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Pulsed electric fields (PEF), pressurized liquid extraction (PLE) and combined PEF + PLE process evaluation: Effects on *Spirulina* microstructure, biomolecules recovery and Triple TOF-LC-MS-MS polyphenol composition

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ABSTRACT

This study aims at evaluating the impact of different processes-pulsed electric fields (PEF), pressurized liquid extraction (PLE) and a multistep process combining PEF + PLE on the yield of antioxidant compounds (protein, polyphenols, chlorophyll *a*, chlorophyll *b*, and carotenoids) from *Spirulina*. Firstly, the effects of PEF or PLE treatment on the extraction yield of *Spirulina* biomolecules were evaluated. To further increase the extraction yield, PEF + PLE was used, as an innovative extraction approach. The results showed that PEF + PLE greatly improved the extraction yield compared with the PEF or PLE treatments alone. Compared with Folch extraction (conventional control technique), PEF + PLE significantly ($P < 0.05$) shortened the extraction time (−165 min) and increased the protein, polyphenol, chlorophyll *a* and antioxidant capacity values of *Spirulina* extracts by 1328%, 979%, 11% and 47% respectively. Furthermore, Triple TOF-LC-MS-MS results showed that PEF + PLE increased both the type and content of phenolic compounds. The above results were attributed to PEF-induced damage on *Spirulina* helical structure, which was verified by fluorescence and scanning electron microscopy.

1. Introduction

In recent years, marine microalgae biomass have attracted much attention due to their high content of high-added-value compounds such as nutrients (proteins, fatty acids, carbohydrates, vitamins and minerals) as well as antioxidant compounds (polyphenols and pigments such as chlorophylls, carotenoids, etc.) with antioxidant, antibacterial and anti-inflammatory properties, among others (Costa, Freitas, Moraes, Zaparoli, & Morais, 2020; Junior et al., 2020). Among microalgae biomasses, a growing interest has been shown over the last years in *Spirulina*, as it is included in the catalog of the European Commission as a novel food for human consumption.

Although the whole biomass can be consumed after different preparation steps, especially to avoid microbial contamination, the different biomolecules of *Spirulina* are also of a great interest and several traditional extraction methods mainly based on solid-liquid or liquid-liquid extractions (i.e., Soxhlet extraction, Folch, etc.) have been used to recover the different high-added value compounds from these microalgae (Lee, Yoo, Jun, Ahn, & Oh, 2010). However, there is a need to develop and optimize new extraction approaches as the traditional methods involve the use of high amount of solvent, which can be toxic, long extraction times and high temperatures (Chen, Liu, Song, Sommerfeld, & Hu, 2020; Mansour, Abo El-Enin, Hamouda, & Mahmoud, 2019). Moreover, some of these biomolecules are labile and can be easily

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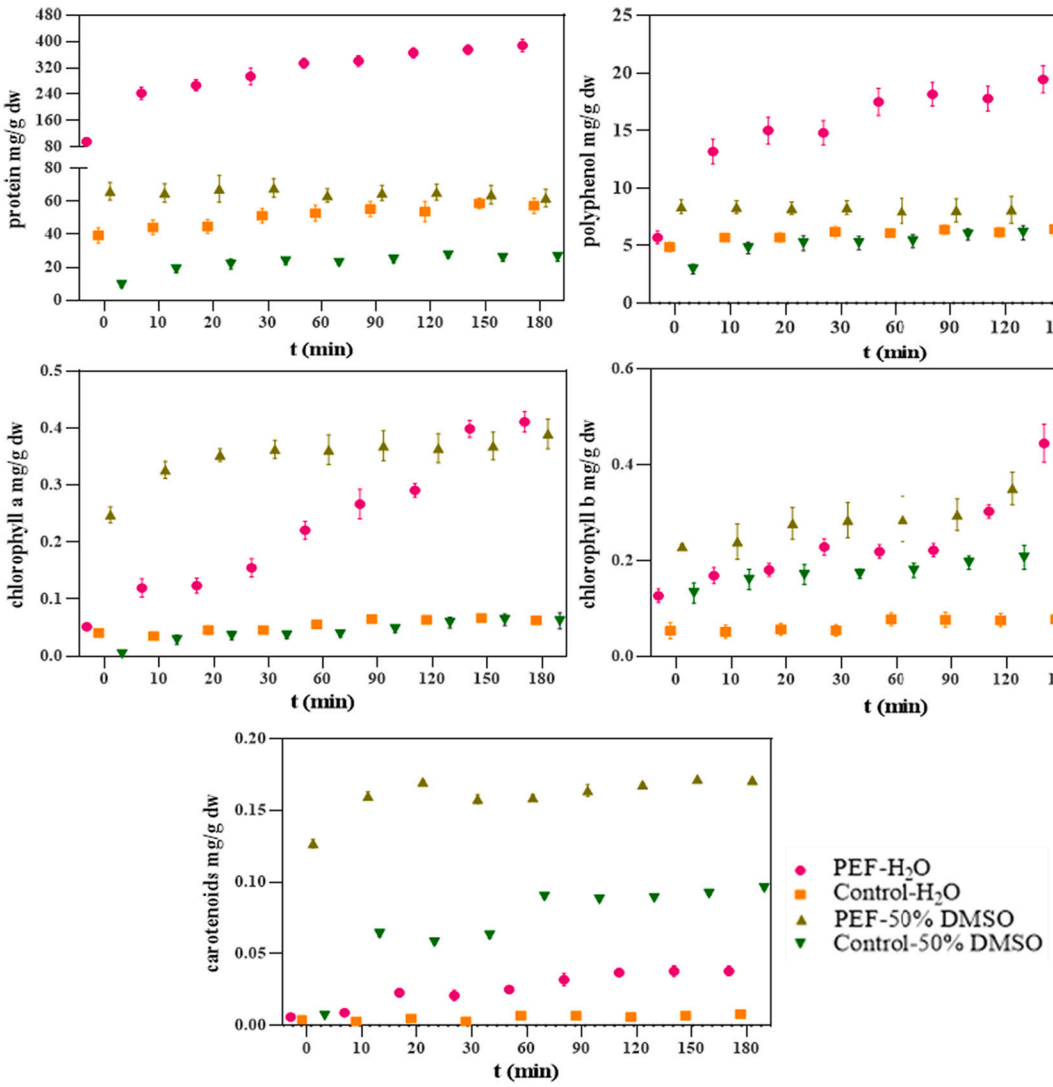

Fig. 1. Protein, polyphenol, chlorophyll *a*, chlorophyll *b* and carotenoids yield (dw) from *Spirulina* treated with PEF/without PEF (control) under different extraction times (0– 180 min) and solvents (H_2O, 50% DMSO).

destroyed under such extraction conditions.

Considering the current focus on sustainability in agreement with Sustainable Development Goals (SDGs), from the perspective of improving extraction efficiency, preserving the biological activity of the compounds and environmental protection, this requires the extraction conditions to be as gentle, efficient, and environmentally friendly as possible (Zhao, de Alba, Sun, & Tiwari, 2019). Therefore, new extraction technologies are recognized in the extraction of microalgae biomass, including PEF (pulsed electric fields), PLE (pressurized liquid extraction), UAE (ultrasound-assisted extraction), MAE (microwave-assisted extraction), etc. (De Sousa et al., 2017; De Sousa et al., 2018; Guo et al., 2019; Kokkali et al., 2020; Vasistha, Khanra, Clifford, & Rai, 2021). The working principles of these innovative extraction technologies are different, and researchers have already focused on combining multiple innovative extraction techniques to improve the yield of microalgae´ nutrients. For example, the use of UAE + MAE enhanced oil production from *Chlorella* (Ma et al., 2015), the combined US + freezing and thawing method increased the yield of phycocyanin from *Spirulina* (Tavanandi, Mittal, Chandrasekhar, & Raghavarao, 2018), etc. Therefore, combining different extraction technologies in multistep processes seem to be interesting approaches to increase the extraction efficiency, reducing consumption of toxic solvents, extraction time and the use of high temperatures.

In our previous studies, PEF and PLE were used to obtain high-added-value compounds with strong antioxidant properties from microalgae *Tetraselmis chuii* and *Phaedoactylum tricornutum* (Kokkali et al., 2020) and marine fish side streams (Wang, Zhou, Collado, & Barba, 2021). PEF is an innovative technology that can be used to produce safe, high-quality, and nutritious food with excellent flavor and extended shelf life. PEF devices typically include a electrical pulse generator, a treatment chamber, and electrodes, with the electrical pulse placed between or through two electrode (Puértolas, Koubaa, & Barba, 2016). PEF treatment can alter cell membrane properties due to electric field pulse discharges, resulting in increased cell membrane permeability (Blahovec, Vorobiev, & Lebovka, 2017). PLE technology is efficient and produces less waste during the extraction process, which can reduce costs and save time. In the PLE process, the solvent is below the critical point to maintain the liquid phase during extraction, and the pressure and temperature conditions are chosen to increase the mass transfer rate by reducing the solvent surface tension and viscosity and increasing the solubility of the components, which makes the solvent more permeable into the extracted solid matrix (Andrade et al., 2021).

