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Sequential multi-stage extraction of biocompounds from *Spirulina platensis*: combined effect of ohmic heating and enzymatic treatment

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Abstract

A sequential multi-stage procedure was applied on the extraction of biocompounds from *Spirulina platensis*. The process consisted in three steps: 1) aqueous extraction, using conventional thermal extraction (CE), ohmic heating (OH, 7 V/cm), enzymatic treatment (EAE, 0.8 $mg_{Lysozyme}/mL$) or both OH and EAE combined; 2) ethanolic extraction; 3) $CHCl_3$/MeOH extraction. The results evidenced that the combined OH-EAE extraction allowed selective recovery of phycobiliproteins in the 1st step, with increments of more than 100% in yield in comparison with CE. Pigments and lipids were selectively extracted in the 2nd step. The combination of OH and EAE in the 1st step resulted in higher amounts of extracted compounds in the following phases compared to processes using non-combined technologies. Results demonstrate that the intensification of extraction steps facilitates the use of environmentally friendly technologies in a multi-stage process capable of recovering and isolating different fractions with bio-functional properties, targeting waste reduction and circular economy.

Keywords: Microalgae; (bio)functional compounds; electric fields; lysozyme; process efficiency

Industrial relevance

Spirulina plantensis represents a potential biomass feedstock due to its potential as a source of compounds of great economic value (including antioxidants, proteins, lipids and natural pigments, in particular blue colorants). The combined use of ohmic heating and enzymes in the aqueous extraction step fosters the use of environmentally friendly technologies to implement sequential high yield and high purity extraction of the different valuable fractions with bio-functional properties, targeting waste reduction and contributing to the implementation of circular economy strategies. This can be integrated with a design of Industry 4.0 driving the development of new products.

Abbreviations

APC	allophycocyanin
CE	conventional thermal extraction
Chl-a	chlorophyll-a
Chl-b	chlorophyll-b
DAD	diode array detector
EAE	enzyme-assisted extraction
FAMEs	Fatty acid methyl esters
GAE	gallic acid equivalents
HVED	high voltage electrical discharges
MEF	moderate electric fields
OH	ohmic heating
OH-EAE	ohmic heating combined with enzymatic

	treatment
PC	phycocyanin
PE	phycoerythrin
PEF	pulsed electric fields
TPC	total phenolic compounds

1. Introduction

The interest in the use of microalgae has recently boosted. Research on microalgae was initially focused in the biofuels production and in the capture of CO_2 from the environment in response to the uprising energy crisis and climate change, but nowadays microalgae are recognized as an important feedstock that may help to mitigate the predicted depletion of natural sources (Geada et al., 2018; Pierobon et al., 2018). Although it has been previously stated that microalgae may supply up to 25% of the required energy worldwide (Rumin, Nicolau, de Oliveira, Fuentes-Grünewald, & Picot, 2020), it is clear that microalgae are a potential source of compounds with great economic value, including antioxidants, dyes, proteins, phycocolloids, lipids, carbohydrates, vitamins and minerals with applications in food, cosmetic, pharmaceutical, bioplastics, biofertilizers and biofuels industries (Rumin et al., 2020). Furthermore, microalgae can be processed following a cascading biorefinery approach making possible to exploit sequentially different valuable fractions and thus contributing to the circular economy with zero waste generation (Chew et al., 2017).

Spirulina (Arthrospira) platensis is a photosynthetic cyanobacterium (usually considered a microalgae) that is produced commercially for food applications due to its particular nutritional composition (*e.g.* higher content in protein, carbohydrates, lipids, etc.) (Ferreira-Santos et al., 2020) and functional properties. In fact, different authors

have reported several biological activities that can be ascribed to *Spirulina*, including antiviral, anti-inflammatory, anti-cancer and antioxidant (Karkos, Leong, Karkos, Sivaji, & Assimakopoulos, 2011). In this sense, *Spirulina* is claimed to be an ideal food and dietary supplement by Food and Agriculture Organization of the United Nations and World Health Organization (Wang & Zhao, 2005).

On the other hand, in the last decades, the recovery of dyes with high pharmaceutical and food potential and lipids for the production of biofuels using microalgae as feedstock has been one of the main challenges for both research and industry. In this context, *Spirulina* is commercially exploited as a source of C-phycocyanin to be used as a natural blue colorant.

