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Pulsed electric field (PEF) recovery of biomolecules from *Chlorella*: Extract efficiency, nutrient relative value, and algae morphology analysis

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ABSTRACT

This study investigated the effects of pulsed electric field (PEF) (3 kV/cm, 44 pulses, 99 kJ/kg), solvent (H_2O or 50 % DMSO) and time (0, 10, 20, 30, 60, 90, 120 and 180 min) on the extraction of *Chlorella* antioxidant biomolecules and minerals. The results showed that PEF treatment increased the biomolecules recovery. For the extraction time of 120 min, more proteins and polyphenols were obtained using water, while more chlorophyll *a* and b, and carotenoids were obtained using 50 % DMSO as the extraction solvent. The extracts mineral concentration (PEF vs control) were analysed including Mg, P, Ca, Fe and Zn, and the Relative Nutrient Values results indicated that *Chlorella* H_2O-extracts could be used as a mineral source for different populations. Finally, the fluorescence and scanning electron microscopy revealed the electroporation effect of PEF.

1. Introduction

One of the main challenges we face in the 21st century is feeding a growing population with increasingly limited natural resources (Torres-Tiji et al., 2020). FAO and the Green Deal recommend that humanity should move towards a more sustainable and environmentally friendly global food system (Couto et al., 2022). In addition, the increased healthy food demand caused by Covid-19 make it necessary for the food industry to think about how to make good use of the existing natural edible resources to better overcome the potential crisis. Microalgae is an important part of marine and freshwater resources (Zhou et al., 2022). Microalgae belongs to autotrophic organisms, which utilize light energy and inorganic nutrients (carbon dioxide, nitrogen, phosphorus, etc.) to synthesize valuable biomass (such as proteins, polysaccharides, polyphenols, etc.) and accumulate minerals (Markou & Nerantzis, 2013). Microalgae can be produced on a large scale without competing with conventional agriculture land (Liu et al., 2022). The chemical composition of microalgal biomass, including proteins, lipids, pigments, polysaccharides, etc., basically supports the development of microalgae products, which has attracted global interest (Song et al., 2018).

Among thousands of species of microalgae, *Chlorella* is one of the most industrially cultivated microalga since the early 1960s, and it has been consumed as novel foods and studied worldwide (Couto et al., 2022). *Chlorella* is a unicellular green alga classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration, in the European Union is a marketable microalgae subjected to the General Food Law Regulation (Regulation (EC) No. 178/2002 of the European Parliament) (Markou, Chentir, & Tzovenis, 2021) and in Spain the AESAN (Spanish agency for food safety and nutrition) is aligned with the European regulation. Chlorella is a microalgae rich in protein (47.82 % DW), lipids (13.32 % DW), carbohydrates (8.08 % DW), and mineral elements such as magnesium (Mg, 344.3 mg/100 g DW), phosphorus (P, 1761.5 mg/100 g DW), calcium (Ca, 593.7 mg/100 g DW), iron (Fe, 259.1 mg/100 g DW), zinc (Zn, 1.19 mg/100 g DW), and selenium (Se, 0.07 mg/100 g DW) (Song et al., 2018; Tokuşoglu & Uunal, 2003). Consuming *Chlorella* rich in the above nutrients has potential health benefits to the human body, such as antioxidant, anti-inflammatory, anti-cardiac, anti-diabetic and regulating the balance of gut microbiota (Gateau et al., 2016). Considerable research and efforts have been devoted to the use of *Chlorella* for food production, however, the high cost of production, cultivation and downstream processing is the biggest hurdle (Loke Show, 2022). In recent years, with the rise of the microalgae culture industry, the culture technology has gradually matured, and downstream processing, such as the efficient recovery of *Chlorella*

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nutrients, has become the main focus.

Chlorella has a multi-layered cell wall structure with a thickness of 100 ~ 200 nm, which is composed of polysaccharides, proteins, and inorganic salts, as a consequence nutrients in the cytoplasm are not easily available (Ahmed & Kumar, 2022). Traditional extraction techniques such as Soxhlet, Folch, hot water extraction, are used to obtain soluble bioactive compounds from *Chlorella*. Although these techniques can obtain most molecules from *Chlorella*, they are gradually replaced by new extraction techniques due to the disadvantages of long extraction time, high temperature, and the use of toxic reagents (Soleimani Khorramdashti, Samipoor Giri, & Majidian 2021). Compared with the traditional extraction technology, pulsed electric field (PEF) extraction is a novel and non-thermal technology that is being widely used for the recovery of microalgae biomolecules due to its advantages of cleanliness, safety, and high efficiency (Gateau et al., 2021). PEF device (laboratory configuration) typically includes an electrical pulse generator, a treatment chamber, and electrodes, with the electrical pulse placed between or through two electrodes (Naliyadhara et al., 2022). The PEF principle is based on the application of short electrical pulses (from a few nanoseconds to a few milliseconds) of high voltage (from 100 ~ 300 V/cm to 80 kV/cm) to the product between two electrodes (Barba et al., 2015). PEF treatment can alter cell membrane properties due to the high-intensity electric field pulse discharges (electroporation phenomenon), resulting in increased cell membrane permeability and promotion of cytoplasmic dissolution (Zhou et al., 2022).

At present, the studies of PEF assisted extraction of biomolecules from *Chlorella* are mainly focused on biological macromolecules like proteins, polysaccharides (ˆt Lam et al., 2017; Carullo et al., 2018; Scherer et al., 2019) and lipid compounds (Canelli et al., 2022). In addition, *Chlorella* is also rich in minerals and antioxidants (Singh et al., 2018), however, there are not many related studies on this topic. Based on this, the recovery of a variety of biomolecules (proteins, polyphenols, chlorophyll *a*, chlorophyll *b*, carotenoids) and minerals (Mg, P, Ca, Fe, Zn, Se) from *Chlorella* assisted by PEF was carried out in this study. Moreover, the effect of PEF treatment on the permeability of *Chlorella* cells was observed under a scanning electron microscope (SEM) and fluorescence microscope (FM), to comprehensively evaluate the effect of PEF on the extraction of nutrients from *Chlorella*.

2. Materials and methods

2.1. Chemicals and reagents

ABTS (2,2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride), Folin-Ciocalteu, gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), D-glucose, phenol, fluorescein sodium salt, potassium persulfate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). The methanol (>99 %) was purchased from Merck (Whitehouse Station, NJ, USA). Sodium carbonate was acquired from VWR (Saint-Prix, France). Bicinchoninic acid (BCA) kit, sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Deionized water (resistivity > 18 MΩ cm^{-1}) was produced by a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.2. Samples

Chlorella pyrenoidosa from Hainan Island (China, 3°30′~20°17′N, 108°15′~120°15′E) was provided by the company Ecospirulina (Serra, Comunitat Valenciana, Spain). The microalgae was produced in open raceway ponds and average temperatures varying from 21 °C to 33 °C and precipitations of 1600 mm per year. At the time of harvesting, biomass was washed and then spray-dried at 160 to 180 °C for 15 min. The final product was green powder with characteristic smell and taste, which was stored at −20 °C for experimental analysis.