In this line, on the one hand, the application of short electrical pulses can control the thermal effect at a low level, which can protect the antioxidant properties of algae biomass (Gómez et al., 2019). On the other hand, the application of high-pressure of PLE greatly reduces the

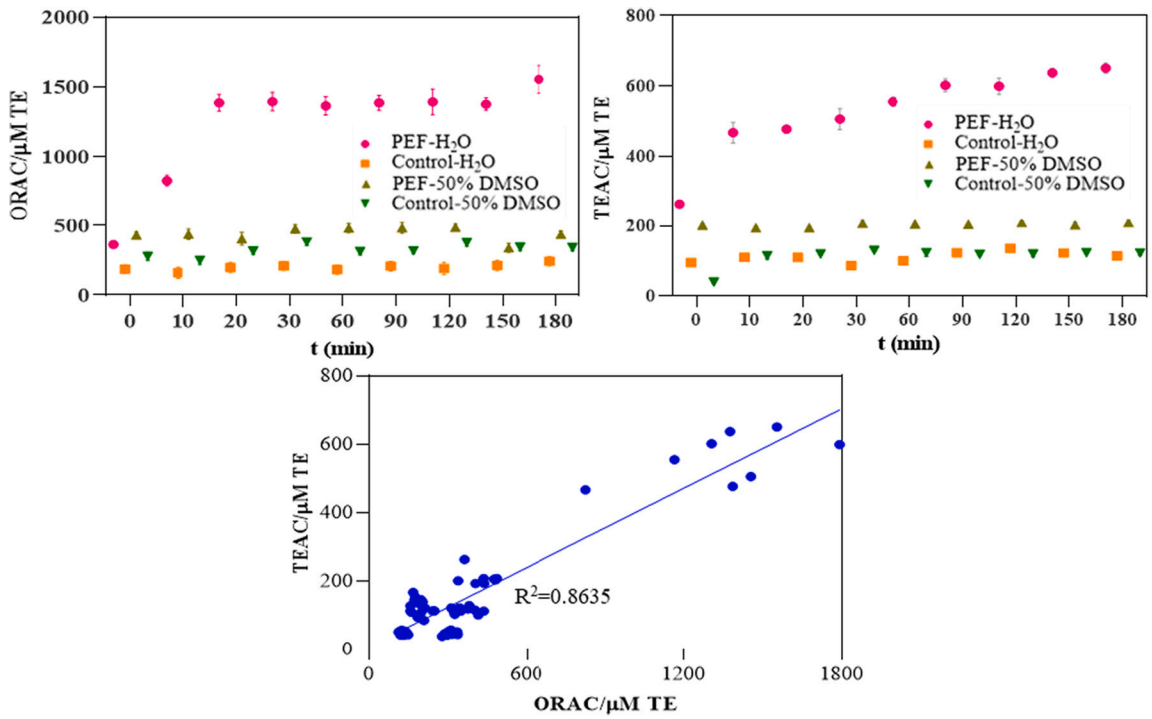

Fig. 2. Antioxidant capacity results of Spirulina extracts treated with PEF/without PEF (control) under different extraction times (0– 180 min) and solvents (H_2O, 50% DMSO). ORAC and TEAC corresponds to oxygen radical antioxidant capacity and Trolox equivalent antioxidant capacity respectively.

strong interaction force between the solute and the matrix, such as van der Waals forces, hydrogen bonds, etc., thereby promoting the diffusion of the solvent into the samples (Zhuang, McKague, Reeve, & Carey, 2004). That is, PEF treatment can lead to changes in the permeability of microalgal cell membranes, while PLE can promote the entry of solvents into the microalgal cytoplasm to dissolve bioactive substances. Therefore, PEF and PLE have a potential 'synergistic' effect in nutrient and bioactives' recovery of *Spirulina*, which is of great significance for the efficient recovery of *Spirulina* biomolecules with good bioactivity.

In this study, PEF, PLE and the combination of PEF + PLE were used to recover protein, polyphenols, pigments (chlorophyll *a*, chlorophyll *b*, carotenoids) from *Spirulina* and the antioxidant capacity of the extracts was evaluated. In addition, from the point of saving reagents and reducing contamination as well as to improve solubility of DMSO, the application of H_2O + organic solvent mixtures (DMSO) was used for biomolecule extraction in this study. The effects of PEF on *Spirulina* microstructure were also analyzed using both fluorescent and scanning electron microscopy. Finally, the Triple TOF-LC-MS-MS phenolic profile of the combined process PEF + PLE was evaluated and compared with that obtained after a conventional Folch extraction process.

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 and $K_2S_2O_8$ were purchased from Sigma-Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3) was acquired from VWR (Saint-Prix, France). Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). Diatomaceous earth and other materials for generation of PLE extracts were bought from Dionex (Dionex, Leeds, UK). Deionized water (resistivity>18 MΩ cm^{-1}) was from Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.2. Samples

Spirulina comes from *Arthrospira platensis* species, strain paracas 15,016, being Paracas the lake where it originally comes from (Lima, Peru). Cultivation took place at Eco*Spirulina* company (Serra, Valencia, Spain) in raceway ponds using a greenhouse under natural sunlight. During the experiment, the day-time temperature was 32 °C and temperature decreased to 24 °C at night. The pH of the culture varied between 9.8 and 10.4, which was controlled by the addition of CO_2 at the time of harvesting daily. *Spirulina* biomass was filtered using a tambor filter of 30 micra mesh. Cultivation medium went back to the cultivation pond, while biomass was vacuum-pressed and then frozen in 50 g portions. The sample was freeze-dried at −40 °C for 72 h to reduce the degradation of poorly resistant biomolecules at higher temperatures before extraction process.

2.3. Microalgae biomolecules extraction

2.3.1. PEF extraction

Spirulina powder and water (~2 g/200 mL) were mixed in the treatment chamber ranging the conductivity between 1000–2000 μS/cm. The minimum electric field strength required to produce changes in the cell is 1 kV/cm, and when the pulse duration is milliseconds, an electric field of 3–4 kV/cm can produce electroporation. Then, the samples were PEF-treated (3 kV/cm, 44 pulses, 99 kJ/kg) according to previous studies (Martí-Quijal et al., 2021). The temperature and conductivity of each sample were measured with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics, Weilheim in Oberbayern, Germany). After PEF treatment, the samples were transferred to a beaker, and 200 mL H_2O or DMSO were added to make the sample-solvent system reach to 2 g algae powder/400 mL solvent. A magnetic stirrer was used to continuously stir the samples at room temperature and the samples were collected at 0, 10, 20, 30, 60, 90, 120 and 180 min, respectively. It should be noted that in PEF extraction,

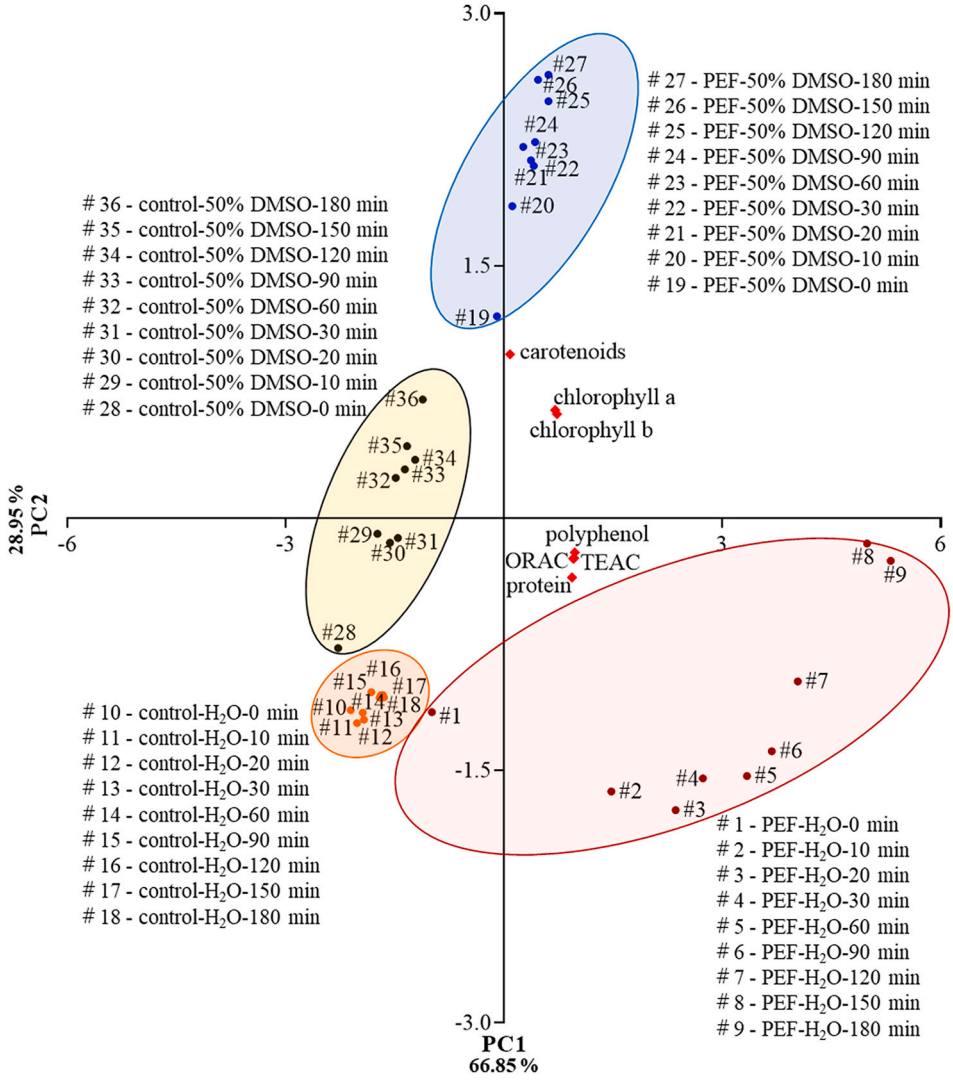

Fig. 3. Principal component analysis (PCA) of the correlation between extraction conditions (PEF-H_2O, PEF-50% DMSO, control-H_2O, control-50% DMSO, extraction time of 0– 180 min), biomolecules yield and antioxidant properties.

pure DMSO cannot be used as an extraction reagent due to its non-conductive properties. The control experiment was carried out as 2 g algae powder/400 mL H_2O or 2 g algae powder/200 mL H_2O + 200 mL DMSO stirred at room temperature and the samples collected at the same time as PEF extraction. Finally, the samples were centrifuged (2504 *g*, 4 °C, 15 min), and the supernatants were collected and stored at −20 °C until needed for analyses.

