was 35.8% (w/w) of total carbohydrates (approximately 5.7% DW biomass) and 5.2% (w/w) of total proteins (approximately 3.2% DW biomass). These values are in the same range of values reported by other authors [6,12,13,22,28]. In the study of Postma et al. [6], for example, it was observed that the application of a PEF treatment at room temperature resulted in an extraction yield of 22–24% for carbohydrates, and 3.2–3.6% for proteins, when the energy input was increased between 50 and 100 kJ/kg at a field strength applied of 17.1 kV/cm. Moreover, no further improvement of the diffusion kinetics of intracellular compounds was detected when PEF effect was combined with the thermal treatments at a higher temperature [6] or elevated pH [23].

These results suggest that PEF was successful in opening pores on membranes of *C. vulgaris* cells (Figs. 1, 3), allowing the selective release of carbohydrates and small-sized cytoplasmic proteins, while hindered simultaneously the diffusion of most proteins, which are likely larger and more bonded to the cell structure. This hypothesis is supported by some literature evidence. In fact, it has been reported that the proteins of *C. vulgaris* species have molecular weights ranging from 12 to

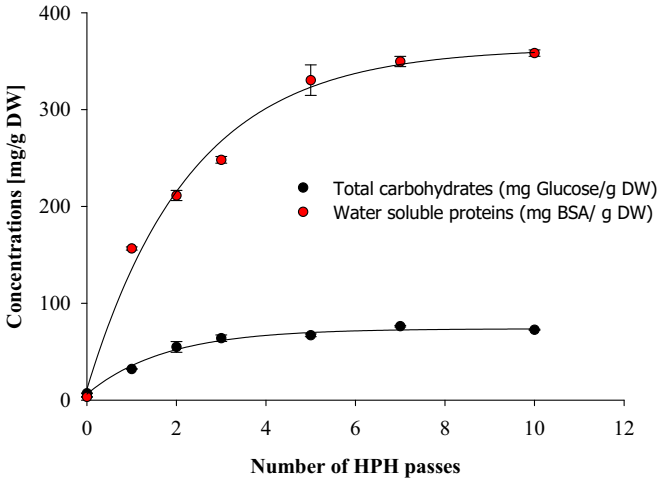

Fig. 6. Concentration of proteins and carbohydrates in the surpenatant of untreated ($n_P = 0$) and HPH ($p = 150$ MPa) treated *C. vulgaris* suspension as a function of the number of passages. Data shown is the mean ± SD, $n = 9$.

120 kDa [26], and that PEF was able to selectively enhance only the extraction of small protein materials, with molecular weight lower than 20 kDa, while larger molecules remained entrapped inside the cells, being unable to cross the permeabilized cell membrane [6]. In contrast, as suggested by the SEM images (Fig. 3), PEF merely electroporated the algae cells without altering the extremely resistant rigid cell wall of *C. vulgaris*, which represents a further barrier against the extraction of proteins [46]. Moreover, it is estimated that 20% of *C. vulgaris* proteins are bonded to the cell wall [47], and therefore they likely remained entrapped in the pellet along with the water-insoluble fraction of proteins. This would contribute to further explain the relatively low amount of proteins released after PEF (Fig. 5b).

Therefore, the disruption of the rigid cell wall of *Chlorella vulgaris* appears to be a crucial step to enhance the protein release [48], hence requiring a more effective cell disruption techniques, such as high pressure homogenization [10].

Fig. 6 reports the amount of carbohydrates and proteins released upon the application of HPH treatment (150 MPa) as a function of the number of passes. In agreement with the results of Fig. 1d, a significant fraction *C. vulgaris* cells was already disrupted after 1 pass and water gained the access to the cytoplasmatic content, allowing the release of a certain amount of carbohydrates and proteins.

The subsequent HPH passes led to the further release of carbohydrates and proteins, whose amount gradually increased up to reaching a saturation value after 5 passes, which was, with respect to the control sample, 9-fold higher for carbohydrates and 200-fold higher for proteins.