2.3. Pulsed electric fields (PEF) extraction process

For the PEF pre-treatment of the *Chlorella* 2 % (w/v) solution was used with the PEF-Cellcrack III equipment (German Institute for Food Technology (DIL)) (ELEA, Germany) located at the Faculty of Pharmacy of the University of Valencia (Burjassot, València, Spain). According to the previous studies in our laboratory (Martí-Quijal et al., 2021), PEF treatment conditions of 3 kV/cm, 44 pulses, 99 kJ/kg were selected to extract antioxidants and minerals from *Chlorella* at room temperature (23 ± 2 °C). The temperature and conductivity of each sample were measured with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). In this study, the aqueous suspension of *Chlorella* (2 g/200 mL) was first treated with PEF. The temperature was changed from 23 ± 2 °C to 31 ± 3 °C and the conductivity was changed from 1400 ± 20 us/cm to 1620 ± 33 us/cm after PEF treatment. Then, the same volume of water or DMSO (200 mL) was added after PEF treatment to make the final sample as 2 g/400 mL water extracts or 2 g/400 mL water: DMSO 1:1 extract (50 % DMSO). Then, a magnetic stirrer was used to continuously stir the samples at room temperature (25 °C) and the samples were collected at 0, 10, 20, 30, 60, 90, 120 and 180 min, respectively. The control experiment was carried out with 2 g *Chlorella* powder/400 mL water or 2 g *Chlorella* powder/400 mL 50 % DMSO stirred at room temperature and the samples were collected at the same time as PEF extraction process. Finally, the samples were centrifuged (2504×g, 4 °C, 15 min) using a 5810R centrifuge (Eppendorf Ibérica, Madrid, Spain), and the supernatants were collected and stored at −20 °C until analyses. Bioactive compounds amount (proteins, polyphenols, pigments) was calculated based on the dry weight (DW) of *Chlorella* powder.

2.4. Bioactive molecules analysis

2.4.1. Protein

The BCA (bicinchoninic acid) working solution was prepared according to the instructions of the BCA kit (Pierce Biotechnology, Inc., Waltham, MA, USA), that was, mixed reagents A and B at a ratio of 50:1 (v/v). 10 μL of samples or bovine serum albumin (BSA) and 200 μL of BCA working solution were added to a 96-well plate, mixed well, and incubated in a 37 °C oven for 30 min and measured the absorbance at 562 nm. The protein content (mg/g DW) was calculated by means of a calibration curve prepared with BSA from 0 ~ 2000 mg/L.

2.4.2. Polyphenol

The Folin-Ciocalteu method was used to analyse the total polyphenol content in the extract (Korzeniowska et al., 2020). Briefly, 0.2 mL samples, 1 mL Folin-Ciocalteu (diluted with water at a ratio of 1:10, v/v) and 0.8 mL sodium carbonate solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 min. Then, the absorbance value was measured at 750 nm using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany). Distilled water was used as a reference. The Gallic acid was used as a standard to calculate the polyphenol content (mg/g DW) in the extracts.

2.4.3. Pigments (chlorophyll a, chlorophyll b, carotenoids)

The chlorophyll *a*, chlorophyll *b* and carotenoids concentration of *Chlorella* extracts were analysed by spectrophotometry. The absorbance values and formulas used to analysed extracts varied with solvent, for 50 % DMSO extracts, the equations were as follows (Wellburn, 1994):

$$C_{ch}^{a} = 12.47*A_{665.1} - 3.62*A_{649.1} \quad (1)$$

$$C_{ch}^{a} = 25.06*A_{649.1} - 6.5*A_{665.1} \quad (2)$$

$$C_{carotenoids} = \left(100*A_{480} - 1.29*C_{ch}^{a} - 53.78*C_{ch}^{b}\right) \quad (3)$$

For H_2O-extracts, the equations were as follows (Kokkali et al., 2020):

$$C_{ch}^{a} = 16.82*A_{665} - 9.28*A_{653} \quad (4)$$

$$C_{ch}^{a} = 36.92*A_{653} - 16.54*A_{665}. \quad (5)$$

$$C_{carotenoids} = \left(1000*A_{470} - 1.91*C_{ch}^{a} - 95.15*C_{ch}^{b}\right)/225 \quad (6)$$

where C_{ch}^{a}, C_{ch}^{b}, and $C_{carotenoids}$ were the concentrations (mg/L) of chlorophyll *a*, chlorophyll *b* and total carotenoids, respectively.

2.4.4. Dynamic proportion

Based on the protein, polyphenols, chlorophyll *a*, chlorophyll *b* and carotenoids content, the dynamic proportion of these compounds in extracts collected at different times (0, 10, 20, 30, 60, 90, 120, 150, 180 min) was further calculated according to Eq. (7) (Zhang et al., 2020).

$$Dynamic\ proportion\ (\%) = (X/T)*100 \quad (7)$$

where X was the content of specific antioxidant compounds (protein, polyphenol, chlorophyll *a*, chlorophyll *b*, carotenoids), T was the total amount of the compounds in the extracts (the sum of protein, polyphenol, chlorophyll *a*, chlorophyll *b*, carotenoids content).

2.4.5. Antiradical properties

The oxygen radical antioxidant capacity (ORAC) (Cao et al., 1993) and the Trolox equivalent antioxidant capacity (TEAC) (Miller et al., 1993) assays were used to evaluate the antioxidant capacity of the *Chlorella* extract. Briefly, 50 μL of extract and fluorescein sodium salt solution were added to a 96-well plate and incubated in a microplate reader at 37 °C for 10 min, then 25 μL AAPH solution was added, and the absorbance was recorded at 520 nm. Each group of samples was tested in 3 wells in parallel, and the experiment was repeated at least three times to make the coefficient of variation value within 10 %. For TEAC experiments, the working solution was prepared as follows. 25 mL of 7 mM ABTS solution were mixed with 440 μL of 140 mM potassium thiosulfate solution (dissolved in distilled water) and incubated under darkness at room temperature for 12 ~ 16 h. During the TEAC test, the working solution was diluted with 96 % ethanol to obtain an absorbance value of 0.700 ± 0.020 at 734 nm. Then, 0.1 mL of the samples or Trolox standard solution were mixed with the above working solution (absorbance value of 0.700 ± 0.020 at 734 nm), and after reacting for 3 min in a dark room, the absorbance at 734 nm was measured. Trolox was used as the standard solution to calculate the antioxidant capacity of the sample, and the unit for ORAC and TEAC was μm Trolox equivalent.

2.5. Mineral analysis

The macro (Mg, Ca, P) and micro (Fe, Zn, Se) minerals were determined according to de la Fuente et al. (2019), with some minor modifications. In brief, 1 mL sample was mixed with 1 mL of concentrated nitric acid (HNO_3, 69 %) and 250 μL H_2O_2, then placed for digestion in a high-pressure microwave digestor (Ethos Easy, Milestone Srl.) at 500 W and 180 °C. After that, the volume was adjusted to 5 mL with ultrapure water, a 100 μL aliquot was taken and the volume was adjusted to 10 mL with ultrapure water. The content of mineral elements was obtained through Agilent model 7990 ICP-MS (Agilent Technologies, CA, USA).

2.6. Nutrient Relative Value (NRV) Analysis (Mg, P, Ca, Fe, Zn, Se)

The contribution of minerals in extracts from 100 g dry *Chlorella* powder (Nutrient Relative Value) towards Dietary Reference Intake (DRI) was calculated as equation (Jalali & Fakhri, 2021) (8):

$$NRV = (X/R)*100 \quad (8)$$

where X corresponded to the mineral content in extracts (water as a solvent) from 100 g *Chlorella* dry powder and R to the Recommended Dietary Allowances (RDAs) presented in Table A (Supplementary Material) (Ramu Ganesan et al., 2020).