2.3.2. PLE extraction

The PLE extraction was based on our previous studies (Zhou et al., 2021). Microalgae samples and diatomaceous earth were thoroughly mixed (0.5 g: 1.5 g) in a mortar and then placed into the PLE extraction tank. An ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) was used to perform the extraction, and the operating conditions were referred to our previous study: preheating period 1 min, heating period 5 min, flush volume 60%, nitrogen purge 60 s, extraction pressure of 103.4 bars, extraction temperature of 40 °C, extraction time of 15 min. Referring to related studies (Parniakov et al., 2015b), different proportions of DMSO (50% DMSO, DMSO) were used for PLE extraction to observe the effect of DMSO concentration on the PLE extraction yield. For 0.5 g *Spirulina* (dw), the final extracts volume was near 20 mL. According to the PLE extracts volume, the control experiment was carried out as 0.5 g algae powder/20 mL solvent (H_2O, 50% DMSO, DMSO) stirred at 40 °C for 15 min. The samples were centrifuged (2504 *g*, 4 °C, 15 min), and the supernatants were stored at −20 °C until needed for analyses.

2.3.3. PEF + PLE extraction

The PEF and PLE extraction processes were then combined in a multistep extraction process to further obtain a higher yield of biomolecules from *Spirulina*. Similar to PEF extraction (Section 2.3.1), *Spirulina* powder and H_2O (~2 g/200 mL) were mixed in the treatment chamber ranging the conductivity between 1000–2000 μS/cm. The samples were PEF-treated (3 kV/cm, 44 pulses, 99 kJ/kg) and transferred to a beaker. Then, according to the selected PEF extraction conditions (Section 2.3.1), 200 mL H_2O were added to make the sample-solvent system reach to 2 g algae powder/400 mL solvent and continuously stirred at room temperature for 120 min. The samples were collected and freeze-dried at 40 °C for 72 h for further PLE extraction. Based on the selected PLE extraction conditions (Section 2.3.2), freeze-dried samples and diatomaceous earth were thoroughly mixed (0.5 g:1.5 g) in a mortar and then placed into the extraction tank. The operating conditions were as follows: preheating period 1 min, heating period 5 min, flush volume 60%, nitrogen purge 60 s, extraction pressure 103.4 bars, extraction temperature 40 °C, extraction time 15 min with DMSO as solvent. Finally collecting the extracts as PEF + PLE

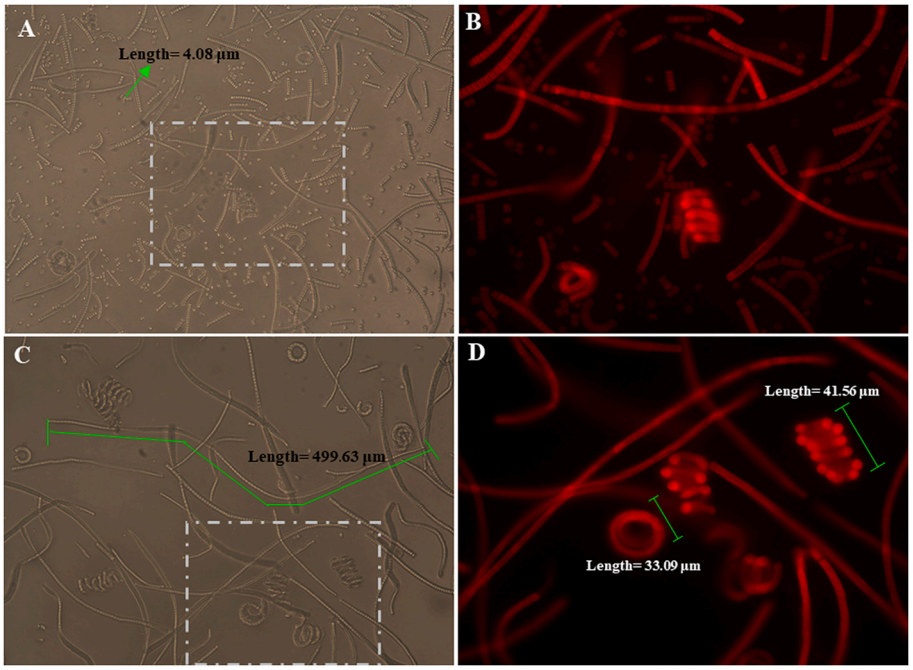

Fig. 4. The effect of pulsed electric fields (PEF) on the microstructure of *Spirulina*. 4A (16×)/4B (32×)-PEF-H_2O extracts, 4C (16×)/4D (32×)-control-H_2O extracts.

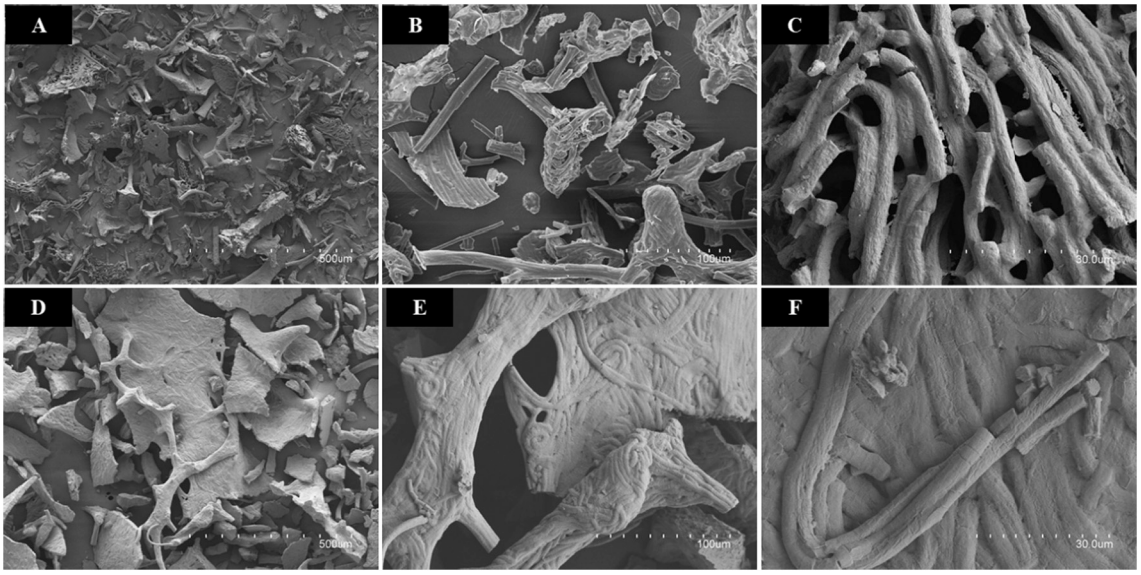

Fig. 5. The effect of pulsed electric fields (PEF) on the microstructure of *Spirulina*. 5A (110×)/5B (450×)/5C (1500×)-PEF-H_2O extracts, 5D (110×)/5E (450×)/5F (1500×)-control-H_2O extracts.

samples for further analysis. The conventional Folch extraction method was used as a control (Folch, Lees, & Sloane Stanley, 1957; Ulmer, Jones, Yost, Garrett, & Bowden, 2018) and the results were compared with those obtained after PEF, PLE and PEF + PLE extractions. For Folch extraction, chloroform and methanol were mixed (5:2, *v*/v) to obtain the extraction reagent. Then, the microalgae powder and the extraction reagent were mixed at the ratio of 1 g/20 mL and stirred at 40 °C under magnetic stirring under darkness for 6 h. The extract was centrifuged (2504 *g*, 4 °C, 15 min) and the supernatant was stored at −20 °C for subsequent experimental analysis.