Concomitantly, sustainable and more environmentally friendly methods should be considered to intensify the processes for obtaining microalgae (bio)products, taking into account the quality of the final products of interest. Several "green techniques", such as microwaves, ultrasounds, electrotechnologies, enzymes, etc., have been applied to facilitate the cell wall disruption and obtain biocompounds from different species of microalgae (Azmi et al., 2020; Chia et al., 2019; Lee, Cho, Chang, & Oh, 2017). Enzyme-assisted extraction (EAE) with lysozyme has been used to disintegrate cell walls and improve the efficiency of pigment extraction (Sobiechowska-Sasim, Stoń-Egiert, & Kosakowska, 2014). Recently, our research group has proposed the use of ohmic heating (OH) as a promising technology to extract C-phycocyanin and other biocompounds from *Spirulina* powders (Ferreira-Santos, Nunes, et al., 2020). OH is an emerging electrotechnology with great potential as it improves the process efficiency, reduces the use of solvents and energy costs, while maintaining the quality and functionality of the obtained products (Rocha et al., 2018; Sastry, 2008). In recent years, several studies have been published on the use of OH (and the associated moderate

electric fields (MEF) effects) for extraction purposes. Complementary to the heating effect, enhanced extraction efficiency has also been partially ascribed to the promotion of electropermeabilization of the cell membranes. Different applications include the extraction of fruit juice, essential oils, pectin, pigments and phenolic compounds from different bioresources (Ferreira-Santos, Genisheva, Pereira, Teixeira, & Rocha, 2019; Gavahian, Farahnaky, Farhoosh, Javidnia, & Shahidi, 2015; Hashemi et al., 2017; Jesus et al., 2020; Loypimai, Moongngarm, Chottanom, & Moontree, 2015; Pereira et al., 2016; Saberian, Hamidi-Esfahani, Ahmadi Gavlighi, & Barzegar, 2017). Besides the traditional application to solvent-based solid-liquid extraction processes, ohmic-assisted hydrodistillation for the recovery of essential oils from plant-based materials has also received some attention in the last years and a review work from Gavahian & Farahnaky is available (Gavahian & Farahnaky, 2018).

The combination of different technologies has emerged as a strategy to increase and accelerate the extraction of different intracellular compounds and make the process more efficient and with less energy consumption (Wen, Zhang, Sun, Sivagnanam, & Tiwari, 2020) taking advantage of the best of each technology and eventually allowing synergies between them. Sequential extraction of the different recoverable (bio)functional fractions will improve the full use of the bioresource, with minimal or zero waste generation while allowing for a fine tuning of the process selectivity. However, the available information about the application of selective multi-stage processes for integral extraction of biocompounds from *Spirulina platensis* is still scarce.

Thus, the objective of the present work was to investigate the influence of OH, EAE and combination of these two technologies in the disintegration and rupture of *Spirulina platensis* cells and, consequently, in the selective recovery of phycobiliproteins as a first

stage of a multi-step extraction process for the integral valorization of the remaining fractions. The influence of the technology employed in the first step on the extraction efficiency and selectivity of the following steps for the extraction of phenolics, other pigments and lipids was also assessed.

2. Materials and methods

2.1. Raw materials and Chemicals

Spirulina platensis biomass used in this work was grown, microbiologically controlled, dried at low temperatures and mechanically ground (particle size between 0.6 to 0.8 mm) by Azienda Agricola Prato della Voja (Bergamo, Italy). Folin-Ciocalteu reagent, sodium chloride, ethanol, sodium carbonate, sulfuric acid, phenol reagent, glucose, chloroform, methanol, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Lysozyme from chicken egg white (≥ 40,000 units/mg protein, EC number 3.2.1.17) and all standards (purity level above 94%) for HPLC and GC were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Reagents were of analytical grade, and ultra-pure water was used throughout the experiments.

2.2. Experimental plan

Figure 1 presents the scheme of the multi-stage extraction procedures applied for biocompounds recovery from *Spirulina platensis*. The multi-stage extraction procedure included three steps, using solvents with different polarity to extract different fractions. The 1st step consisted in one of the following four processes: 1) an aqueous extraction process (CE, used as control heating experiment), or 2) an aqueous extraction assisted by electric fields (OH) or 3) an aqueous extraction assisted by an enzymatic treatment (EAE), or 4) an aqueous extraction assisted by OH combined with enzymatic treatment (OH-EAE). The 2nd step consisted in the ethanolic (95%, v/v) extraction of dried

residual biomass from the 1st step. The 3rd extraction step consisted in the extraction of dried residual biomass from the 2nd step with a (2:1, v/v) mixture of chloroform/methanol ($CHCl_3$:MeOH).

2.2.1. 1st Step

2.2.1.1. Extraction reactor

The 1st extraction step (be it process 1), 2), 3) or 4) mentioned above) was conducted in a double-jacketed glass cylinder (3 mm of internal diameter and 100 mm of height) with an overall volume of 25 mL, containing a stainless-steel electrode at each edge (distance between electrodes is 2.8 cm) insulated with polytetrafluoroethylene (Pereira et al., 2016). For OH experiments, the power source working with a sinusoidal wave at 20 kHz (Agilent 33220A, 1 Hz-25 MHz and ± 10 V; Penang, Malaysia) allowed changing the voltage. Frequency was set at 20 kHz in order to eliminate electrochemical effects such as electrolysis and electrode oxidation, minimizing leakage of metals into the extraction medium. Researchers reported that the use of higher frequencies (in the order of kHz) may markedly suppress corrosion of stainless steel electrodes during OH. Nevertheless, titanium electrodes are still considered to have higher corrosion resistance and biocompatibility characteristics (Pataro et al., 2014) and may be advantageous when equipment able to provide high frequency is not available or economically feasible. Other factors such as extreme pH and electrical conductivity of the extraction medium also influence the metal release rate (these factors were controlled in the experiments). For CE and EAE experiments, temperature was controlled with a thermostatic circulator water system (F25-ED, Julabo, Seelbach, Germany) and measured with a stainless-steel type-K thermocouple (temperature precision of ±1 ºC; Omega Engineering, Inc., Stamford, CT, USA), located at the geometric center of the reactor. The thermocouple

was connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA), and Lab View 7 Express software (National Instruments, NI Data logger) was used to extract the data. A portable oscilloscope (ScopeMeter® 125/S, Fluke, WA, USA) was used to measure electrical frequency, voltage and current intensity during OH treatments.

The reactor was covered with aluminium foil to avoid the direct incidence of light.

2.2.1.2. Extraction conditions

For 1st step extractions, 2 g of dried *S. platensis* were mixed with 40 mL of distilled water in the extraction reactor with an agitation rate of 170 rpm. This ratio was selected taking into account our previous studies (Ferreira-Santos, Nunes, et al., 2020) and studies reported by other authors (Nagar, Sharma, & Kumar, 2018). It imparted to the fluid an electrical conductivity of 2 ± 0.2 mS/cm (measured using a conductivity/TDS/Salinity Meter (HANNA Instruments Inc., edge, HI2003, USA) at room temperature) that ensures a homogeneous current flow, which is relevant for the OH experiments. The optimal parameters of the extraction procedures were selected using previously reported results (Ferreira-Santos, Nunes, et al., 2020) and preliminary EAE experiments for C-phycocyanin recovery (data shown in supplementary material, Figures S1 and S2). The OH treatments were conducted at a voltage gradient of 7 V/cm. A concentration of 0.8 mg/mL of lysozyme was used for EAE. The extraction process was performed at 37 ºC during different periods of time (10, 20, 30, 40, 50 and 60 min). All the mixtures were centrifuged (9500 *g* for 10 min at 4 ºC) to obtain the liquid extracts that were analyzed for the content of phycobiliproteins and soluble phenolics. The solid fraction (exhausted biomass) was freeze-dried and subjected to the subsequent extraction step.

2.2.2. 2nd Step

For this step, the extractions were carried out using 1.5 g of dried biomass from the 1st step mixed with 30 mL of ethanol 95% (v/v) for 60 min at 60 °C in a cylindrical reactor duly protected from light. A temperature-controlled water bath (SW22, Julabo, Seelbach, Germany) with shaking (170 rpm) was used to maintain the extraction temperature. The mixtures obtained after the extraction were centrifuged as described for the 1st step and the resulting liquid extracts were filtered (Whatman nº 2) before analysis of chlorophylls (a and b), carotenoids, polyphenols, carbohydrates and residual lipids content. Lipids were re-extracted with a modification of the Bligh and Dyer method (Bligh & Dyer, 1959) using 2 mL of each ethanolic extract previously dried under a nitrogen atmosphere.

The solid fraction (exhausted biomass) was then freeze-dried and subjected to the subsequent extraction step.

2.2.3. 3rd Step

This step was aimed to extract the remaining lipids by applying a modification of the Bligh and Dyer method (Bligh & Dyer, 1959), extracting 300 mg of the dried biomass from the 2nd step with 5 mL of $CHCl_3$:MeOH (2:1 v/v) for 6 h at room temperature and agitation of 200 rpm in the same apparatus used for the 2nd step. The lower organic phase was collected and re-extracted 3 times with the same ratio of chloroform-methanol. Finally, the resulting organic phase was filtered, and the total lipid content was determined gravimetrically after evaporation of organic solvents under a nitrogen atmosphere.

The ash contents of the final residues were determined in accordance to the National Renewable Energy Laboratory (NREL) official protocol (NREL/TP-510-42622).

2.3. Extracts analysis

2.3.1. Extraction yield

The extraction yields of the various steps is a measure of the solvent efficiency to extract specific fractions from the original dry material. Yield calculation (presented in %) was based on the cumulative mass of extract using the following equation (Eq. (1)):

$$\text{Yield (\%)} = \frac{\text{extracted solids (g)}}{\text{initial dry material (g)}} \times 100 \quad (1)$$

2.3.2. Phycobiliproteins quantification

Phycobiliproteins concentration was determined using a UV-Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths of 562, 615 and 652 nm, since C-phycocyanin has a maximum absorbance that is proportional to its concentration in the visible range between 610 and 620 nm, and 652 nm is the wavelength where the class of allophycocyanin absorbs. The amounts of phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE) were calculated according to Eqs. (2), (3) and (4), respectively (Arashiro et al., 2020).

$$PC(mg/mL) = [A615 - (0.474 \times A652)]/5.34 \quad (2)$$

$$APC(mg/mL) = [A652 - (0.208 \times A615)]/5.09 \quad (3)$$

$$PE(mg/mL) = \frac{A562 - (2.41 \times PC) - (0.849 \times APC)}{9.62} \quad (4)$$

where A562, A615 and A652 are the absorbances measured at the respective wavelengths.