An asymptotic behavior in the extraction yield of intracellular compounds, such as chlorophyll and carotenoids, as a result of the increased degree of cell disruption with increasing the number of passes has previously been shown by Xie et al. [49]. These authors reported that the release of these pigments from HPH-processed *Desmodesmus* microalgae could be enhanced by increasing the number of passes up to a saturation value above which no additional leakage of interest compounds could be achieved.

From the results of Fig. 6, using the same assumption for the composition of *C. vulgaris* cells used for PEF [6], the amount of carbohydrates and proteins released after 5 HPH passes was 41.9% (w/w) of total carbohydrates (approximately 6.7% DW biomass) and 54.1% (w/w) of total proteins (approximately 33.0% DW biomass).

Similarly, Safi et al. [26,48] found that, among the different cell disruption techniques, including the chemical treatments, ultrasonication, and manual grinding, HPH was the most efficient one, and that after an HPH treatment ($p = 270$ MPa, $n_p = 2$) water gained rapid access to the cytoplasmic proteins and infiltrated the chloroplast to recover 50–66% of proteins from the total protein content of *C. vulgaris* cells. However, even from these results it appears that, despite the high cell disruption efficiency of the HPH treatment, the complete release of all the proteins contained in the algae could not be reached, because of the rigidity of the cell wall [50], as well as the insoluble nature of some proteins that remained in the pellet [51]. In this frame, it has been demonstrated that the combination of higher HPH pressure than that used in our work with chemical cell lysis could further improve the extractability of protein from algae cells. In particular, Ursu et al. [52] observed that 2 HPH passes at 270 MPa allowed the recovery of 98% of total protein content of the microalgae *C. vulgaris* when the pH of the suspension was maintained at 12.

The comparison between the results of Figs. 5 and 6 highlights the capacity of PEF to efficiently release low molecular weight molecules, such as carbohydrates, to an extent comparable to the one obtained from HPH treatment for a sufficiently high number of passes (85.4%). This selectivity of PEF toward the carbohydrates could be advantageously exploited for specific applications [41]. In contrast, despite the huge increase in protein extraction caused by PEF processing with respect to untreated microalgae suspension, the protein yields are still relatively low being 10 fold lower than that detected in HPH treated samples.

However, next to the extraction yield of valuable intracellular compounds, the feasibility of a cell disintegration technique should also take into account the total energy consumed. In this work, to enable the comparison between PEF and HPH, on the basis of the work of Günerken et al. [7], the total energy consumed (in kWh/kg_{DW}) was calculated as the energy to disrupt 1 kg of dry microalgae biomass (= consumed energy/(treated biomass·cell disruption yield)), considering a cell disruption yield of, respectively, 100% for 5 passages HPH treatment ($Z_P = 1$), and 81% ($Z_P = 0.81$) for PEF treatment (20 kV/cm, 100 kJ/kg). For HPH, an overall efficiency of the pumping system of 87% was considered.

The results showed that, at the low solids concentration used in this work (1.2%, w/w), HPH is always an energy intensive cell disintegration technique, with a total consumed energy 20.0 kWh/kg_{DW}, whereas PEF, despite the lower yields is characterized by a total consumed energy of 2.9 kWh/kg_{DW}. These results are in contrast with the findings of Safi et al. [28], who demonstrated that PEF was energetically less efficient (10.42 kWh/kg_{DW}) than HPH (0.32 kWh/kg_{DW}) after only one passage at 100 MPa when applied for the recovery of proteins from suspensions of *Nannochloropsis gaditana* microalgae with a cell concentration of, respectively, 60 g/L and 100 g/L. Probably, this difference can be somehow explained in terms of the peculiarity of the tested microalga, the different biomass concentrations as well as on the different PEF and HPH systems. For example, in agreement with previous findings [53], it is likely that the energy efficiency of the continuous flow PEF system used in the present work is higher than that of the batch chamber (electroporation cuvette with a maximum capacity of 400 μL) used in the work of Safi et al. [28]. On the other hand, it has been reported that processing biomass with higher solid concentrations than the diluted suspension used in our work, could positively affect the energy efficiency of both HPH and PEF treatment. To this regard, for example, when Yap et al. [15] processed suspensions of *Nannochloropsis* sp. by HPH at different concentrations, they found the same extent of cell rupture, but the energy demand of HPH was about 28 kWh/kg_{dw} at 0.25% (w/w) solids and 0.28 kWh/kg_{dw} at 25% (w/w) solids. Moreover, they also demonstrated that large scale HPH equipment is considerable more energy efficient than lab-scale apparatus. Thus, from these results it appears that processing of concentrated algae biomass using large scale HPH equipment could require up to 10 fold less energy than that required in our experiments where diluted suspensions were processed in a lab-scale PEF unit.