2.4. Microalgae cell structure

2.4.1. FM (Fluorescence Microscope)

The samples obtained after PEF treatment (3 kV/cm, 44 pulses, 99 kJ/kg) (see Section 2.3.1) were centrifuged at 157 *g*/10 min. After centrifugation, the supernatant was removed to collect the precipitate, and it was washed with 90% methanol and centrifuged. The sample was repeatedly washed until the supernatant was colorless, and the precipitate was collected and diluted with deionized water. Then the cell structure of the microalgae was observed under a fluorescence microscope. The control group was set as a mixed extract of microalgae and H_2O without PEF treatment (see Section 2.3.1). The bright-field digital images were collected through an Eclipse 90i Nikon microscope (Nikon corporation, Japan) with an 3/16 x objective, equipped with a digital

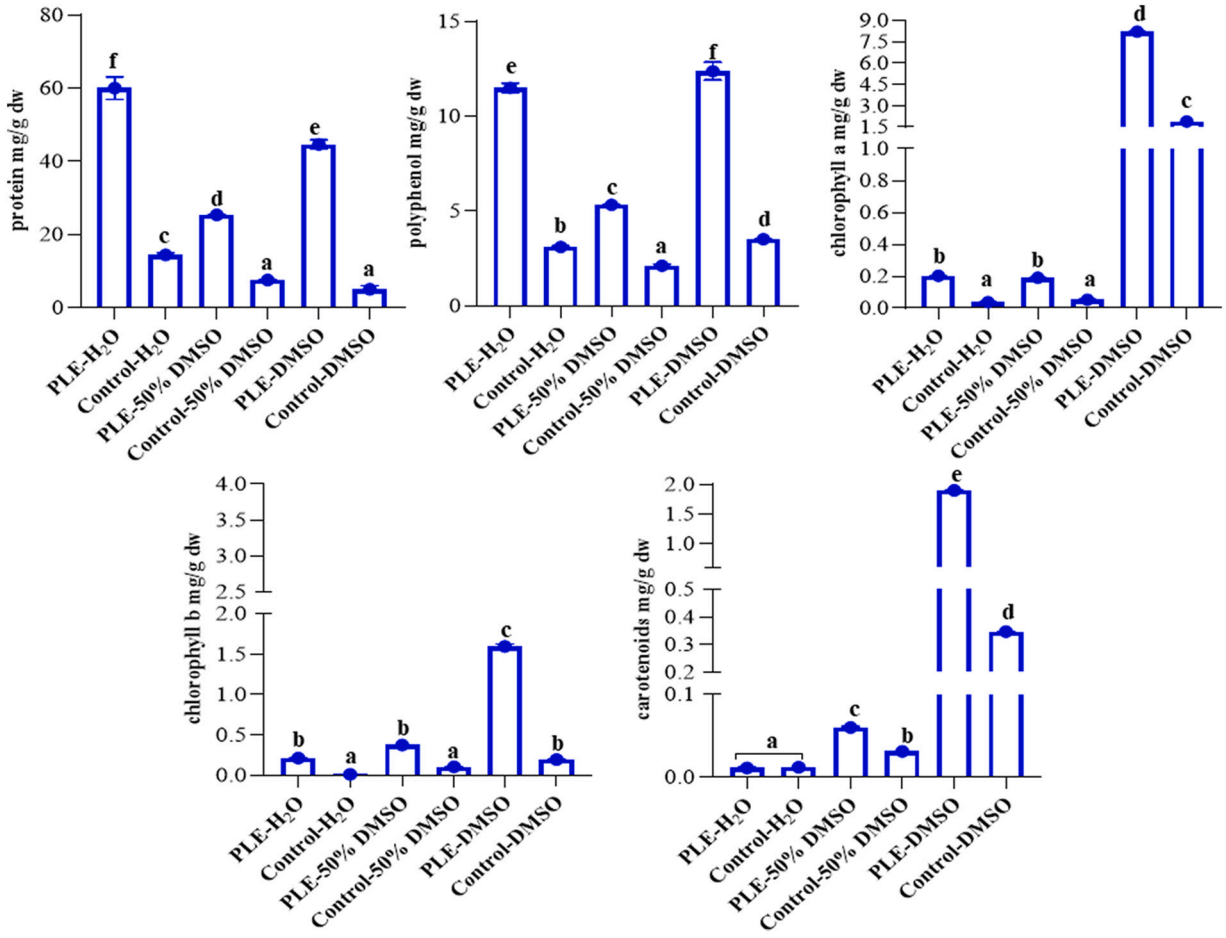

Fig. 6. Effect of PLE, control (without PLE) and different solvents (H_2O, 50% DMSO, DMSO) on the yield (mg/g dw) of protein, polyphenol, chlorophyll *a*, chlorophyll *b* and carotenoids. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$).

camera (Nikon DS-5Mc). Images were processed and analyzed by the Nis Elements BR 2.32 software (Nikon corporation, Japan) software.

2.4.2. SEM (scanning electron microscopy)

A scanning electron microscope (S-4800) was used to analyze the microstructure of freeze-dried samples of microalgae (Fang, Xu, Kawashima, Hata, & Kijima, 2021). Freeze dried microalgae samples in Section 2.4.1 were then mounted on specimen stubs with colloidal silver, sputter-coated with gold-palladium and imaged with a SEM (S-4800) at magnifications of 110×, 450× and 1500×.

2.5. Biomolecules (protein, polyphenol, pigments) and antioxidant properties analyses

The bicinchoninic acid (BCA) method was used to analyze the protein content of the extracts (Al Khawli, Martí-Quijal, Pallarés, Barba, & Ferrer, 2021) The protein content was determined using a calibration curve (0–2000 mg/L) with bovine serum albumin (BSA) as a standard. Ten microliters of samples or BSA and 200 μL of BCA working solution were added to a 96-well plate, mixed well, and incubated at 37 °C for 30 min. Then, the absorbances were measured at 562 nm.

On the other hand, spectrophotometry was used to analyze the pigments concentration of *Spirulina* extracts. The absorbance values and formulas used to analyze extracts varied with solvent, for DMSO and 50% DMSO extracts, the equation was as follows (Wellburn, 1994):

$$Ca = 12.47 \times Abs_{665.1nm} - 3.62 \times Abs_{649.1nm}$$

$$C_b = 25.06 \times Abs_{649.1nm} - 6.5 \times Abs_{665.1nm}$$

$$C_{Carotenoids} = (100 \times Abs_{480nm} - 1.29 \times C_a - 53.78 \times C_b)/220$$

For H_2O extracts, the equation was as follows (Kokkali et al., 2020):

$$C_a = 16.82 \times Abs_{665nm} - 9.28 \times Abs_{653nm}$$

$$C_b = 36.92 \times Abs_{653nm} - 16.54 \times Abs_{665nm}$$

$$C_{Carotenoids} = (1000 \times Abs_{470nm} - 1.91 \times C_a - 95.15 \times C_b)/225$$

where C_a and C_b corresponded to the concentrations (mg/L) of chlorophyll *a* and chlorophyll *b* respectively. The final yield of pigments was calculated based on the dry weight of *Spirulina* (mg/g dw).

The oxygen radical antioxidant capacity (ORAC) (Gregório et al., 2020) and the Trolox equivalent antioxidant capacity (TEAC) (Sridhar & Charles, 2019) assays were used to evaluate the antioxidant capacity of the extract. In the ORAC assay, Trolox and 2,2′-Azobis(2-methylpropionamidine)dihydrochloride (AAPH) were used both as antioxidants and oxygen free radicals, respectively, while phosphate buffer was used as a blank control. To carry out the assay, 50 μL of extract and 50 μL of the 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 were 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 three times to make the coefficient of variation value less than 10%.

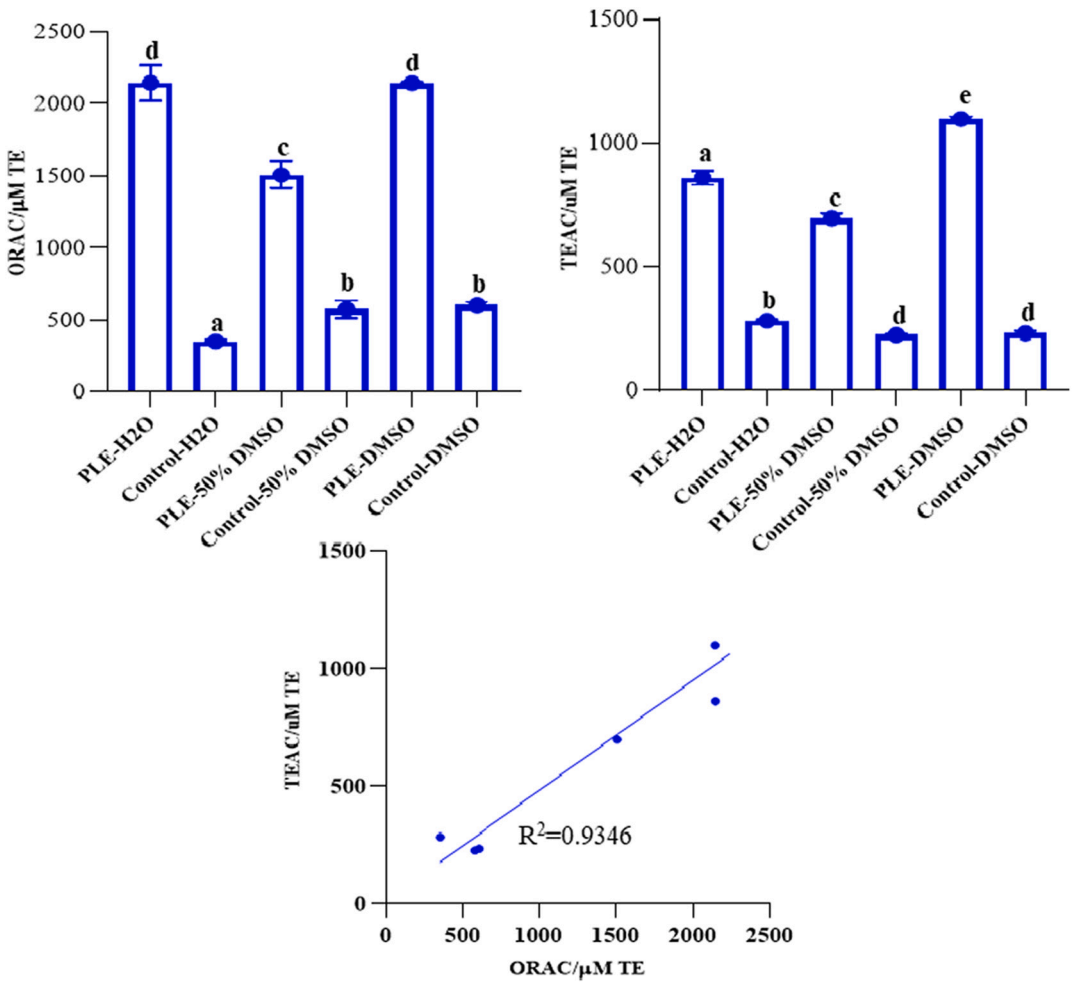

Fig. 7. Effect of PLE, control (without PLE) and different solvents (H_2O, 50% DMSO, DMSO) on the antioxidant capacity of *Spirulina* extracts. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$). ORAC and TEAC corresponds to oxygen radical antioxidant capacity and Trolox equivalent antioxidant capacity respectively.