2.3.3. Phenolic compounds

The concentration of total phenolic compounds (TPC) was measured using the Folin–Ciocalteu method, which is based on the colorimetric reduction/oxidation reaction of phenols (Ferreira-Santos et al., 2019). For all analyses, 5 µL of extract (water or ethanol

95 % for control) were mixed with 15 µL of Folin–Ciocalteu reagent and 60 µL of Na_2CO_3 (75 g/L). The prepared solution was kept at 60 ºC for 5 min. Absorbance was measured at 700 nm by an UV/vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). A calibration curve was prepared using a standard solution of gallic acid (1500 to 50 mg/L, R^2 = 0.99). Final values were expressed as gallic acid equivalents (GAE) (mg GAE/g_{dm} *Spirulina*).

The identification and quantification analysis of phenolic compounds present in *S. platensis* extracts were performed as described previously (Ferreira-Santos et al., 2019) using a Shimatzu Nexpera X2 UPLC chromatograph equipped with Diode Array Detector (DAD) (Shimadzu, SPD-M20A). Separation was performed on a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size; from Waters) and a pre-column of the same material at 40 ºC. The HPLC grade solvents used were water/formic acid (0.1%) and acetonitrile as eluents and the flow rate was 0.4 mL/min. Phenolic compounds were identified by comparing their UV spectra and retention times with those of corresponding standards. Quantification was carried out using calibration curves for each compound analyzed using concentrations between 250-2.5 mg/mL (250, 125, 100, 50, 25, 10, 5, 2.5 mg/mL). In all cases, the coefficient of linear correlation was R^2 > 0.99. Compounds were quantified and identified at different wavelengths (208–370 nm).

2.3.4. Chlorophylls and carotenoids

Chlorophyll-a (Chl-a), Chlorophyll-b (Chl-b) and carotenoids contents were determined using a UV-Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths 470, 649 and 664 nm. The amounts of Chlorophyll-a, Chlorophyll-b and carotenoids were calculated according to Eqs. (5), (6) and (7), respectively (Lichtenthaler & Wellburn, 1983).

$$\text{Chl} - \text{a}\ (\mu g/mL) = (13.36 \times \text{A664}) - (5.19 \times \text{A649}) \quad (5)$$

$$\text{Chl} - \text{b}\ (\mu g/mL) = (27.43 \times \text{A649}) - (8.12 \times \text{A664}) \quad (6)$$

$$\text{Carotenoids}\ (\mu g/mL) = \frac{(1000 \times A470 - (2.13 \times \text{Chl} - \text{a}) - (93.63 \times \text{Chl} - \text{b})}{209} \quad (7)$$

where A470, A664 and A664 are the absorbances measured at the respective wavelengths.

2.3.5. Fatty acids

In addition to fatty acids, the lipidic fractions obtained with mixture of polar and non-polar organic solvents contain pigments (carotenoids, chlorophylls, etc.), proteins, amino acids and other compounds present in the microalgae biomass.

The analysis of fatty acid composition of *S. obtusiensis* lipidic fractions (from 2nd and 3rd step) was performed after purification of the extracted lipids by liquid-liquid microextraction in organic solvent (chloroform/methanol), adding water to form a biphasic system and simultaneous methylation with a mixture of methanol acidified by sulfuric acid (15:85, v/v) (Lopes, Miranda, Alves, Pereira, & Belo, 2019). The fatty acid methyl esters (FAMEs) in organic phase were quantified by gas chromatography in a CP-3800 gas chromatograph (Varian Inc., USA) fitted with a FID detector and a TRACSIL TR-WAX capillary column (30 x 0.25 x 0.25mm, Teknokroma, Spain) using pentadecanoic acid (C15:0) as internal standard. The injector and detector temperatures were 220 and 250 °C, respectively, and helium was used as carrier gas at 1 mL/min. The initial oven temperature was 50 °C, maintained for 2 min, followed by a 10 °C/min ramp up to 225 °C, and the final isothermal conditions were maintained for 10 min. A mixture of fatty acid methyl ester standards was used for identification purposes, comparing the respective retention times. Relative amount of each long chain fatty acid

(%) was expressed as the ratio between its concentration (g/L) and the sum of the concentrations of all fatty acids analyzed.

2.3.6. Determination of antioxidant activity

Two different methods of measuring the antioxidant activity were used: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH assay) and Ferric reducing antioxidant power (FRAP assay) was determined as described by Ballesteros *et al.* (Ballesteros, Ramirez, Orrego, Teixeira, & Mussatto, 2017), with some modifications.