On the other hand, it has been also reported that the energy demand of PEF could be reduced by increasing the biomass content of the

suspension. For example, Goettel et al. [18] using a lab-scale PEF unit found that for an algae suspension containing 100 g_{dw}/kg_{sus} algae the energy demand was 0.44 kWh/kg_{dw}, while for a suspension containing 167 g_{dw}/kg_{sus} algae, the energy demand of PEF was reduced up to 0.25 kWh/kgdw. Similarly, Safi et al. [28] found that increasing the biomass concentration from 45 to 60 g/L resulted in an almost double amount of released proteins (from about 5% w/w to 10% w/w).

Thus, as previously observed for HPH [15], it cannot be excluded that also for PEF the processing of high biomass concentration could positively affect the extraction yield of intracellular compounds and reduce the energy requirements per unit biomass. Further research is, therefore, needed in order to achieve for both PEF and HPH optimal conditions in terms of extraction yield and energy consumption as well as to achieve a more general conclusion about the energy efficiency of PEF and HPH.

Moreover, for the first time, the comparison between PEF and HPH has also been carried out in terms of the energy consumed to extract 1 kg of carbohydrates or proteins, which were, respectively, 40.5 kWh/kg of glucose equivalent and 72.3 kWh/kg of BSA equivalent for PEF, and 311.8 kWh/kg of glucose equivalent and 60.4 kWh/kg of BSA equivalent for HPH. Obviously the validity of this analysis is confined to the range of solids concentration used in these experiments (1.2% w/w). The estimated energy consumptions apparently show that the carbohydrates can be recovered through PEF treatment at comparable yields with HPH, but with higher purity and lower energy consumption, with the perspective, in the case these results can be replicated at higher solid concentrations, of positively affecting the fractionation in the later biorefinery stages.

In the case of proteins, instead, HPH is more energetically efficient than PEF, because of the significantly higher yields. However, our results suggest that the PEF treatment offers the advantage of higher purity than HPH. In addition, further studies are required to investigate the effect of microalgae pretreatment on the molecular composition of the protein extract, considering that preliminary experiments, carried out at the same operating conditions, show that the extracts obtained by PEF and HPH treatments significantly differ in composition.

4. Conclusions

The present study provides additional insights into the impact of PEF and HPH treatments on the disintegration efficiency of *C. vulgaris* cells and into the subsequent recovery of intracellular compounds, namely carbohydrates and proteins.

PEF resulted in being a relatively mild cell disruption method, which merely electroporates the algae cells without the formation of any cell debris, allowing to selectively enhance the extraction yield of small ionic substances and carbohydrates to an extent comparable to that achieved by HPH. The extraction efficiency of proteins, instead, was relatively low and did not exceed 5.2% of the total.

HPH, instead, was able to disrupt completely the microalgae cells, favoring an instantaneous and efficient release of all the intracellular material, including a large amount of proteins, whose release was 10.3 fold higher than by PEF. However, despite the higher extraction efficiency, the formation of large amounts of finely sized cell debris by HPH significantly complicates any downstream separation process.