Regarding TEAC assay, first the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) working solution was prepared. For that purpose, 25 mL of 7 mM ABTS were mixed with 440 μL of 140 mM potassium thiosulfate solution and incubated under darkness at room temperature for 12–16 h to obtain the working solution. 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 and 2 mL of the working solution were mixed, 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.

The Folin-Ciocalteu method was used to analyze the total polyphenol content in the extracts (Korzeniowska, Łęska, & Wieczorek, 2020). That is, 0.2 mL of sample, 1 mL of Folin-Ciocalteu (diluted with water at a ratio of 1:10, *v*/v) and 0.8 mL of a sodium carbonate solution (75 g/L) were mixed and incubated in a water bath at 50 °C for 10 min. Then, the absorbances were measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard to prepare the calibration curve to quantify the amount of total polyphenols in the extracts.

Finally, the phenolic profile of *Spirulina* extracts was evaluated using a TripleTOF™ 5600 (ABSCIEX) LC/MS/MS system equipped with Agilent 1260 Infinity (Agilent, Waldbronn, Germany). The chromatographic separation was carried out on a Waters UPLC C18 column 1.7 μm (2.1 × 50 mm) Acquity UPLC BEH·C18 from Waters (Cerdanyola del Vallès, Spain). The mobile phase is composed of water (0.1% CH_2O_2, A) and methanol (0.1% CH_2O_2, B). The gradient elution of the mobile phase is as follows: 0–13 min, 90% (A) and 10% (B); 13–15 min 100% (B); 15.1–22 min, 90% (A) and 10% (B). The flow rate and injection volume are 0.4 mL/min and 5 μL. The MS acquisition is under a mass range of 80–1200 *m/z*. The calibration was carried out using an external calibration delivery system, which infuses the calibrating solution before samples introduction. The MS is operated using an Information Dependent Acquisition (IDA) with the survey scan type (TOF-MS) and the dependent scan type (Product Ion) at −50 V of collision energy. The MS parameters are ion spray voltage of −4500 V, decluttering potential of 90 V, collision energy of −50 V, temperature at 400 °C with curtain gas of 25 psi, ion source gas 1 at 50 psi and ion source gas 2 at 50 psi, IDA MS/MS is performed using the following criteria: ions that exceeded 100 CPS, ion tolerance 50 m Da, collision energy fixed at 25 V and dynamic background subtraction activated. For the quantification, an external calibration curve using a representative polyphenol of each group of phenolic compounds potentially found in the samples was prepared, being the following polyphenols, the ones selected for each specific group: phenolic acids (gallic acid); flavonoids (flavones: apigenin; flavonols: kaempferol; flavanones: naringenin; flavanols: catechin); stilbenes (resveratrol); isoflavonoids (genistein); phenylethanoids: (hydroxytyrosol).

2.6. Statistical analysis

One-way ANOVA with Dunnett's multiple comparisons test was performed using Statgraphics® Centurion XV (Statpoint Technologies,

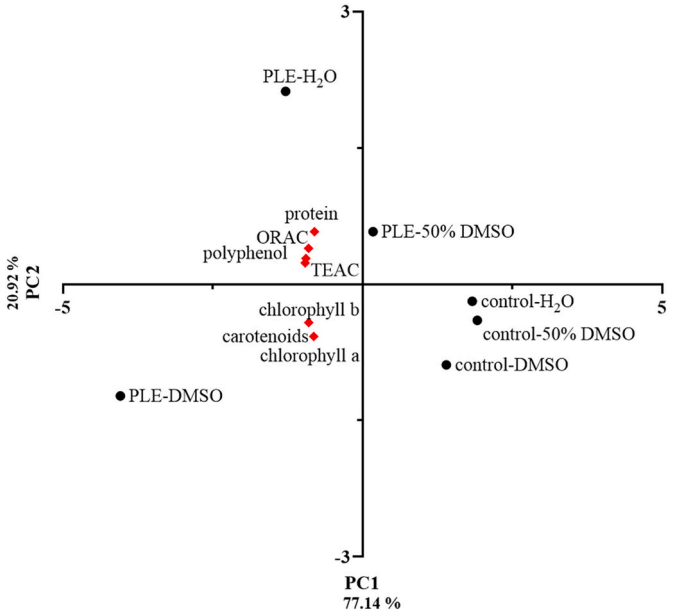

Fig. 8. Principal component analysis (PCA) of the correlation between extraction conditions (PLE-H_2O, PLE-50% DMSO, PLE-DMSO, control-H_2O, control-50% DMSO, control-DMSO), biomolecules yield and antioxidant properties.

Inc., USA) and it was used to detect statistically significant differences of results between the different extraction technologies on the following: yields of proteins, polyphenols, chlorophyll *a*, chlorophyll *b*, carotenoids, and antioxidant properties. Statistical significance was accepted at $p < 0.05$. At least three replicates were carried out for all the experiments.

3. Results and discussion

3.1. PEF extraction- Spirulina biomolecules yield

The results of protein, polyphenol, chlorophylls a and b and carotenoids of the *Spirulina* extracts obtained at different times (0–180 min) assisted by PEF pretreatment and different solvents (H_2O, 50% DMSO) were shown in Fig. 1. As it was shown in the figure, the *Spirulina* protein yield ranged between 10 and 400 mg/g dw, observing increased values of protein (up to 300 mg/g dw) when PEF was applied compared to the samples without PEF pretreatment, independently of the solvent used. It should be also noted that the protein extraction increased until 60 min, then the values reached a plateau, and it was not observed a significant increase in the protein recovery with the further extend of extraction time. Moreover, it was observed that the protein extraction was higher when H_2O was used compared to 50% DMSO. The polyphenol yield from *Spirulina* ranged between 3–20 mg/g dw. Similar to the results found for protein, PEF pretreatment increased the polyphenol content of the extracts compared to untreated samples, independently of the solvent used. Moreover, compared with 50% DMSO, H_2O is more effective in promoting polyphenol recovery, observing a rapid increase polyphenol yield (from 7.5 to 17.5 mg/g dw) within 0–60 min in PEF-H_2O extracts, being the final yield near 20 mg/g dw at 180 min.

The results showed a low content of chlorophyll *a*, chlorophyll *b* and carotenoids in *Spirulina* extracts, corresponded to 0.05–0.4 mg/g dw, 0.1–0.4 mg/g dw and 0–0.2 mg/g dw, respectively. PEF treatment increased the pigment content of the *Spirulina* extract in both H_2O and 50% DMSO extracts. Considering the effect of time, chlorophyll *a*, chlorophyll *b* and carotenoids yield curves after PEF increased slowly with the elapse of extraction time up to 180 min, while the yield in the control group remained stable, especially in the control-H_2O group, which was hardly affected by the extraction time.

Similar results were found in other previous studies for protein and polyphenol extraction kinetics. For example, Parniakov et al. (2015a, 2015b) explored the impact of PEF on the recovery of biomolecules of microalgae when prolonged extraction times with H_2O as a solvent, showing that the biomolecules content in microalgae extracts gradually increased over time, especially increasing rapidly before 3600 s, and observing a slow increase rate until extraction time of 10,800 s (Parniakov et al., 2015a). These results could be explained as the lower solution saturation in the early stage of extraction was conducive to the dissolution of biomolecules into the solvent. However, as the extraction time was further extended, higher solution saturation might lead to a decrease in the extraction rate.

Obviously, the extraction solvent in this study affected the extraction results of *Spirulina* biomolecules. For the low extraction yield of protein in 50% DMSO extracts, another study attributed the phenomenon to the precipitation of proteins at high concentration of organic solvent during the extraction process (Arakawa, Kita, & Timasheff, 2007). Previous studies have shown that polyphenols solubility decreased with the DMSO concentration increased from 0–50%, and then increased with the DMSO concentration increased from 50% to 100%, indicting the low solubility of biomolecules in 50% DMSO, which was consistent to our research (Parniakov et al., 2015b). However, the pigments extraction trend was different from protein and polyphenol. The higher contents of chlorophyll *a*, chlorophyll *b* and carotenoids presented in 50% DMSO was related to solvent dissolution properties. DMSO $(CH_3)_2SO$) had a hydrophilic sulfinyl group and two hydrophobic methyl groups, which could dissolve both water-soluble compounds and fat-soluble compounds, resulting in the increase of fat soluble pigments content in a 50% DMSO extracts (Mueller, Trapp, & Neubert, 2019). Overall, the results indicated that both PEF and solvent had important effect on protein, polyphenol, and pigments yield, which together determined the extraction efficiency.