DPPH assay consists in the reduction of the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH•) radical in the presence of hydrogen-donating antioxidant and in the formation of the non-radical DPPH-H form at the end of the reaction. The reaction was carried out in a 96-well microplate containing 30 µl of all extract and 270 µl of 150 µM DPPH solution (dissolved in 80% methanol to an absorbance of 0.700 ± 0.01 at 515 nm). The solution was mixed and allowed to stand for 1 h in the dark at room temperature. Then the absorbance was measured at 515 nm by an UV/vis spectrophotometer (Synergy HT, BioTek Instruments, Inc., USA). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard reference.

The scavenging effect was calculated by equation Eq. (8):

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A0 - A1)}{A0} \times 100 \qquad (8)$$

where A0 is the absorbance of blank control, and A1 is the absorbance of sample at 515 nm.

FRAP assay consists in the ability of extracts to reduce ferric ions (Fe^{3+} to Fe^{2+}), in the form of ferric 2,4,6-tripyridyl-s-triazine (TPTZ). FRAP values were expressed as µmol Fe^{2+}/L. For this, 10 µL of extract (properly filtered) were mixed with 290 µL of FRAP

reagent in a 96-well microplate. The reaction mixture was subsequently incubated at 37 ºC for 15 min. After that, the absorbance was determined at 593 nm in a spectrophotometric microplate reader, against a blank that was prepared using the corresponding solvent. A calibration curve was prepared using an aqueous solution of ferrous sulphate $FeSO_4.7H_2O$.

2.4. Optical microscopy

After each extraction process, *Spirulina* biomass samples were observed with a microscope BX51 (Olympus, Japan). Images were captured with DP72 digital camera (Olympus, Japan) at magnification 100X.

2.5. Statistical analysis

The extractions and analyses were performed in triplicate and the data is presented as mean ± standard deviation (SD) values. GraphPad Prism® software (version 6.0; GraphPad Software, Inc., San Diego CA, USA) was used for statistical analyses. The analysis of variance (ANOVA) and the least significant difference test were used to determine statistically different values at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Extraction yields

In the last few years our research group has studied the influence of electric fields in the recovery of compounds with high added value from natural sources, such as plant by-products, macro and micro-algae or food waste (Coelho, Pereira, Rodrigues, Teixeira, & Pintado, 2019; Ferreira-Santos et al., 2019; Ferreira-Santos, Nunes, et al., 2020; Jesus et al., 2020; Pereira et al., 2016). In addition, enzymatic hydrolysis has been reported as an efficient technology to extract phenolic compounds from different matrices (Bonifácio-

Lopes, Teixeira, & Pintado, 2020; Fernández, Vega, & Aspé, 2015). Considering that the combination of technologies can greatly reduce the costs associated with extraction process and improve the process efficiency (Rajha et al., 2018), it is expected that, in this particular case, by combining OH with EAE, a decrease in the amount of enzyme required and also a reduction in processing time will occur, as well as an improvement in the recovery of the compounds of interest.

Results on the influence of the extraction steps conditions on the extraction yields are shown on Table 1. The application of physical/thermal (CE and OH) and/or biological (EAE) treatments (1st step) can significantly increase the extraction yield values compared to CE ($p < 0.05$). Similarly, in the second extraction step, where ethanol is used as solvent, the pretreatments applied in the first step with enzyme and/or electric fields that already resulted in significant differences in the extraction yields, had a similar effect in the subsequent steps, mostly non-polar molecules (such as chlorophyll, carotenoids and lipids) being recovered. In addition, the combination of OH and EAE, as a treatment in the aqueous extraction (1st step) or pre-treatment for the 2nd step, resulted in the greatest increase in the extraction yields ($p < 0.01$). These results suggest that treatments using electric fields and lysozyme cause lysis of the cell wall of *Spirulina*, promoting the extraction of intracellular compounds (shown below in the microscopy results, section 3.7).

In the 3rd step, the biomass was subjected to an extraction with chloroform-methanol and the extraction yield was significantly lower compared to the previous extraction steps. This may be due to the high amount of extractives obtained in the previous stages of extraction, where most of the lipids have been extracted (total lipids present in this *Spirulina* powder is approx. 11.3%, as previously reported by us (Ferreira-Santos, Nunes, et al., 2020)) when ethanol 95% was used (results reported in section 3.5). These

results suggest that, unless the extract profile in this 3rd step is unique and potentially with very high added value, it will be of no use to include it in a sequential extraction approach to recover the different algae fractions.

The ash content (inorganic fraction) was determined in the final biomass residue, from the 3rd step. The results showed that the final biomass pre-treated with CE presents a greater quantity of inorganic matter compared to biomass pre-treated with OH and EAE. Interestingly, the combination of technologies has showed to be effective in recovering inorganic compounds, such as minerals, throughout the process, with less quantity in the final residue. In addition, the safe and inexpensive disposal of all waste products generated during the process must be considered. Residual biomass at the end of the multi-step extraction was 54-42%, depending on the treatment applied. A portion of this biomass can go to an anaerobic digester to generate biogas, and the rest can be used as nutrients, after a hydrolysis step, to liberate fermentable sugars to feed a bioreactor, e.g. for the heterotrophic (or mixotrophic) production of microalgae again. The final residue, due to its high mineral content, can also be considered as biofertilizer and biostimulant in agriculture, providing ecological and economical alternatives to synthetic products (Rumin et al., 2020).