2.7. Microalgae morphology

2.7.1. Fluorescence microscope

The samples obtained after PEF treatment (3 kV/cm, 44 pulses, 99 kJ/kg) were centrifuged at 157×g for 10 min. After centrifugation, the supernatant was removed to collect the pellet, and it was washed with 90 % methanol and centrifuged to collect the precipitate again (157×g/10 min). The sample was repeatedly washed until the supernatant was colourless, and the precipitate was collected and diluted with water. The control group was set as a mixed extract of microalgae and water without PEF treatment. To characterize *Chlorella* morphology, these samples were observed by means of an Eclipse 90i Nikon widefield microscope (Nikon corporation, Japan) equipped with 5-megapixels cooled digital colour camera Nikon Digital Sight DS-5Mc (Nikon corporation, Japan). All microscopy images were acquired and processed by using Nis-Elements Br 3.2 Software (Nikon corporation, Japan). Nikon objective used for all images was CFI Plan Fluor DIC M/N2 40X (MRH00401). An optical zoom factor of 0.8x or 2x was combined with this objective. Brightfield images were acquired by illuminating with a halogen lamp for transmitted visible light, while fluorescent images were acquired by illuminating with a mercury lamp. Filter blocks used in fluorescent images were for red (Nikon reference G-2E/C) [EX 540/25, DM 565, BA 605/55], for ultraviolet excitation (Nikon reference UV2-A) [EX 330-380, DM 400, LP 420]. Main image properties were: RGB 24 bits; Frame size of 2560*1920 pixels; image dimensions of field of view were 270*200 μm for 32x images (0.11 μm/pixel) and 105*80 μm for 80x images (0.04 μm/pixel). Image with annotations has a frame size 1075*806 pixels and 0.25 μm/pixel, but same field of view dimensions of 32x original image.

2.7.2. Scanning electron microscopy (SEM)

A Hitachi S-4800 (Tokyo, Japan) scanning electron microscope was used to analyse the microstructure of *Chlorella* samples (aqueous extraction) after freeze drying (FreeZone 2.5 L, Labconco, MI, USA) for 72 h at a cold trap temperature of −65 °C. Freeze-dried *Chlorella* samples were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with an SEM microscope (S-4800) at magnifications of 110×, 450× and 1500× (Fang et al., 2021).

2.8. Statistical analysis

All experiments and measurements of characteristics were repeated at least three times. One-way analysis of variance (ANOVA) was used for determining the significant differences among samples using the software Statgraphics plus (version 5.1, Statpoint Technologies Inc., Warrenton, VA). For each analysis, a significance level of 5 % was assumed, a p-value < 0.05 was considered statistically significant. The error bars presented on the figures correspond to the standard deviations, letters were used to label the significance of the difference. The unsupervised principal component analysis (PCA) was performed by exporting the dataset in the software GraphPad 9 (GraphPad Software, San Diego, California, USA).

3. Results and discussion

3.1. Biomolecules' content and composition ratio analysis

The effects of PEF (3 kV/cm, 44 pulses, 99 kJ/kg), solvent (water or 50 % DMSO) and extraction time (0, 10, 20, 30, 60, 90, 120 and 180 min) on the extraction of *Chlorella* components were shown in Fig. 1. The results showed that the extraction of *Chlorella* protein was 12 ~ 42 mg/g DW, polyphenols 3.5 ~ 5.5 mg/g DW, chlorophyll *a* 0 ~ 0.5 mg/g DW, chlorophyll *b* 0 ~ 0.6 mg/g DW, carotenoids 0 ~ 0.15 mg/g DW. Compared with the biomolecules content of *Chlorella* powder: protein

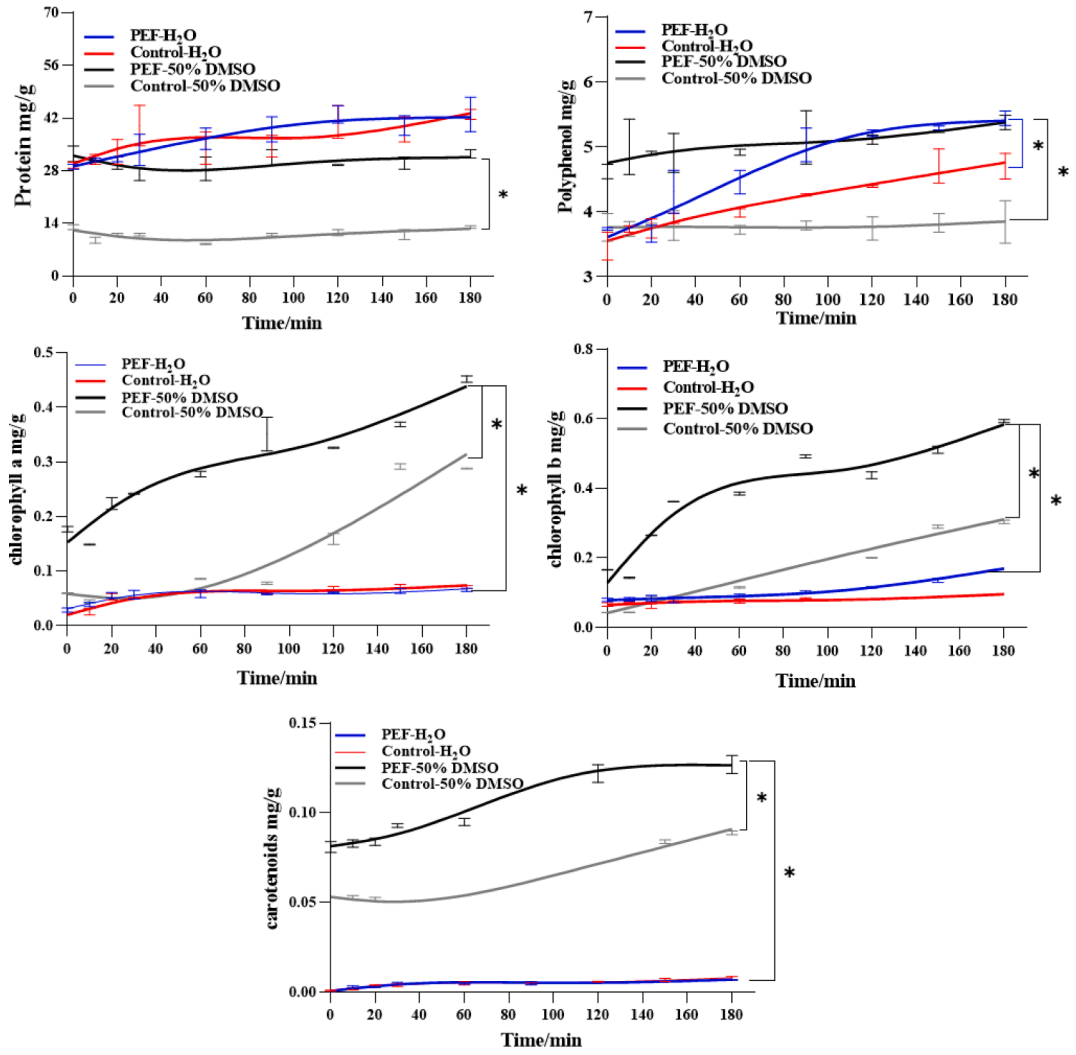

Fig. 1. Biomolecules extraction recovery (mg/g dw) from *Chlorella* treated with PEF/without PEF (control) under different extraction times (0 to 180 min) and solvents (H_2O, 50 % DMSO). *:The significant difference level is $p < 0.05$.