3.2. Effects of PEF on antioxidant properties

ORAC and TEAC assays were used to analyze the antioxidant capacity of *Spirulina* extracts. As it was shown in Fig. 2, the antioxidant capacity of PEF extracts was higher than that of control group (no PEF treatment). The antioxidant capacity evaluated by ORAC and TEAC were both as PEF-H_2O > PEF-50% DMSO>control-H_2O/control-50% DMSO, which was in the same order as the polyphenol content, indicating that the polyphenols played an important role in the total antioxidant capacity of the extracts.

From the perspective of the effect of extraction time on the total antioxidant capacity, PEF-H_2O extracts showed a significant increase of ORAC and TEAC value over time (0–30 min), which could be attributed to the increase of polyphenols and chlorophylls content during this process. The antioxidant value of other extraction curves did not change significantly within 0–180 min.

Specifically, TEAC and ORAC assays evaluated the capacity of the samples to remove 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) and 2,2′-azobis-2-methyl-propanimidamide respectively (Şen, Bener, Bekdeşer, & Apak, 2021). Considering the possible evaluation differences caused by the different radical ions in ORAC and TEAC assays, we further analyzed the correlation coefficient between these two experiments. The results showed that there was a good positive correlation ($R^2 = 0.8635$) between TEAC and ORAC, indicating that they were highly consistent in the evaluation of antioxidant properties. In addition, correlation analysis based on extraction conditions (PEF, time, solvent), biomolecules (proteins, polyphenols, pigments) and antioxidant properties (ORAC, TEAC) was further performed using PCA (principal component analysis), and the results were shown in Fig. 3. The proportion of variance of the two principal components was 66.85% for PC1 and 28.95% for PC2, respectively. Different extraction condition groups were divided into four categories by extraction technology (PEF,

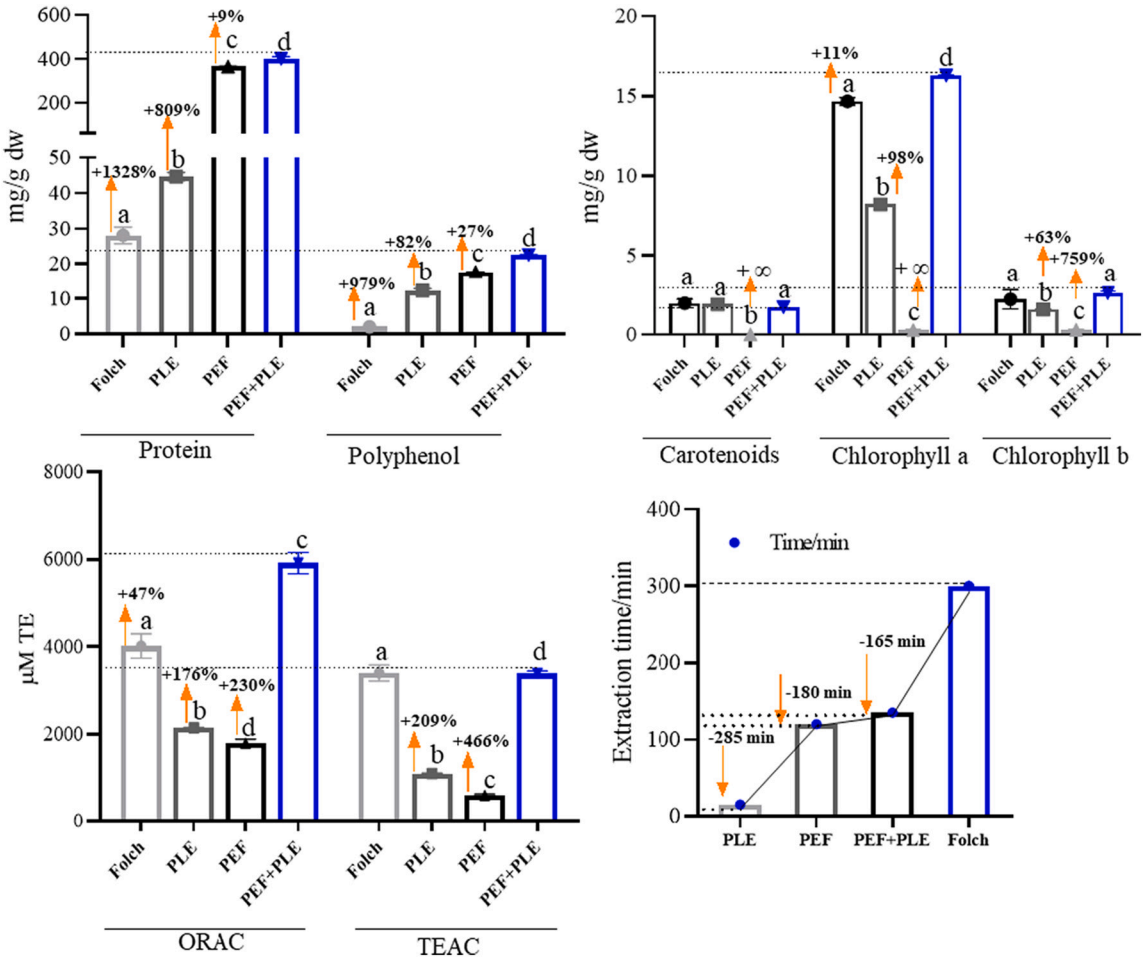

Fig. 9. Protein, polyphenol, carotenoids, chlorophyll *a*, chlorophyll *b*, carotenoids content and antioxidant properties of *Spirulina* extracts as well as extraction time (refers to the extraction time for the above results, not the extraction time under the same yield) required for PEF + PLE, PEF, PLE and Folch extraction processes. Same lowercase letters indicate no significant differences ($p > 0.05$) while different lowercase letters indicate significant differences ($p < 0.05$).

control) and solvent (H_2O, 50% DMSO), indicating that the extraction results were mainly affected by extraction technology and solvent in this study. Moreover, TEAC, ORAC, proteins and polyphenols were closely distributed in the same quadrant, indicating that the antioxidant properties were mainly related to the protein and polyphenol contents in the extracts. Finally, in terms of the biomolecules yields and antioxidant properties, PEF-H_2O-120 min was selected as the parameters in the further PEF extraction process.

3.3. Effects of PEF on microstructure of Spirulina

3.3.1. Fluorescence microscope

Fig. 4 showed the effect of PEF treatment on the microstructure of *Spirulina*. When comparing Fig. 4A and C, it could be observed that the PEF treatment destroyed the microstructure of *Spirulina*. The fragmented *Spirulina* filaments were clearly observed in Fig. 4A, while the complete algae filament structure was shown in Fig. 4C. The length of *Spirulina* filaments was usually about 400–600 μm, which was observed in Fig. 4C (499.63 μm), and it was significantly longer than the fragments (4.08 μm) in Fig. 4A.

After magnifying the partial of Fig. 4A/C, the classic spiral structure of *Spirulina* could be observed. As it was shown in the scale of Fig. 4D, the spiral length was 41.56 μm and the bottom spiral diameter was 33.09 μm, which was consistent with previous reports (Akao et al., 2019). Fluorescence microscopy images also showed that PEF treatment could only destroy partially *Spirulina* filaments, because complete *Spirulina* filaments and helical structures were still found after PEF treatment, which indicated that expanding the voltage intensity and the number of pulses of PEF might further break the *Spirulina* filaments and increase the yield of biomolecules. A related study compared the effects of PEF (25 kV/cm for 150 μs) and bead-milling treatment on the microstructure of *Spirulina*, showing the microscopic results that PEF treatment resulted in the separation of cylindrical filament cells (trichomes), while bead-milling disrupted the intact cell structure, which resulted in a higher purity C-phycocyanin in the PEF extract (Martínez, Luengo, Saldaña, Álvarez, & Raso, 2017). This was consistent with the results of Fig. 4A, B in our study, that was, PEF treatment dispersed the *Spirulina* 'filament' structure, resulting in an increase in the yield of biomolecules. In addition, another study has shown that PEF could cause the damage to the microalgae (*Chlorella vulgaris*) cell membrane at a microscopic level. Scherer et al. (2019) stained PEF-treated *C. vulgaris* cells with Evans blue, a reagent that did not penetrate intact cells, and found that Evans blue was able to penetrate cells immediately after PEF treatment, indicating that PEF treatment penetrated *C. vulgaris* cells (Scherer et al., 2019). According to Parniakov et al. (2015a, 2015b), these results could be attributed to the electroporation or electropermeabilization effect of PEF, i.e., the action of short-duration and high-field-strength electrical pulses caused the microstructure damage or formation of pores of microalgae (Parniakov et al., 2015a).