3.2. Phycobiliproteins extraction (1st step)

The main objective of this study is to promote the efficient extraction of high added value intracellular compounds from *Spirulina* without changing their characteristics, enhancing their application in the food, nutraceutical and pharmaceutical industries.

In this sense, the recovery of phycobiliproteins (pigments used as natural colorants) from microalgae is extremely valuable due to their high commercial value. The extraction of these molecules has already been carried out by various physical,

mechanical, chemical and biological methods (Izadi & Fazilati, 2018; Jaeschke et al., 2019; Kissoudi, Sarakatsianos, & Samanidou, 2018; Pan-utai & Iamtham, 2019). However, their total extraction is usually hampered by the rigidity of the cell wall, as well as by the extraction conditions (biomass to solvent ratios, type of solvent and previous biomass processing). Furthermore, the initial extractable amount of phycobiliproteins in the cells depends on the growth conditions and harvest stage, and absolute values may not be comparable (Vernès et al., 2015). Therefore, the application of strategies that allow full access to the intracellular medium is essential to facilitate the extraction of specific components. Although traditional methods (e.g., sonication and freeze-thawing methods) induce cell wall disruption, they have several drawbacks because of high energy consumptions and long extraction times (Kissoudi et al., 2018; Vernès et al., 2015). Moreover, cell disintegration should be done avoiding the use of processing conditions that could negatively affect the quality of the extracts and have a negative impact on the product value (Postma et al., 2016).

Figure 2 presents the contents of phycobiliproteins (PC, APC and PE) after water extraction for 10 to 60 min (1st step). The data is presented for CE, OH, EAE, and OH combined with EAE. The obtained data shows that the most representative phycobiliprotein in *S. platensis* is PC followed by APC and PE, ranging from 25.5–56.6, 5.4–14.7, 1.9–8.6 mg/g, respectively. These results are in agreement with those reported by Arashiro and co-workers (Arashiro et al., 2020), also confirming the commercial potential of *Spirulina* as a source of natural blue dyes.

It is possible to observe that the single OH and EAE treatments led to a significant increase in phycobiliproteins recovery compared to CE. Furthermore, this increase was markedly greater ($p < 0.01$) when the combination of the enzymatic treatment and electric fields (OH-EAE) was used. In addition, higher amounts of phycobiliproteins

were obtained in less time for all the tested technologies in comparison with CE, being again this time reduction more evident for the combined process.

Recently, the use of green methodologies such as electro-technologies (pulsed electric fields (PEF), high voltage electrical discharges (HVED) and OH) to obtain high value products from microalgae has been the focus of research, allowing the reduction of the amount of toxic solvents and extraction times, increasing the extraction efficiency, and targeting its industrial feasibility (’t Lam et al., 2017; Ferreira-Santos, Nunes, et al., 2020; Jaeschke et al., 2019; Käferböck et al., 2020; Postma et al., 2016; Zhang, Lebovka, Marchal, Vorobiev, & Grimi, 2020). Moreover, it is known that OH reduces energy consumption compared to the traditional processes, leading to a more energy efficient process (Ferreira-Santos et al., 2019). Also, we have previously shown that, comparing with CE, OH displays a greater efficiency in the recovery of intracellular compounds, such as pine bark’s polyphenols and Spirulina’s C-phycocyanin (Ferreira-Santos et al., 2019; Ferreira-Santos, Nunes, et al., 2020).

Jaeschke *et al.* (Jaeschke et al., 2019) applied PEF treatment with higher intensities (E = 40 kV/cm and W_{spec} = 112 kJ/kg) and used sodium phosphate buffer instead of tap water as solvent in subsequent extraction, yielding 76% of PC (corresponding to 94 mg/g of *Spirulina)*. In addition, EAE with lysozyme and a combination with mechanical grinding has been used to disintegrate cell walls and improve the efficiency of pigment extraction (Sobiechowska-Sasim et al., 2014). The results obtained in our work are in agreement with these findings. The use of enzymes or electric fields alone led to an increase of extraction efficiency of phycobiliproteins, and the combination of the two technologies showed a synergic positive effect. Besides the additive effect of using both technologies, a positive effect of OH upon the enzyme activity may also be possible. In fact, several studies reported that the application of low intensity electric fields can lead

to a modification in the structure of certain enzymes, improving their activity (Poojary et al., 2017; Wang et al., 2015; Xie, Liao, & Zhou, 2013). This effect may contribute to the improved recovery of intracellular compounds by the combination of OH and EAE, and also for the increase in the rate of extraction, making it more advantageous. These interactions can be the focus of study for future investigations.

3.3. Total phenolic compounds of aqueous and ethanolic extracts (1st and 2nd step)

Phenolic compounds are known to have important bioactive and functional properties. For example, some phenolic compounds show potent activities such as antioxidant, anti-microbial, anti-cancer, among others, which make these compounds interesting for commercial applications in the food, health and pharmaceutical industries (Dai & Mumper, 2010; Ferreira-Santos et al., 2019).