480 ~ 600 mg/g DW (Liu et al., 2021), polyphenols 7.06 ~ 19.16 mg/g DW (Jelínek et al., 2015), chlorophyll *a* 3.04 ~ 7.69 mg/g DW, chlorophyll *b* 0.39 ~ 10.34 mg/g DW, carotenoids 0.29 ~ 11.83 mg/g DW (Hynstova et al., 2018), the extraction yield of this study could theoretically be further improved. Fig. 1 showed that PEF treatment increased the extraction of biomolecules compared to the control group. For example, the protein content of PEF-H_2O was higher than that of control-H_2O for extraction times from 80 to 120 min, and the protein yield of PEF-50 % DMSO was significantly higher ($p < 0.05$) than that of control-50 % DMSO (from 0 to 180 min). Similarly, the polyphenol extraction results also showed that the yield of PEF-H_2O and PEF-50 % DMSO extracts were significantly higher ($p < 0.05$) than those of control-H_2O and control-50 % DMSO extracts, respectively, which could be attributed to the electroporation phenomenon of PEF. However, it should be emphasized that the content of biomass extracted by PEF is not high compared with the total biomass in *Chlorella*, especially the protein yield, which indicates that the use of PEF technology alone to recover the protein of *Chlorella* is not enough.

Compared with the effect of PEF on protein and polyphenol yield, the extraction of chlorophyll *a*, chlorophyll *b* and carotenoids were more affected by solvent. The results showed that the extraction of pigments was higher in 50 % DMSO extracts than in water extracts, regardless of whether a PEF treatment was applied or not. As we know, chlorophyll *a*, chlorophyll *b* and carotenoids are fat-soluble pigments, while DMSO ($(CH_3)_2SO$) has one hydrophilic sulfinyl group and two hydrophobic methyl groups, which can dissolve water-soluble and fat-soluble compounds and increase pigment extraction (García-Vaquero et al., 2021; Mueller et al., 2019). However, the opposite result was shown in protein yield, i.e., 50 % DMSO decreased protein yield compared with water. This may be attributed to the precipitation of protein caused by increasing organic reagent concentration, thereby reducing the protein content in the extract (Arakawa et al., 2007). Similar to the present study, Parniakov et al (2015) investigated the potential of PEF-assisted extraction of nutrients from microalgae *Nannochloropsis spp*. using a mixture of organic solvents (DMSO) and water, and the results showed that PEF increased the yield of microalgae proteins, polyphenols, and pigments, and 50 % DMSO was beneficial to increase the extraction yield of pigment, which was consistent with the results of our study (Parniakov et al., 2015b).

The extraction time and biomolecules content determined the final extraction efficiency. The results in Fig. 1 showed that the protein and polyphenol content reached a stable level at 120 min, and there was no significant difference between the extraction yields at 180 min. The contents of chlorophyll *a*, chlorophyll *b* and carotenoids in the 50 % DMSO extract continued to increase from 0 to 180 min, whereas the pigment content in the water extract was almost unchanged (0 ~ 180 min). In order to better describe the dynamic proportion of these components in the extract, we further analysed the proportion of specific

biomolecules at each time point according to Eq. (7), and the results were shown in Fig. 2. The results in Fig. 2 showed that the proportions of molecules in different extraction techniques and solvents were different at specific times. Specifically, as the extraction time of PEF-H_2O and control-H_2O was extended to 180 min, the proportions of polyphenols and chlorophyll (chlorophyll *a* and chlorophyll *b*) slightly decreased and increased respectively, while the proportions of protein and carotenoids remained stable. However, different dynamic ratio changes occurred when 50 % DMSO was used as the extraction solvent. From the PEF-50 % DMSO and control-50 % DMSO extracts, it was observed that the prolonged extraction time was accompanied by a significant increase in the proportions of chlorophyll *a*, chlorophyll *b* and carotenoids and a significant decrease in the proportion of polyphenols (Schoefs, 2003). Chlorophylls and carotenoids were sensitive to heat, light, acid, and alkali, observing an increased reduction of pigments' content in the extract when other techniques such as ultrasound-assisted extraction was applied (Parniakov et al., 2015a). Fig. 2 showed that the elapse of the extraction time up to 180 min did not reduce the chlorophyll content in the extract, which was mainly attributed to the fact that the PEF treatment conditions (3 kV/cm, 44 pulses, 99 kJ/kg) of this study did not cause thermal effects to the extraction process (the temperature increase was from room temperature (23 ± 2 °C) to a final temperature of 31 ± 3 °C), thus avoiding the thermal decomposition of heat-sensitive components. The dynamic components proportion results provided a reference for selecting recovery conditions for different antioxidant components (single or composite component), which facilitated the development of further processes such as separation and purification of antioxidant biomolecules from the *Chlorella* extracts after the extraction stage.

3.2. Antioxidant capacity and multi-factor correlation analysis

The measurement of the antioxidant capacity of the samples depended on the technique and free radical generator or oxidant used, so it is necessary to use different methods to evaluate the antioxidant properties of the extract (Siddeeg et al., 2021). In this study, oxygen radical antioxidant capacity (ORAC) and Trolox equivalent antioxidant capacity (TEAC) was used to analyse the effects of PEF, solvent, and extraction time on the antioxidant properties of *Chlorella* extracts. The results presented in Fig. 3 showed that the antioxidant capacity of PEF extracts was higher than that of the control group in both ORAC and TEAC analyses, these results could be attributed to the higher biomolecules content (*section 3.1*) in the *Chlorella* PEF extracts.

However, the results of ORAC and TEAC were inconsistent in the evaluation of the antioxidant capacity of *Chlorella* extracts. In terms of antioxidant capacity, ORAC results showed that PEF-50 % DMSO > control-50 % DMSO > PEF-H_2O > control-H_2O, while TEAC results showed that PEF-H_2O > PEF-50 % DMSO > control-H_2O/control-50 % DMSO. Combined with the PCA loading plot in Fig. 3, ORAC was strongly correlated with carotenoids, chlorophyll *a*, and chlorophyll *b*, while TEAC was strongly correlated with proteins and polyphenols. This fact can be attributed to the different antioxidant capacities observed by the different antioxidant compounds. For example, chlorophyll *a*, b and carotenoids were better recovered in the PEF-50 % DMSO extracts while polyphenols were the predominant compounds in the PEF-H_2O extracts. In addition to this study, inconsistencies in the results of ORAC and TEAC have also been reported in other studies. For example, studies have shown that the antioxidant capacity of carotenoids measured by TEAC and ORAC corresponded to β-carotene > lutein > zeaxanthin and lutein > zeaxanthin > β-carotene respectively, and these differences could be attributed to the different reaction mechanisms of TEAC and ORAC (Barba et al., 2013). Methods for measuring antioxidant capacity were divided into two categories: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET). The assay principle of HAT was followed as antioxidants and substrates competed for thermally generated peroxyl radicals through the decomposition of azo compounds, such as ORAC, while the assay of ET measured the ability of antioxidants to reduce oxidants, such as TEAC (scavenging ABTS cationic radicals) (Barba et al., 2013). Therefore, when the samples are complex or contain different kinds of antioxidants (proteins, polyphenols, pigments, etc.), the ORAC and TEAC methods could be less relevant due to different kinetics and reaction mechanisms.