In the ongoing work, the optimal cell disruption conditions identified in this work for individual PEF ($E = 20$ kV/cm; $W_T = 100$ kJ/kg_{SUSP}) and HPH ($n_P = 5$) treatment, are tested in a cascade biorefinery, in order to maximize in a selective and sustainable way the extraction yield of target compounds, by reducing the overall processing costs, which nowadays represent the main bottleneck to the full exploitation of microalgal biomass.

References

[1] M. Yamamoto, M. Fujishita, A. Hirata, S. Kawano, Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae), J. Plant Res. 117 (2004) 257–264, http://dx.doi.org/10.1007/s10265-004-0154-6.

[2] M.F. Demirbas, Biofuels from algae for sustainable development, Appl. Energy 88 (2010) 3473–3480, http://dx.doi.org/10.1016/j.apenergy.2011.01.059.

[3] S.-H. Song, I.H. Kim, T.J. Nam, Effect of hot water extract of *Chlorella vulgaris* on proliferation of IEC-6 cells, Int. J. Mol. Med. 29 (2011) 741–746, http://dx.doi.org/10.3892/ijmm.2012.899.

[4] C. Safi, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Morphology, composition, production, processing and applications of *Chlorella vulgaris*: a review, Renew. Sust. Energ. Rev. 35 (2014) 265–278, http://dx.doi.org/10.1016/j.rser.2014.04.007.

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

[6] P.R. Postma, G. Pataro, M. Capitoli, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, G. Olivieri, G. Ferrari, Selective extraction of intracellular components from the microalga *Chlorella vulgaris* by combined pulsed electric field-temperature treatments, Bioresour. Technol. 203 (2016) 80–88, http://dx.doi.org/10.1016/j.biortech.2015.12.012.

[7] E. Günerken, E. D'Hondt, M.H.M. Eppink, L. Garcia-Gonzalez, K. Elst, R.H. Wijffels, Cell disruption for microalgae biorefineries, Biotechnol. Adv. 33 (2015) 243–260, http://dx.doi.org/10.1016/j.biotechadv.2015.01.008.

[8] E. Luengo, J.M. Martınez, A. Bordetas, I. Alvarez, J. Raso, Influence of the treatment medium temperature on lutein extraction assisted by pulsed electric fields from *Chlorella vulgaris*, Innov. Food Sci. Emerg. Technol. 29 (2015) 15–22, http://dx.doi.org/10.1016/j.ifset.2015.02.012.

[9] G. Pataro, M. Goettel, R. Straessner, C. Gusbeth, G. Ferrari, W. Frey, Effect of PEF treatment on extraction of valuable compounds from microalgae *C. vulgaris*, Chem. Eng. Trans. 57 (2017) 67–72, http://dx.doi.org/10.3303/CET1757012.

[10] M.M. Poojary, F.J. Barba, B. Aliakbarian, F. Donsi, G. Pataro, D.A. Dias, P. Juliano, Innovative alternative technologies to extract carotenoids from microalgae and seaweeds, Mar. Drugs 14 (2016) 1–34, http://dx.doi.org/10.3390/md14110214.

[11] C. Joannes, C.S. Sipaut, J. Dayou, S.M. Yasir, R.F. Mansa, The potential of using pulsed electric field (PEF) technology as the cell disruption method to extract lipid from microalgae for biodiesel production, Int. J. Renew. Energy Res. 5 (2015) 598–621.

[12] F.J. Barba, N. Grimi, E. Vorobiev, New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae, Food Eng. Rev. 7 (2015) 45–62, http://dx.doi.org/10.1007/s12393-014-9095-6.

[13] C. Grosso, P. Valentão, F. Ferreres, P.B. Andrade, Alternative and efficient extraction methods for marine-derived compounds, Mar. Drugs 13 (2015) 3182–3230, http://dx.doi.org/10.3390/md13053182.