As mentioned before, pigments, phenolic compounds and proteins are some of the metabolites present in the cytoplasm or internal organelles which makes difficult their extraction due to the resistance of the cell wall (Chen et al., 2020; Zhu et al., 2007). In our study, a better efficiency in the recovery of phenolic compounds was observed for OH and EAE treated biomass as compared with CE treated samples (Figure 3), both in step 1 and in step 2, possibly ascribed to a positive effect in the cell wall disruption.

For example, in the 1st step, the recovery of phenolic compounds was increased ≈1.8-fold and ≈1.9-fold by OH and EAE, respectively, in comparison to CE (Figure 3A). Moreover, in the 2nd step, the physical (OH) and the enzymatic pre-treatment (EAE) used in the 1st extraction also allowed for a significant increase in the extraction of phenolic compounds ($p < 0.05$) (Figure 3B) when ethanol extraction was applied. These results can be explained by the affinity to the solvent used, as phenolic compounds

differ on their solubility in water and ethanol (Ferreira-Santos, Zanuso, Genisheva, Rocha, & Teixeira, 2020): there are phenolics of polar character extracted in the 1st step and others of a less polar or apolar character, extracted in the 2nd step. This is reflected in the differences in the specific phenolic compounds profile (Table 2). In addition, the technology used for extraction helps to diffuse these compounds across cell membranes and the global extraction of phenolics was significantly affected by the technology used. This behaviour was also observed for other matrices, such as pine bark and rice bran. For instance, the combination of OH/ethanol (50% v/v) showed potential for a sustainable and selective recovery of pine bark phenolic compounds (Ferreira-Santos et al., 2019). Also, Loypimai and co-workers (Loypimai et al., 2015) reported that anthocyanins extraction from rice bran is favored by a combined process of OH with water.

The combination of the OH and EAE significantly enhanced the extraction process of phenolic compounds (1st step, 15.6 ± 0.6 mg GAE/g and 2nd step, 10.05 ± 0.5 mg GAE/g *Spirulina*) when compared to its isolated use, as already happened for phycobiliproteins.

In the literature, it is possible to observe that the application of electric fields for biocompounds recovery from microalgae was already studied, but mostly with pulses and rarely with OH. Postma *et al.* (Postma et al., 2016) used PEF (at 25-55 ºC) for carbohydrates extraction, and their results indicate that a small fraction (< 5 %) of the carbohydrates present in the *Chlorella vulgaris* was released using conventional treatments, while the application of PEF allowed for significantly higher (22-25 %) extraction yields. Recently, our research group optimized the extraction process of high value biocompounds from *Spirulina* powder using OH in which the values obtained were similar to those obtained in this work (Ferreira-Santos, Nunes, et al., 2020). It was

also shown that OH enhanced the recovery of high value compounds, when compared to CE.

The use of enzymes is considered a green process that reduces extraction time and solvent use, improving the extraction yield of specific biocompounds (Rajha et al., 2018). The combination of OH with enzymatic extraction promotes the fragilization of the structure of the *Spirulina* cell wall, increasing the exchange surface area with the solvent, ameliorating thus the release of biocompounds. These facts have been reported by other authors, showing that the combination of emerging technologies increases the extraction of biocompounds, improving the efficiency of extraction. Rajha et al. (Rajha et al., 2018) reported a selective multistage extraction process by combination of electro-technologies (HVED) with enzymes, favoring the process of phenolic compounds recovery from vine shoots. Recently, Zhang and co-workers (Zhang et al., 2020) proposed a multi-stage aqueous and non-aqueous extraction of biomolecules (carbohydrates, protein, lipids and pigments) from the microalgae *Phaeodactylum tricornutum* using emerging technologies like HVED and high pressure. They concluded that multi-stage extraction with several solvents benefits the integral recovery of bioactive compounds with high commercial interest.

Liquid chromatographic analysis was applied in order to identify and quantify the main individual phenolic compounds and to evaluate the influence of the technique and the selectivity of each solvent used in the extraction process over the chemical profile of the *S. platensis* extract (see Table 2). In total, ten phenolic compounds were tentatively identified in the extracts of *S. platensis* according to their corresponding standards.

The 1st extraction step (aqueous extraction) resulted in higher extraction of phenolic compounds. Chlorogenic acid (from 131.3 to 154.8 µg/g of dry extract) was only detected in the ethanolic extracts resulting from the 2nd extraction step as well as

kaempferol and hesperidin that were extracted between 2.4 to 2.7 fold and 4.5 to 7.7 fold more, respectively, in the 2nd extraction step compared to the 1st extraction step. Ellagic acid, ferulic acid, taxifolin and quercetin were compounds found only in aqueous extracts. Ellagic acid was found in concentrations ranging from 424.7 to 502.2 µg/g of dry extract, showing to be the most representative compound in the aqueous extract. On the other hand, kaempferol is the most representative phenolic compound in the ethanolic extract resulting from the 2nd extraction step (244.0 to 375.6 µg/g of dry extract). All other identified compounds were quantified with a concentration between 11.0 to 181.8 µg/g of dry extract (see Table 2).