The antioxidant capacity of PEF-50 % DMSO extract in ORAC results and PEF-H_2O extract in TEAC results showed an increasing trend with time, while other curves remained stable. On the one hand, the ORAC results in this study were correlated with pigments content in PCA

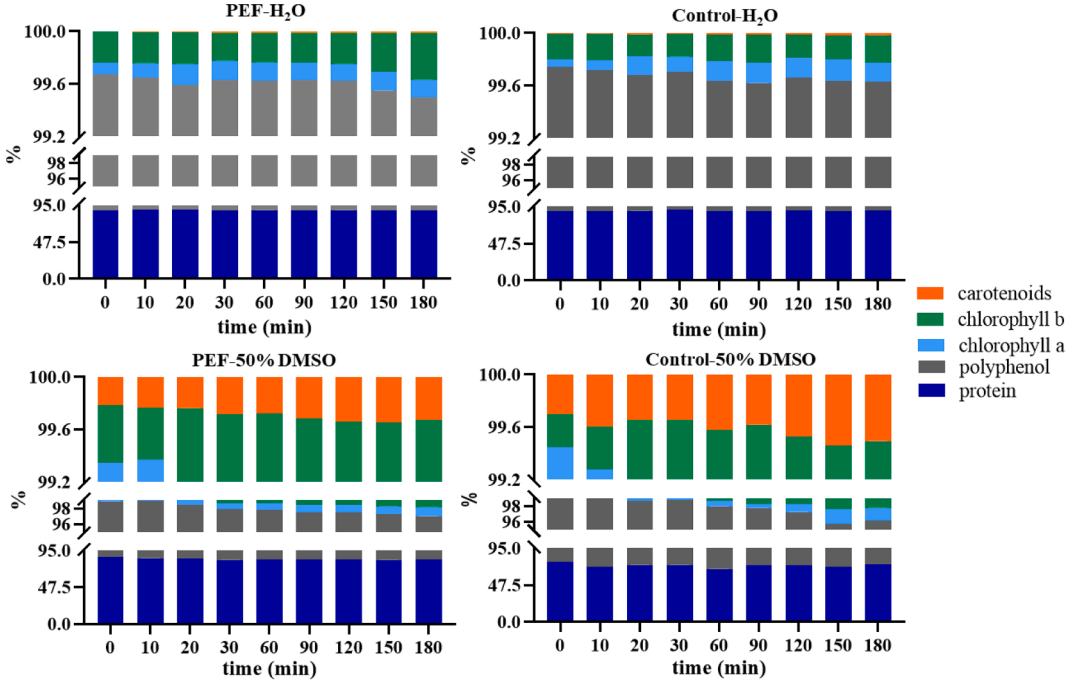

Fig. 2. Dynamic proportion of biomolecules at different time point (0 to 180 min).

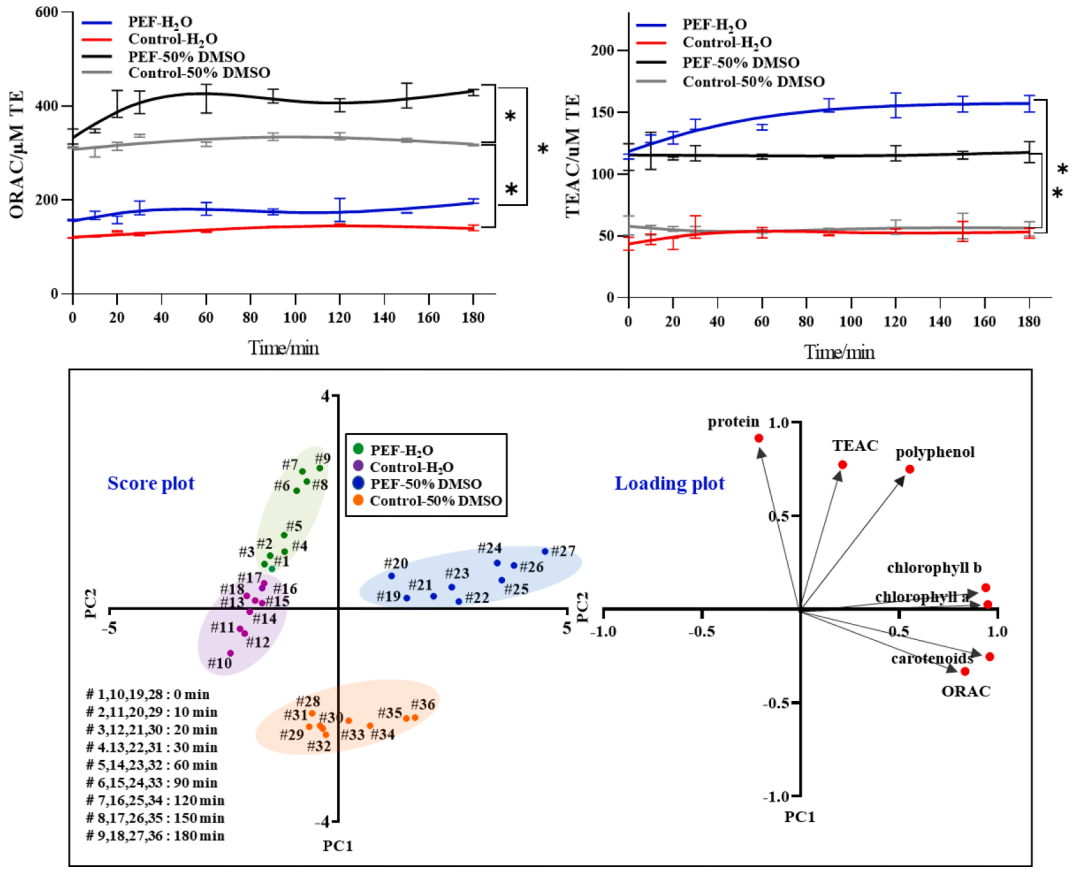

Fig. 3. Antioxidant results ((A) ORAC, (B) TEAC) and principal component analysis (PCA) (C) of *Chlorella* extracts. *:The significant difference level is $p < 0.05$.

Table 1
Mg, P, Ca, Fe, Zn, Se (Nd) concentration and Nutrient relative value (NRV) analysis.

Mineral	Mg		P		Ca		Fe		Zn	
Powder (mg/kg dw)	3660 ± 30		10894 ± 180		1593 ± 30		679 ± 6		21.8 ± 0.3	
Reference data (mg/kg dw)	3443 ± 0.1		1762 ± 0.2		5937 ± 0.7		2591 ± 0.4		11.9 ± 0.7	
Extracts	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-
Concentration (mg/kg dw)	352 ± 2 4[a]	364.8 ± 4.4[a]	1132 ± 20[a]	940 ± 40[b]	70 ± 5[a]	200 ± 8[b]	3.98 ± 0.03[a]	23.0 ± 0.1[b]	5.31 ± 0.07[a]	3.07 ± 0.07[b]
	NRV: contribution of minerals in extracts (extract from 100 g dry *Chlorella* powder) towards DRI									
Mineral	Mg		P		Ca		Fe		Zn	
Life stages	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-	PEF-	control-
Baby (6 ~ 12 months)	46.9	48.7	41.2	34.2	2.7	7.7	3.6	20.9	17.7	10.3
Children (1 ~ 3 years)	44.0	45.6	24.6	20.4	1.0	2.9	5.7	32.9	17.7	10.3
Children (4 ~ 8 years)	27.1	28.1	22.6	18.8	0.7	2.0	4.0	23.0	10.6	6.2
Males (9 ~ 13 years)	14.7	15.2	9.1	7.5	0.5	1.5	5.0	28.8	6.6	3.9
Males (14 ~ 18 years)	8.6	8.9	9.1	7.5	0.5	1.5	3.6	20.9	4.8	2.8
Males (19 ~ 30 years)	8.8	9.1	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
Males (31 ~ 50 years)	8.4	8.7	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
Males (51 ~ 70 years)	8.4	8.7	16.2	13.4	0.7	2.0	5.0	28.8	4.8	2.8
Males (>70 years)	8.4	8.7	16.2	13.4	0.6	1.7	5.0	28.8	4.8	2.8
Females (9 ~ 13 years)	14.7	15.2	9.1	7.5	0.5	1.5	5.0	28.8	6.6	3.9
Females (14 ~ 18 years)	9.8	10.1	9.1	7.5	0.5	1.5	2.7	15.3	5.9	3.4
Females (19 ~ 30 years)	11.4	11.8	16.2	13.4	0.7	2.0	2.2	12.8	6.6	3.9
Females (31 ~ 50 years)	11.0	11.4	16.2	13.4	0.7	2.0	2.2	12.8	6.6	3.9
Females (51 ~ 70 years)	11.0	11.4	16.2	13.4	0.6	1.7	5.0	28.8	6.6	3.9
Females (>70 years)	11.0	11.4	16.2	13.4	0.6	1.7	5.0	28.8	6.6	3.9
Pregnant (19 ~ 30 years)	10.1	10.4	16.2	13.4	0.7	2.0	1.5	8.5	4.8	2.8
Breastfeed (19 ~ 30 years)	11.4	11.8	16.2	13.4	0.7	2.0	0.1	0.7	4.4	2.6