[14] N. Grimi, A. Dubois, L. Marchal, S. Jubeau, N.I. Lebovka, E. Vorobiev, Selective extraction from microalgae *Nannochloropsis* sp. using different methods of cell disruption, Bioresour. Technol. 153 (2014) 254–259, http://dx.doi.org/10.1016/j.biortech.2013.12.011.

[15] B.H.J. Yap, G.J. Dumsday, P.J. Scales, G.J.O. Martin, Energy evaluation of algal cell disruption by high pressure homogenization, Bioresour. Technol. 184 (2015) 280–285, http://dx.doi.org/10.1016/j.biortech.2014.11.049.

[16] F. Donsì, M. Annunziata, G. Ferrari, Microbial inactivation by high pressure homogenization: effect of the disruption valve geometry, J. Food Eng. 115 (2013) 362–370, http://dx.doi.org/10.1016/j.jfoodeng.2012.10.046.

[17] N. Samarasinghe, S. Fernando, R. Lacey, W.B. Faulkner, Algal cell rupture using high pressure homogenization as a prelude to oil extraction, Renew. Energy 48 (2012) 300–308, http://dx.doi.org/10.1016/j.renene.2012.04.039.

[18] M. Goettel, C. Eing, C. Gusbeth, R. Straessner, W. Frey, Pulsed electric field assisted extraction of intracellular valuables from microalgae, Algal Res. 2 (2013) 401–408, http://dx.doi.org/10.1016/j.algal.2013.07.004.

[19] J. Raso, W. Frey, G. Ferrari, G. Pataro, D. Knorr, J. Teissie, D. Miklavcic, Recommendation guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes, Innov. Food Sci. Emerg. Technol. 37 ((2016) 312–321, http://dx.doi.org/10.1016/j.ifset.2016.08.003.

[20] Y.S. Lai, P. Parameswaran, A. Li, M. Baez, B.E. Rittmann, Effects of pulsed electric field treatment on enhancing lipid recovery from the microalga, Scenedesmus, Bioresour. Technol. 173 (2014) 457–461, http://dx.doi.org/10.1016/j.biortech.2014.09.124.

[21] M.D.A. Zbinden, B.S.M. Sturm, R.D. Nord, W.J. Carey, D. Moore, H. Shinogle, S.M. Stagg-Williams, Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae, Biotechnol. Bioeng. 110 (2013) 1605–1615, http://dx.doi.org/10.1002/bit.24829.

[22] E. Luengo, J.M. Martınez, M. Coustets, I. Alvarez, J. Teissie, M.P. Rols, J. Raso, A comparative study on the effects of millisecond and microsecond-pulsed electric field treatments on the permeabilization and extraction of pigments from *Chlorella vulgaris*, J. Membrane Biol. (2015) 883–891, http://dx.doi.org/10.1007/s00232-015-9796-7.

[23] O. Parniakov, J.F. Barba, N. Grimi, L. Marchal, S. Jubeau, N. Lebovka, E. Vorobiev, Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae Nannochloropsis, Algal Res. 8 (2015) 128–134, http://dx.doi.org/10.1016/j.algal.2015.01.014.

[24] F. Donsì, G. Ferrari, E. Lenza, P. Maresca, Main factors regulating microbial inactivation by high-pressure homogenization: operating parameters and scale of operation, Chem. Eng. Sci. 64 (2009) 520–532, http://dx.doi.org/10.1016/j.ces.