3.3.2. Scanning electron microscope

To observe the effect of PEF on the cell surface structure of *Spirulina*,

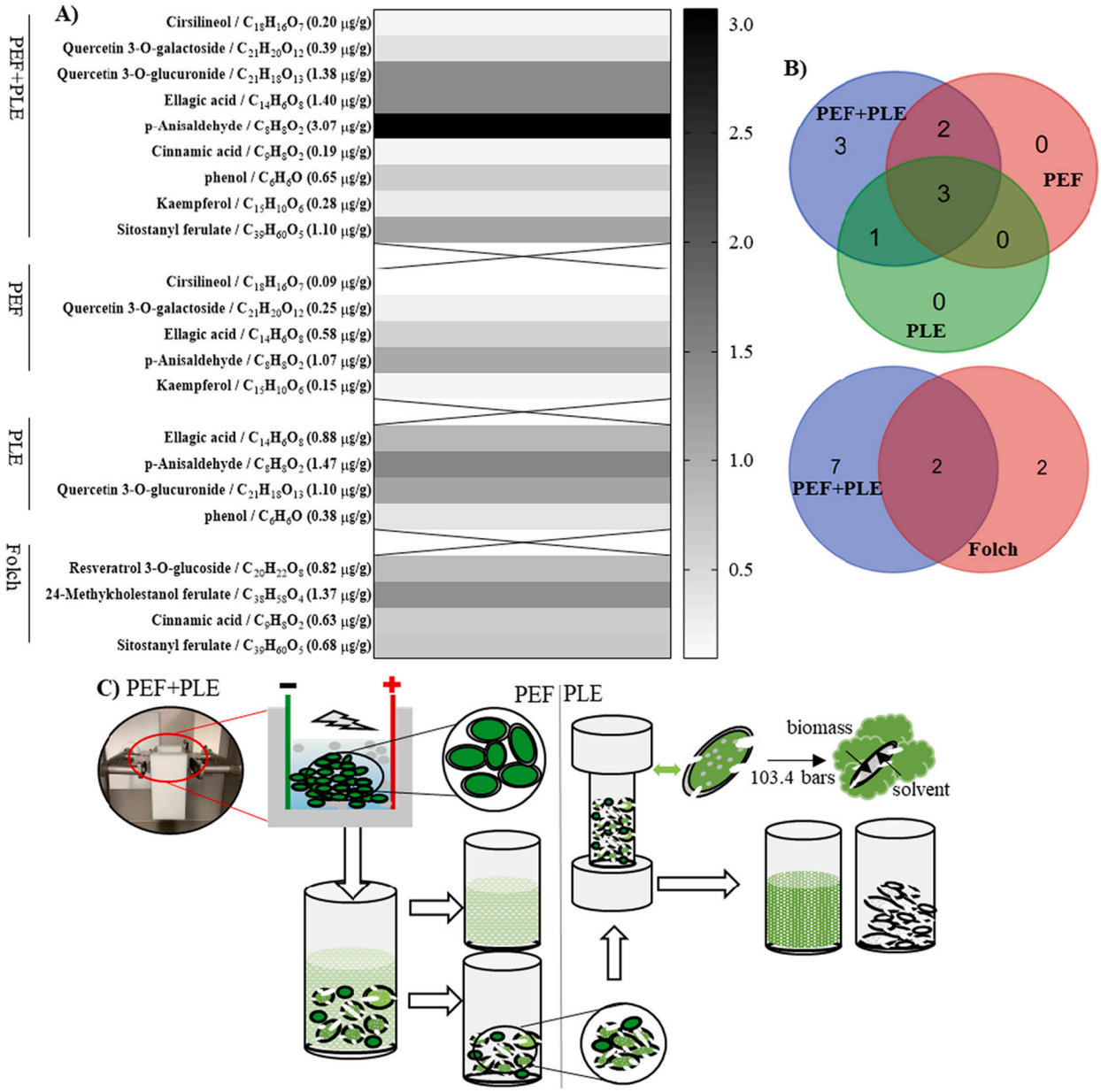

Fig. 10. A) Phenolic profile and content of extracts obtained from *Spirulina* biomass by PEF + PLE and Folch extraction; B) Venn diagram-same (overlapping)/ different (non-overlapping) polyphenol types; C) Schematic diagram of the effect of PEF + PLE on *Spirulina*.

this study further used SEM to analyze the freeze-dried *Spirulina* samples. In Fig. 5A/5D, when the magnification was 110× (500 μm), the PEF-treated sample exhibited a more fragmented sheet structure, while the control group had a relatively complete block structure. In Fig. 5B/E, when the magnification was 450× (100 μm), it could be clearly observed that the fragmentation of *Spirulina* was more obvious after PEF treatment. Moreover, in the control group, complete filaments of *Spirulina* were observed, which appeared to be closely arranged together with a smooth surface. In Fig. 5C/5F, rod-shaped *Spirulina* filaments could be observed in both PEF and non-PEF treated samples, and the surface structure of non-PEF treated samples was still smooth and tightly arranged (Fig. 5F). However, after PEF treatment, some of the rod-shaped *Spirulina* appeared to be broken, and the surface structure appeared loose and porous (Fig. 5C). Carullo, Donsì, Ferrari, and Pataro (2021) conducted a similar study and their results showed that PEF treatment resulted in a rough surface structure of *Spirulina* with the formation of cracks and depressions, which was caused by electroporation and subsequent leakage of intracellular material (Carullo et al., 2021). In addition, another study carried out by Käferböck et al. (2020) showed that the trichome structure of *Spirulina* was damaged after PEF treatment, which promoted an increase in the extraction yield of valuable components, such as C-phycocyanin, etc. (Käferböck et al., 2020). Combined with the results of fluorescence microscopy and SEM experiments, the increase in the yield of *Spirulina* biomolecules in this study was related to the electroporation effects of PEF.

3.4. Effects of PLE on Spirulina biomolecules yield

To further apply PEF + PLE to recovery biomolecules from microalgae, PLE extraction process was then determined. Fig. 6 showed that PLE extraction significantly increased the protein, polyphenol, chlorophyll *a*, chlorophyll *b* and carotenoids content when compared with the control group ($p < 0.05$). Specifically, the yield of protein was between 5 and 60 mg/g dw, among which the highest content was in PLE-H_2O group (60 mg/g dw), followed by the PLE-DMSO group (45 mg/g dw).

Previous studies showed that using H_2O as the extraction solvent

could recover proteins, polyphenols, and pigments from microalgae assisted by PLE (Zhou et al., 2021), but there were few reports about using DMSO as a solvent in PLE extraction process. In this study, using H_2O or DMSO as the solvent could significantly increase the protein yield assisted by PLE ($p < 0.05$). For polyphenols, compared with H_2O or 50% DMSO, using DMSO as a solvent significantly increased the content of polyphenols ($p < 0.05$). Moreover, the polyphenol content of PLE-DMSO and PLE-H_2O group corresponded to 12.5 mg/g dw and 12.0 mg/g dw respectively, which was almost three times that of control-DMSO and control-H_2O group respectively ($P < 0.05$). Therefore, both PLE and solvent had an important effect on the yield of protein and polyphenols. For pigments yield, the solvent had a greater impact than PLE effects, ie., regardless of whether PLE was used or not, the pigments yield with DMSO as the extraction solvent was significantly higher than that of H_2O and 50% DMSO extracts. Among them, the best results were shown in PLE-DMSO extracts, corresponded to 7.5 mg/g dw of chlorophyll *a*, 1.7 mg/g dw of chlorophyll *b* and 2.0 mg/g dw of carotenoids respectively. Moreover, higher content of pigments was obtained in 50% DMSO extracts than H_2O extracts, which was similar to the results of PEF extraction that the increase proportion of DMSO (50% DMSO) was conducive to the extraction of pigments.

3.5. Effects of PLE on antioxidant properties

As shown in Fig. 7, the results of ORAC (oxygen radical antioxidant capacity) and TEAC (Trolox equivalent antioxidant capacity) presented a good correlation ($R^2 = 0.9346$). The antioxidant capacity of PLE extract was 2–3 times that of the control group, which was due to the higher biomolecules content in PLE extracts. Both ORAC and TEAC results showed that the antioxidant capacity of PLE-H_2O and PLE-DMSO extracts were stronger than PLE-50% DMSO extracts, which could be attributed to the results that PLE-H_2O extracts contained more protein/polyphenols and PLE-DMSO extracts contained more chlorophyll *a*/chlorophyll *b*/carotenoids. In this line, the correlation between the antioxidant properties and biomolecules was analyzed using a PCA study (Fig. 8). The proportion of variance of the two principal components in PCA was 77.14% for PC1 and 20.92% for PC2, respectively. All biomolecules were distributed on the same side of PC1 with PLE-H_2O/PLE-DMSO, and on the opposite side with PLE-50% DMSO, indicating that 50% DMSO as a solvent is not suitable for recovering biomolecules from *Spirulina* when using PLE, which was consistent with previous studies (Parniakov et al., 2015b). Moreover, the biomolecules were distributed on the same side of PC1 as TEAC/ORAC, indicating that all these biomolecules had a positive effect on the antioxidant properties. Among them, proteins and polyphenols were closely distributed with ORAC/TEAC, which indicated that proteins and polyphenols were more strongly associated with the antioxidant capacity of the extracts in this study.

Previous studies have used ethanol and hexane to extract antioxidant compounds from *Spirulina* and found that polar reagents were more conducive to obtaining *Spirulina* biomass, which was due to the high protein and carbohydrate content of *Spirulina*, corresponding to 50–70% and ~15% respectively (Herrero, Álvarez, Señoráns, Cifuentes, & Ibáñez, 2005). Moreover, the DMSO used in this study could dissolve both water-soluble and fat-soluble compounds (Mueller et al., 2019). Therefore, in addition to water-soluble proteins and carbohydrates, fat-soluble pigments were also recovered by DMSO, which further increased the antioxidant capacity of *Spirulina* extracts. Combined the results of biomolecules content and antioxidant properties, DMSO was more suitable for the recovery of *Spirulina* biomolecules in PLE extraction process.