Note. The Nutrient Relative Value (NRV)– contribution of minerals in extracts (extract from 100 g dry *Chlorella* powder) towards DRI was calculated as: NRV = X/R × 100 %, Where X and R corresponded to the mineral content in *Chlorella* extracts (from 100 g *Chlorella* dry powder) and Recommended Dietary Allowances (RDAs) respectively. Different lowercase letters indicate significant differences ($p < 0.05$), Nd-not detected.

analysis, so the increased antioxidant activity of PEF-50 % DMSO extract could be related to the increase in chlorophyll and carotenoid content over time. The TEAC results were related to polyphenol content in the PCA analysis, so the increased antioxidant activity of the PEF-H_2O extract was related to the increased polyphenol content over time, which corresponded to the results in Fig. 3. Furthermore, the score plot in Fig. 3 showed that all extraction conditions were divided into 4 groups, based on the presence or absence of PEF treatment and solvent type (water, 50 % DMSO), indicating that the extraction efficiency was more affected by extraction technique and solvent than extraction time, which provided a reference for selecting the recovery conditions of *Chlorella* biomolecules. Finally, considering the extraction yield of all biomolecules and the cleanliness of the solvent, water was selected as the solvent in this study, and the extraction time of 120 min was used as the experimental condition to analyse the effect of PEF treatment on the recovery of trace minerals and morphology of *Chlorella*.

3.3. Minerals yield and analysis of NRV (Nutrient relative value)

Minerals are necessary for culture development due to their diverse functionalities in body metabolism and homeostasis and are important for the maintenance of hormonal and regulatory functions of the body as well as build of muscles and bones. Moreover, minerals are essential nutrients because they cannot be synthesized in the body and must be obtained through food or as supplements to meet daily requirements.

On this line, this study analysed the content of minerals in *Chlorella* dry powder and *Chlorella* extract, including magnesium, phosphorus, calcium, iron, and zinc, and further calculated the NRV of minerals (extracted from 100 g *Chlorella* dry powder) with reference to RDAs, the results were shown in Table 1. The results showed that *Chlorella* powder was rich in Mg, P, Ca, Fe and Zn, and compared with the average values in other studies, i.e., Mg (3443 mg/kg DW), P (17615 mg/kg DW), Ca (5927 mg/kg DW), Fe (2591 mg/kg DW), Zn (11.9 mg/kg DW) (Tokuşoglu & Uunal, 2003), the P, Ca and Fe contents of the *Chlorella* powder in this study were relatively low, while the contents of Mg and Zn were relatively high, which could be attributed to the differences of cultivation conditions.

The mineral content results showed that PEF treatment had no significant effect on Mg ($p > 0.05$), significantly increased P and Zn content ($p < 0.05$) and decreased Ca and Fe concentration respectively ($p < 0.05$). The PEF induced phenomenon that reduced Ca and Fe yield was worth considering, as most reports showed that PEF treatment disrupted microalgae cells and increased biomolecule yields, and the following explanations could be given according to the related studies (Parniakov et al., 2015b). Ca and Fe can be present in microalgal cells by chelating with biomolecules, such as proteins (Yang et al., 2022), and electrostatic interactions induced by PEF treatment may alter the spatial structure of proteins, such as unfolding and aggregation (Gateau et al., 2021), which altered the functional properties of proteins (solubility, etc.) (Dong et al., 2020), resulting in Ca or Fe-containing proteins sedimentation during extraction, which was one possibility leading to this result.

In this study, water was used as the mineral extraction solvent, which was safe and edible. On these basics, the contribution of macro (Ca, Mg, P) and trace minerals (Fe, Zn, Se) in *Chlorella* extracts (NRV (Nutrient relative value), extracted from 100 g dry *Chlorella* powder) towards Recommended Dietary Allowances (RDAs) was calculated using Eq. (8), the results are shown in Table 1.

Mg is an indispensable mineral required in the human diet for processing ATP (adenosine triphosphate) and bones. Mg deficiency could cause various diseases, such as type-2 diabetes, metabolic syndrome, hypertension, atherosclerotic vascular disease, etc (Eggleston et al., 2022). *Chlorella* extract (control extracts) met more than 45 % of the Mg RDAs for infants (6 ~ 12 months) and children (1 ~ 3 years old), and about 30 % Mg RDAs for children (4 ~ 8 years old), as well as more than 8 % Mg RDAs for male/female (>9 years old, 8.7 ~ 15.2 %) and pregnant/breastfeed female (19 ~ 30 years old, 10.4 ~ 11.8 %). P is a multifunctional component, which is an important component of bones and cells, and also plays an important role in the body's energy processing due to its presence in ATP and DNA (Eggleston, Triplett, Bett-Garber, Boue, & Bechtel, 2022). The NRV results showed that *Chlorella extracts* (PEF extracts) met 41.2 % of the P RDAs for infants (6 ~ 12 months), and more than 20 % of the P RDAs for children (1 ~ 8 years), as well as more than 9 % P RDAs for male/female (>9 years old, 9.1 ~ 16.2 %) and pregnant/breastfeed female (19 ~ 30 years old, 16.2 %). Ca is the most abundant mineral in the body, which is essential for muscle, bones, teeth, heart, and digestive system health, as well as the synthesis and function of blood cells (Michos, Cainzos-Achirica, Heravi, & Appel, 2021). The NRV results showed that the Ca NRV in the *Chlorella* extracts (control extracts) was relatively low, specifically, it met 7.7 % of the Ca RDAs for infants (6 ~ 12 months), and 2 ~ 3 % calcium RDAs for children (1 ~ 8 years old), as well as 1.5 ~ 2.0 % Ca RDAs for male/female (>9 years old) and pregnant/breastfeed female (19 ~ 30 years old). Fe is an essential metal for biological processes, which is a fundamental inorganic nutrient in the human body, playing an important role in DNA synthesis and repair, ATP production and oxygen transport (Salnikow, 2021). The NRV results showed that *Chlorella* extracts (PEF extracts) could meet 20 ~ 30 % of Fe RDAs for infants (6 ~ 12 months), children (1 ~ 8 years old), males (>9 years old) and females (9 ~ 13, > 51 years old), as well as 8.5 % and 0.7 % Fe RDAs for pregnant and breastfeed female respectively. Zn is an essential micronutrient in our diet, which is a key component for the function of numerous proteins, including Zn-containing metalloenzymes and zinc-associated transcription factors, and is an essential micronutrient required for numerous cellular processes and immune system development (Ho et al., 2022). NRV results showed that chlorella extracts (PEF extracts) met 17.7 % of the Zn RDAs for infants (6 ~ 12 months) and children (1 ~ 3 years old), and 10.6 % of the Zn RDAs for children (4 ~ 8 years old), as well as 4.8 ~ 6.6 % of the Zn RDAs for male/female (>9 years old) and pregnant/breastfeed female (19 ~ 30 years old). Among them, the NRV of Mg, P and Fe were relatively high, and the NRV of Ca and Zn were relatively low, which depended on the mineral content of *Chlorella* and the extraction process. From these results, *Chlorella* extract could be used as a source of minerals. Moreover, the bioavailability of minerals in *Chlorella* extracts should be considered, and the corresponding research has been gradually carried out in our laboratory. At present, there are few reports on the use of PEF to extract minerals from *Chlorella*. This study showed that PEF increased the yield of some minerals, which provided a possibility for the application of PEF in the recovery of microalgae minerals in the future.