2008.10.002.
[25] F. Donsì, G. Ferrari, P. Maresca, High-pressure homogenisation for food sanitisation, in: G.V. Barbosa-Canovas, D. Lineback, A. Mortimer, W. Spiess, K. Buckle, P. Colonna (Eds.), Global Issues in Food Science and Technology, Elsevier, 2009, pp. 309–352, , http://dx.doi.org/10.1016/B978-0-12-374124-0.00019-3.
[26] C. Safi, C. Frances, A.V. Ursu, C. Laroche, C. Pouzet, C. Vaca-Garcia, P.Y. Pontalier, Understanding the effect of cell disruption methods on the diffusion of *Chlorella vulgaris* proteins and pigments in the aqueous phase, Algal Res. 8 (2015) 61–68, http://dx.doi.org/10.1016/j.algal.2015.01.002.
[27] C. Shene, M.T. Monsalve, D. Vergara, M.E. Lienqueo, M. Rubilar, High pressure homogenization of Nannochloropsis oculata for the extraction of intracellular components: effect of process conditions and culture age, Eur. J. Lipid Sci. Technol. 118 (2016) 631–639, http://dx.doi.org/10.1002/ejlt.201500011.
[28] C. Safi, L. Cabas Rodriguez, W.J. Mulder, N. Engelen-Smit, W. Spekking, L.A.M. van den Broek, G. Olivieri, L. Sijtsma, Energy consumption and water-soluble protein release by cell wall disruption of *Nannochloropsis gaditana*, Bioresour. Technol. 239 (2017) 204–210, http://dx.doi.org/10.1016/j.biortech.2017.05.012.
[29] C.R. Thomas, D. Geer, Effects of shear on proteins in solution, Biotechnol. Lett. 33 (2010) 443–456, http://dx.doi.org/10.1016/10.1007/s10529-010-0469-4.
[30] F. Donsì, B. Senatore, Q. Huang, G. Ferrari, Development of novel pea protein-based Nanoemulsions for delivery of nutraceuticals, J. Agric. Food Chem. 58 (2010) 10653–10660, http://dx.doi.org/10.1021/jf101804g.
[31] R.H. Wijffels, M.J. Barbosa, M.H.M. Eppink, Microalgae for the production of bulk chemicals and biofuels, Biofuels Bioprod. Biorefin. 4 (2010) 287–295, http://dx.doi.org/10.1002/bbb.215.
[32] H.W. Bischoff, H.C. Bold, Some soil algae from enchanted rock and related algal species, Phycological Studies IV, University of Texas, Austin, Tex., 1963, pp. 1–95.
[33] E.Y. Ortiz Montoya, A.A. Casazza, B. Aliakbarian, P. Perego, A. Converti, J.C. Monteiro de Carvalho, Production of *Chlorella vulgaris* as a source of essential fatty acids in a tubular photobioreactor continuously fed with air enriched with CO_2 at different concentrations, Biotechnol. Prog. 30 (2014) 916–922, http://dx.doi.org/10.1002/btpr.1885.
[34] O. Tastan, G. Ferrari, T. Baysal, F. Donsì, Understanding the effect of formulation on functionality of modified chitosan films containing carvacrol nanoemulsions, Food Hydrocoll. 61 (2016) 756–771, http://dx.doi.org/10.1016/j.foodhyd.2016.06.036.
[35] F. Donsì, F. Ferrari, G. Pataro, Applications of pulsed electric field treatments for the enhancement of mass transfer from vegetable tissue, Food Eng. Rev. 2 (2010) 109–130, http://dx.doi.org/10.1007/s12393-010-9015-3.
[36] R. Bobinaite, G. Pataro, N. Lamanauskas, S. Šatkauskas, P. Viškelis, G. Ferrari, Application of pulsed electric field in the production of juice and extraction of bioactive compounds from blueberry fruits and their by-products, J. Food Sci. Technol. 52 (2014) 5898–5905, http://dx.doi.org/10.1007/s13197-014-1668-0.
[37] O. Kunrunmi, T. Adesalu, S. Kumar, Genetic identification of new microalgal species from Epe Lagoon of West Africa accumulating high lipids, Algal Res. 22 (2017) 68–78, http://dx.doi.org/10.1016/j.algal.2016.12.009.