3.6. Effects of PEF + PLE on Spirulina biomolecules yield and antioxidant properties

The previous results showed that both PEF and PLE extraction recovered a certain proportion of proteins, polyphenols, and pigments from *Spirulina*. However, compared with the total biomass content of *Spirulina*, 17% ~ 57% protein (Lafarga, Fernández-Sevilla, González-López, & Acién-Fernández, 2020), 6.0–20.0 mg/g chlorophyll (Carlota De Oliveira Danesi, Dalva Godoy Danesi, Monteiro de Carvalho, & Sato, 2004) and 25–33.2 mg GAE/g polyphenol (Alberto, Francesco, Aliakbarian, Converti, & Perego, 2015), the yield of extracts obtained by using PEF or PLE alone could be further increased. Therefore, PEF + PLE, a novel extraction technology which has not been reported yet was used to obtain biomolecules from *Spirulina*, and the conventional extraction method-Folch extraction was used as a control. As it was shown in Fig. 9, the biomolecules obtained by PEF + PLE corresponded to 400 mg/g dw protein, 9.2 mg/g dw polyphenols, 16.3 mg/g dw chlorophyll *a*, 4.6 mg/g dw chlorophyll *b* and 1.7 mg/g dw carotenoids. It was obvious that the application of PEF + PLE further increased biomolecules yield compared to use PEF or PLE extraction alone in Fig. 9. It was worth noting that, compared with traditional extraction methods-Folch extraction, PEF + PLE increased the protein, polyphenol, chlorophyll *a* and ORAC values of *Spirulina* extracts by 1328%, 979%, 11% and 47% respectively. In addition, PEF + PLE greatly shortened the extraction time of Folch extraction by 165 min. On this line, PEF + PLE met the requirements of short extraction time with high biomolecules content in microalgae nutrients recovery industry.

Moreover, the combined use of PEF + PLE in this study achieved an improved extraction compared to the extraction assisted by PEF or PLE alone found in other studies. For example, Martí-Quijal et al. (2021) applied PEF to obtain *Spirulina* extracts containing carotenoids (0.50 mg/g dw, 60 min), chlorophyll *a* (0.60 mg/g dw, 120 min) and polyphenol (19.75 mg GAE/g dw, 180 min) (Martí-Quijal et al., 2021). Zhou et al. (2021) applied PLE to obtain *Spirulina* extracts which contained protein (210 mg/g dw), chlorophyll *a* (1.46 mg/g dw), carotenoids (0.12 mg/g dw) and polyphenols (11.49 mg/g dw) (Zhou et al., 2021). These studies showed that the biomolecules recovery effect of using PEF or PLE alone was not as good as PEF + PLE. Moreover, the biomolecules recovered by PEF + PLE from *Spirulina* in this study was also higher than other extraction techniques. For example, Vernès et al. used ultrasound technology (probe, 20 kHz) to obtain 229% higher protein content (28.42 ± 1.15 g/100 g dw) from *Spirulina* than the traditional extraction method (8.63 ± 1.15 g/100 g dw) (Vernès et al., 2019), which was still relatively lower to the content of 400 mg/g dw protein in the PEF + PLE *Spirulina* extract.

In addition, PEF + PLE existed a potential protective effect on the antioxidants. In general, the feature of PLE came from the destructive effect of high pressure/high temperature on the raw materials during the extraction process, therefore, the biological activity of biomolecules in microalgae could inevitably damage at high temperature (Ji et al., 2020). PEF destroyed the microalgae cell structure and thus reduced the dependence of PLE on high temperature, allowing PLE could be performed at room temperature, which not only improved the extraction efficiency, but also protected the antioxidants. In this study, PEF + PLE played the role of 'one plus one was greater than two', and PEF-'electroporation' combined with PLE-'pressure fluid' made it more efficient and environmentally friendly compared with traditional methods or using PEF or PLE alone. Briefly, PEF + PLE had great potential in the development of microalgae industry.

3.7. Effects of PEF + PLE on Spirulina phenolics composition

Polyphenols had an important impact on the antioxidant capacity of biomass in this study, this was attributed to the ability of polyphenol to capture free radicals, active oxygen and chelate metal ions, which was depend on both content and type of polyphenols (Da Silva Port's, Chisté, Godoy, & Prado, 2013). The above experimental results showed that PEF or PLE could increase the polyphenol content in the extract, which was not equal to the increase of phenolic types. Therefore, Triple TOF-LC-MS-MS was used to analyze the polyphenol components of PEF + PLE,

PEF, PLE and Folch extracts, to further analyze whether extraction technology affected the polyphenol composition of *Spirulina* extract, the results were shown in Fig. 10.

The results showed that the polyphenol type identified from the PEF + PLE, PEF, PLE and Folch extracts of *Spirulina* in this study corresponded to 9, 5, 4 and 4, respectively. Specifically, the polyphenols of PEF + PLE extracts were mainly p-anisaldehyde (3.07 μg/g), ellagic acid (1.40 μg/g), quercetin 3-o-glucuronide (1.38 μg/g) and sitostanyl ferulate (1.10 μg/g), while for PEF, PLE, Folch extracts, the main polyphenols were corresponding as *p*-anisaldehyde (1.07 μg/g), *p*-anisaldehyde(1.47 μg/g)/quercetin 3-o-glucuronide (1.10 μg/g) and 24-methylcholestanol ferulate (1.37 μg/g) respectively. Fig. 10B shows the amount of the same (overlapping) and different (non-overlapping) polyphenol types obtained by different extraction methods through a Venn diagram. It can be seen from Fig. 10B that the type of polyphenols obtained by PEF PLE, PEF + PLE and Folch was different, which was influenced by both extraction technology and the solvent. In general, the results of Triple TOF-LC-MS-MS confirmed that PEF + PLE extraction not only increased the content of polyphenols, but also increased the type of polyphenol profile, which was valuable for the utilization of microalgae polyphenols.

According to related reports, there were more than 1100 kinds of algae in the ocean, which were perfect natural sources of polyphenols (Jimenez-Lopez et al., 2021). Researchers classified polyphenols in microalgae according to different molecular structures: simple phenols (phenols, catechol, hydroquinone, phloroglucinol, etc.), C6-C1 (phenolics acids, aldehydes), C6-C2 (phenylethanoids, phenylacetic, acetophenone, phenethyl) alcohol), C6-C3 (hydroxycinnamic acids, cinnamic aldehydes, monolignols, phenyl propenes, coumarins, etc.), C6-C1-C6 (xanthonoids), C6-C2-C6 (stilbenoids, anthraquinones, anthrones), C6-C3-C6 (flavonoids), C6-C7-C6 (diarylheptanoids), lignans, lignins, tannins and phenolic terpenoids (Jimenez-Lopez et al., 2021). This study identified several above typical polyphenols, such as phenol (simple phenol), cinnamic acid (C6-C3) and quercetin (C6-C3-C6). Moreover, polyphenols with relatively complex structures were also detected, such as syringaresinol, which could resist oxidation and regulate the intestinal flora, however, reports on the extraction and application of syringaresin from microalgae were rare (Cho, Song, Yoon, Park, & Kim, 2018). Based on the results of this study, the principle of PEF + PLE improving the yield of microalgae biomolecules and polyphenol types was shown in Fig. 10C. PEF treatment could cause an increase of cell membrane permeability and consequently caused an increase of the cell membrane and tissue conductivity (Maza et al., 2020). Combined FM/SEM results, the PEF extraction process destroyed the *Spirulina* filaments and causes damages to the cell structure, which helped to the diffusion of DMSO into microalgae during the PLE high-pressure extraction process, thereby to achieve efficient recovering biomolecules from *Spirulina*. It should be noted that different microalgae, such as green algae, brown algae, and cyanobacteria had different cell structures, cell wall thickness, and conductivity of the cell suspension, which might lead to different effects of PEF treatment. Therefore, considering the diversity of microalgae, it is necessary to study the optimal extraction parameters of PEF, PLE and PEF + PLE when applied to different types of microalgae.

4. Conclusions

This study is the first report regarding the combined application of pulsed electric fields (PEF) and pressurized liquid extraction (PLE) in a multistep process to recover biomolecules from *Spirulina* observing promising results regarding high yield of biomolecules obtained. The main effect of PEF in the extraction of *Spirulina* is attributed to its ability to destroy the intact filaments of the microalgae and damaging the cell wall structure, thus promoting a further PLE efficient extraction. The PEF + PLE extraction not only increases the content but also the types of polyphenols in the extract, which has a potential economic value for the industrial application of microalgae polyphenols. Compared with other traditional extraction reagents, such as chloroform, methanol, n-hexane, etc., DMSO has the advantages of low toxicity, high fluidity, and good selectivity at a normal temperature and pressure. The final extract contains DMSO, which can be removed by vacuum distillation, freeze-drying, etc. Besides, the extract in this study contains complex biomolecules, which can be further separated and purified by organic reagent precipitation, membrane separation, ion exchange column, etc. to obtain purified individual compounds. Overall, the application of PEF + PLE to recover high-added-value compounds from different microalgae biomass can be considered as a promising tool.

CRediT authorship contribution statement

Jianjun Zhou: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Min Wang:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Writing – review & editing. **Houda Berrada:** Methodology, Software, Supervision, Writing – review & editing. **Zhenzhou Zhu:** Supervision, Writing – review & editing. **Nabil Grimi:** Methodology, Software, Supervision, Writing – review & editing. **Francisco J. Barba:** Conceptualization, Visualization, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

All the authors declare that they do not have any conflict of interest.

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