3.4. Fluorescence microscope (FM)

Previous reports have shown that high electric field strength altered cell membrane properties during PEF treatment, resulting in increased membrane permeability and enhanced cytoplasmic extraction (Saulis, 2010). However, the effect of PEF treatment on the microalgae morphology depended on the cell structure (cell wall thickness) and treatment conditions (pulse, electric field strength, time, etc.), which should be specifically explored. In this study, microscopy was used to analyse the effect of PEF (3 kV/cm, 44 pulses, 99 kJ/kg) on the morphology of *Chlorella*, and the results were shown in Fig. 4.

Fig. 4A (16×) and 4B (64×) were the morphology of *Chlorella* in no-PEF treated (control) suspension, showing no single cells but circular aggregates of multiple *Chlorella* in this field of view. Fig. 4C (16×) and 4D (64×) were *Chlorella* in a PEF-treated suspension, and some 'cracks' in the circular *Chlorella* aggregates could be observed. To confirm that the increased bioactive compounds (proteins, polyphenols, pigments) yield of *Chlorella* is related to the change of cell morphology by the electric field effect of PEF, this study further focused on observing the single *Chlorella* cells, the results were shown in Fig. 4E and 4F. Fig. 4E (32×) and 4F (160×) were single *Chlorella* cell morphology in PEF-treated suspension. Fig. 4F shows that *Chlorella* cells were

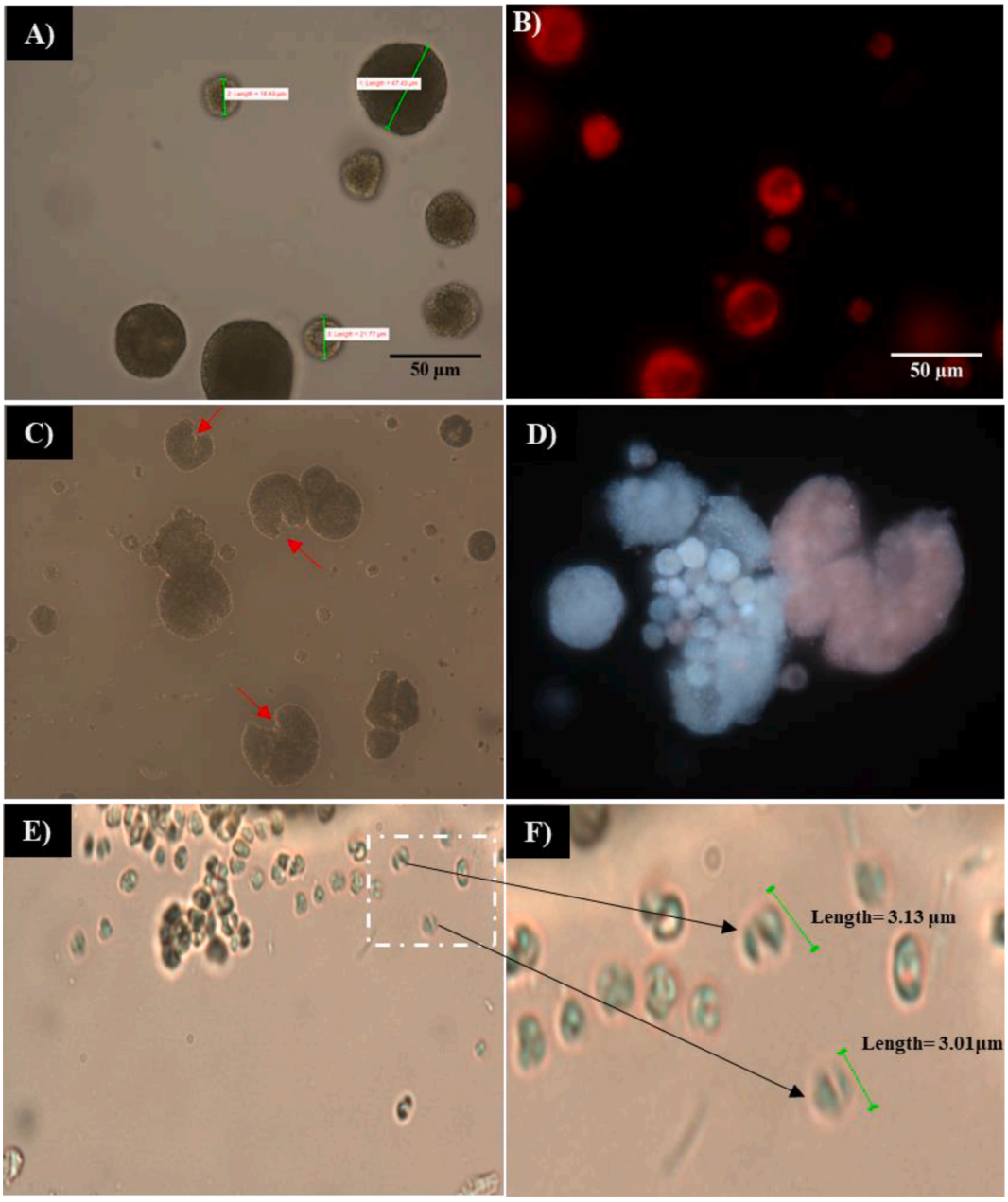

Fig. 4. Microstructure of *Chlorella* under fluorescence microscope. A (16×)/B (64×)-control extraction, C (16×)/D (64×)-PEF extraction, E (32×)/F (160×)-PEF extraction.

approximately 3 μm in diameter, which was consistent with previous reports on *Chlorella* (3 ~ 10 μm) (Lee et al., 2020). Fig. 4E and 4F showed that *Chlorella* cells were ruptured or perforated after PEF treatment, indicating that the 'electroporation phenomenon' occurred during the PEF extraction process, increased the recovery of *Chlorella* biomolecules. Similarly, Scherer et al. (2019) analysed the effect of PEF on the permeability of *Chlorella* cells through a microscope (63×). And their studies showed that PEF-treated *Chlorella* cells could be stained with Evans blue, a dye that could accumulate in permeabilized cells, indicating that PEF treatment caused the rupture of *Chlorella* cells (Scherer et al., 2019). In this study, although the aggregation of *Chlorella* made it impossible to count specific cell numbers, the results still showed that PEF treatment destroyed part of the cell morphology of *Chlorella*.

3.5. Scanning Electron Microscopy (SEM)

In this study, SEM was used to observe the effect of PEF on the cell structure of *Chlorella*, and the results are shown in Fig. 5. The size of *Chlorella* should be 3–10 μm, and from the scale in Fig. 5, the 'globular cells' should be formed by the aggregation of several *Chlorella*, which may be caused by the harvesting, drying and extraction process.