[38] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[39] O. Folin, V. Ciocalteau, On tyrosine and tryptophane determinations in proteins, J. Biol. Chem. 73 (1927) 627–650.
[40] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1957) 350–356, http://dx.doi.org/10.1021/ac60111a017.
[41] G.P. t Lam, P.R. Postma, D.A. Fernandes, R.A.H. Timmermans, M.H. Vermue, M.J. Barbosa, M.H.M. Eppink, R.H. Wijffels, G. Olivieri, Pulsed electric field for protein release of the microalgae *Chlorella vulgaris* and *Neochloris oleoabundans*, Algal Res. 24 (2017) 181–187, http://dx.doi.org/10.1016/j.algal.2017.03.024.
[42] E.M. Spiden, B.H.J. Yap, D.R.A. Hill, S.E. Kentish, P.J. Scales, G.J.O. Martin, Quantitative evaluation of the ease of rupture of industrially promising microalgae by high pressure homogenization, Bioresour. Technol. 140 (2013) 165–171, http://dx.doi.org/10.1016/j.biortech.2013.04.074.
[43] E. Suali, R. Sarbatly, S.R.M. Shaleh, Characterisation of Local *Chlorella* sp. Toward Biofuel Production, International Conference on Applied Energy ICAE, 2012, pp. 2965–2970 (ID: ICAE2012-A10331).
[44] W.Y. Choi, H.Y. Lee, Effective production of bioenergy from marine *Chlorella* sp. by high-pressure homogenization, Biotechnol. Biotechnol. Equip. 30 (2016) 81–89, http://dx.doi.org/10.1080/13102818.2015.1081407.
[45] D. Wang, Y. Li, X. Hu, W. Su, M. Zhong, Combined enzymatic and mechanical cell disruption and lipid extraction of green alga *Neochloris oleoabundans*, Int. J. Mol. Sci. 16 (2015) 7707–7722, http://dx.doi.org/10.3390/ijms16047707.
[46] M. Coustets, N. Al-Karablieh, C. Thomsen, J. Teissie, Flow process for electro-extraction of total proteins from microalgae, J. Membr. Biol. 246 (2013) 751–760, http://dx.doi.org/10.1007/s00232-013-9542-y.
[47] M.D. Berliner, Proteins in *Chlorella vulgaris*, Microbios 46 (1986) 199–203.
[48] C. Safi, A.V. Ursu, C. Laroche, B. Zebib, O. Merah, P.Y. Pontalier, C. Vaca-Garcia, Aqueous extraction of proteins from microalgae: effect of different cell disruption methods, Algal Res. 3 (2014) 61–65, http://dx.doi.org/10.1016/j.algal.2013.12.004.
[49] Y. Xie, S.H. Ho, C.N.N. Chen, C.Y. Chen, K. Jing, I.S. Ng, J. Chen, J.S. Chang, Y. Lu, Disruption of thermo-tolerant *Desmodesmus* sp. F51 in high pressure homogenization as a prelude to carotenoids extraction, Biochem. Eng. J. 109 (2016) 243–251, http://dx.doi.org/10.1016/j.bej.2016.01.003.
[50] M.J. Scholz, T.L. Weiss, R.E. Jinkerson, J. Jing, R. Roth, U. Goodenough, M.C. Posewitz, H.G. Gerken, Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall, Eukaryot. Cell 13 (2014) 1450–1464, http://dx.doi.org/10.1128/EC.00183-14.
[51] C. Safi, G. Olivieri, R.P. Campos, N. Engelen-Smit, W.J. Mulder, L.A. van den Broek, L. Sijtsma, Biorefinery of microalgal soluble proteins by sequential processing and membrane filtration, Bioresour. Technol. 225 (2017) 151–158, http://dx.doi.org/10.1016/j.biortech.2016.11.068.
[52] A.-V. Ursu, A. Marcati, T. Sayd, V. Sante-Lhoutellier, G. Djelveh, P. Michaud, Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*, Bioresour. Technol. 157 (2014) 134–139, http://dx.doi.org/10.1016/j.biortech.2014.01.071.
[53] G. Pataro, B. Senatore, G. Donsi, G. Ferrari, Effect of electric and flow parameters on PEF treatment efficiency, J. Food Eng. 105 (2011) 79–88, http://dx.doi.org/10.1016/j.jfoodeng.2011.02.007.