Fig. 5A (110×) and 5B (450×) are *Chlorella* samples before PEF extraction. Most of the *Chlorella* aggregates were spherical and some were shrunken, which may be related to the dehydration process during harvesting.

Fig. 5C and 5D are the morphology of *Chlorella* samples after PEF extraction. From Fig. 5C there are some 'non-spherical' fragments (marked by red circles), which are cell fragments after PEF extraction. Fig. 5E, 5F show similar debris generation in the control samples. When compared with Fig. 5A and 5B, the number of aggregates in Fig. 5C, 5D,

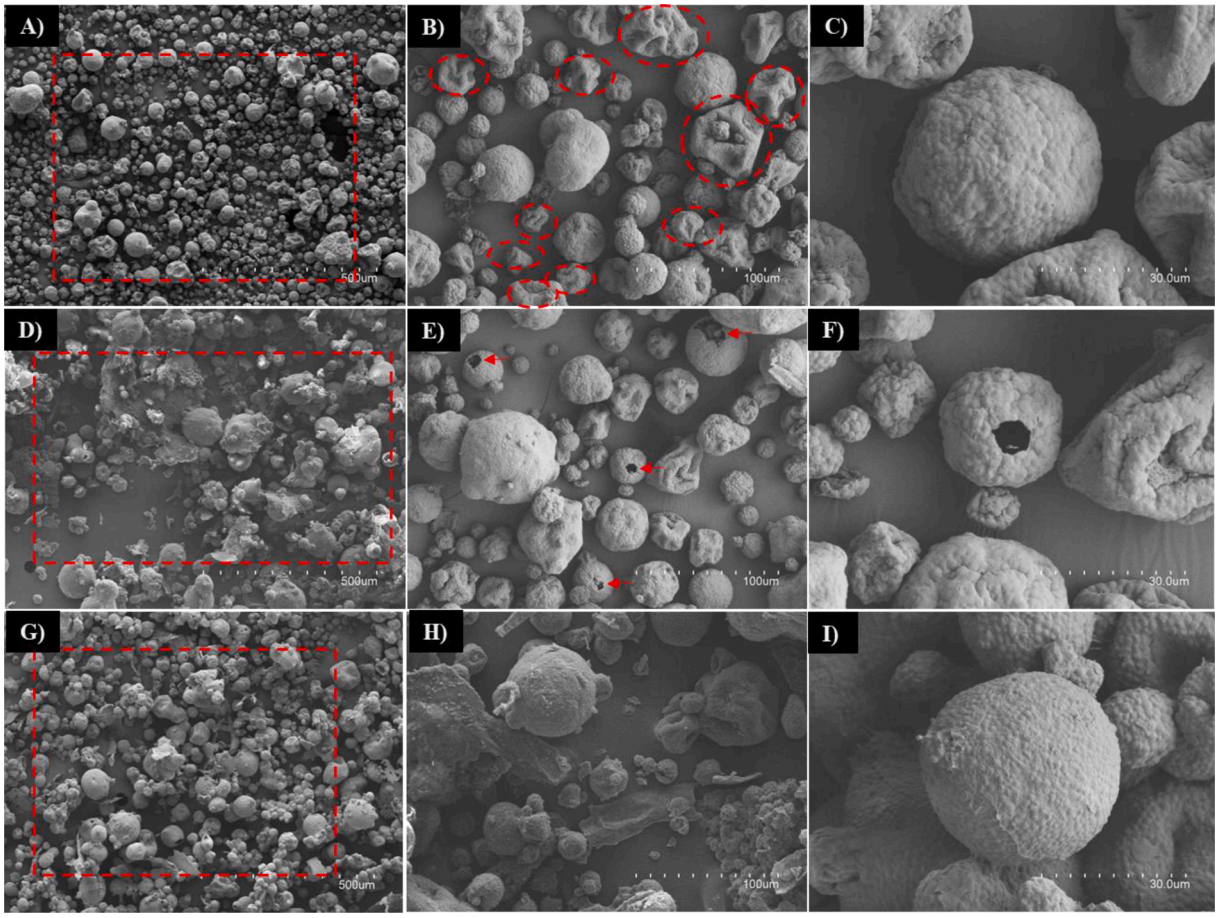

Fig. 5. Microstructure of *Chlorella* under SEM (scanning electron microscope). A (110×)/B (450×)/C (1500×)-*Chlorella* powder (before extraction), D (110×)/5E (450×)/F (1500×)-PEF-H_2O extraction, G (110×)/H (450×)/I (1500×)-control-H_2O extraction.

5E and 5F decreased, indicating that these aggregates were dispersed in the solvent (H_2O or 50 % DMSO) during extraction. Fig. 5 shows that there was no significant difference in the 'fragmentation' phenomenon between PEF and the control group. However, combined with the results of fluorescence microscopy, PEF treatment can indeed destroy the *Chlorella* aggregates to a certain extent, which is also present in the SEM image, but it is not obvious. Furthermore, fluorescence microscopy results (4E and 4F) showed that PEF treatment may cause perforation of microalgal cells, which was not easily observed due to the packing and aggregation of cells in the SEM images of our study. Similarly, other studies have reported the effect of PEF on the morphology of *Chlorella*, with inconsistent conclusions due to differences in PEF treatment parameters. A recent study also analysed the effect of PEF on the surface structure of *Chlorella*, however, the SEM results showed that PEF treatment (5 μs at 20 kV cm^{-1}, 31.8 kJ kg^{-1}) had no visible effect on the cell structure of *Chlorella* (Canelli et al., 2022). While in another study, Carullo et al. (2018) found that PEF-treated *Chlorella* cells were deformed (shrunken), which was attributed to the increased permeability of cells membrane by PEF treatment and the release of biomass (Carullo et al., 2018). These studies showed that the effect of PEF on the surface structure of *Chlorella* was different, which was related to the processing parameters of PEF, such as the number of pulses, electric field strength, processing time, etc. For example, during PEF treatment, high-intensity voltage and more pulses may aggravate microalgal cell rupture, while low-intensity voltage and relatively few pulses may not cause significant damage to microalgae, so different PEF conditions correspond to different results. Moreover, the properties of microalgae, such as cell size, cell wall thickness, microalgae harvesting process, etc., will also affect the effect of PEF extraction, which should be analysed comprehensively.

4. Conclusions

The recovery of *Chlorella* biomolecules was affected by different factors (PEF, extraction solution and time), and the proportion of biomolecules changed dynamically with the extraction time during the extraction process. The PEF treatment increased the yield of antioxidant biomolecules in *Chlorella*, and microscopic analysis indicated that this was mainly related to the PEF electroporation mechanism. *Chlorella* contained various minerals, and the NRV values calculated based on RDAs suggest that *Chlorella*-water PEF-extract could be used as a mineral source for different populations, and similar studies have not been reported yet. In addition, the content of antioxidant biomolecules and minerals in PEF extract relative to *Chlorella* total nutrient content could be further improved, which could be achieved by changing the processing conditions of PEF or combining with other extraction technologies.

CRediT authorship contribution statement

Min Wang: Investigation, Formal analysis, Visualization, Writing – original draft. **Jianjun Zhou:** Conceptualization, Methodology, Writing – review & editing. **Juan Manuel Castagnini:** Writing – review & editing. **Houda Berrada:** Writing – review & editing. **Francisco J. Barba:** Conceptualization, Methodology, Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134615.

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