Previous studies carried out by other authors have shown that *Spirulina* contains phenolic compounds with a high antioxidant capacity (Machado, Pinheiro, Vicente, Souza-Soares, & Cerqueira, 2019; Matos, Cardoso, Falé, Afonso, & Bandarra, 2020; Seghiri, Kharbach, & Essamri, 2019). Casazza *et al.* (Casazza, Ferrari, Aliakbarian, Converti, & Perego, 2015) reported a maximum phenolic yield of 33.2 mgGAE/g *Spirulina*, and Matos *et al.* (Matos et al., 2020) showed concentrations of approximately 3.34 mgGAE/g *Spirulina* for aqueous extracts and 2.05 mgGAE/g *Spirulina* for ethanolic extracts. Authors showed that phenolic acids are the main group of phenolics present in *Spirulina* (Goiris et al., 2014; Machado et al., 2019; Seghiri et al., 2019). These data are in accordance with those obtained in this work.

3.4. Chlorophyll and carotenoids quantification of ethanolic extracts (2nd step)

Chlorophylls and carotenoids are lipophilic compounds authorized in food applications with different technological features, such as coloring in the manufacture of cold drinks, ice creams, among others ("Directive 94/36/CE of the European Parliament and the

Council, 30 June 1994, about Dyes used in Food Products.," n.d.). The consumption of these compounds has been linked with a number of health benefits, including anti-inflammatory activity, cancer chemoprotection, prevention of cardiovascular and metabolic diseases (e.g. cholesterol, hypertension, diabetes, *etc.*) and degenerative diseases (e.g., Alzheimer's disease) (Amorim-Carrilho, Cepeda, Fente, & Regal, 2014; Ferreira-Santos, Aparicio, Carrón, Montero, & Sevilla, 2020). Almost all carotenoids, to a greater or lesser degree, show scavenging properties against different free radicals (Santocono, Zurria, Berrettini, Fedeli, & Falcioni, 2007) and this antioxidant capacity has been extensively investigated (Amorim-Carrilho et al., 2014).

Figure 4 presents the content of photosynthetic compounds (chlorophylls and carotenoids) from the 2nd step of extraction. These compounds were not quantified in the 1st extraction step because they are non-extractable with water. Our results clearly show that chlorophyll-a is the most prevalent photosynthetic compound in ethanolic extracts, being recovered in amounts ranging from 8.4 to 19.1 mg/g from *Spirulina*, corresponding to concentrations of 37 to 158 mg/g in the dry extract. Based on the obtained results, it is possible to observe that previous biomass treatments from 1st step helped to increase the extraction efficiency of chlorophyll and carotenoids in the 2nd step. For chlorophyll-a, the use of OH and EAE significantly (p <0.001) increased the amount of this green pigment with recoveries 44.5% and 44.8% higher compared to the obtained from biomass pretreated with the traditional methodology (CE). Moreover, when biomass was pretreated with lysozyme (EAE) combined with moderate electric fields (OH) the recovery was 128% higher.

Similar results were obtained for chlorophyll-b and carotenoids, in which the combined pretreatment of EAE and OH showed larger efficiency in the recovery of these compounds when compared with the extracts obtained from the biomass pre-treated by

the CE and by the individually applied EAE and OH. For example, the amount of recovered carotenoids was 2 times higher in the extracts obtained from the biomass pre-treated by EAE-OH, when compared with CE.

These results confirm results reported by several authors showed that some microalgae/cyanobacteria, such as *Chlorella vulgaris* and *Spirulina platensis*, contain high concentrations of chlorophylls, mainly chlorophyll-a, and carotenoids (zeaxanthin, lutein, astaxanthin, canthaxanthin, β-carotene) (Hynstova et al., 2018).

3.5. Total lipids and fatty acid profile of ethanolic and chloroform/methanol resulting extracts (2nd and 3rd step)

A number of methods can be used to achieve oil's extraction from microalgae, including solvent extraction, supercritical extraction, pyrolysis, enzymatic hydrolysis, microwaves, ultrasounds, among others. Moreover, the development of an efficient method for lipid extraction and purification from microalgae biomass is one of the critical steps in the downstream processes (Jeevan Kumar, Vijay Kumar, Dash, Scholz, & Banerjee, 2017). Organic solvent extraction (e.g., hexane, ethanol, methanol, acetone, petroleum ether, and a mixture of chloroform and methanol) is one of the most well-known methods for the extraction of lipids from vegetable, flower, and oil seeds. Furthermore, various organic solvents have been used for extraction of lipids from microalgae (Chaiklahan, Chirasuwan, Loha, & Bunnag, 2008). It has been found that a mixture of non-polar and polar organic solvents will ensure complete extraction of all neutral lipids. On the other hand, in addition to the solvent, the use of pretreatments to facilitate disintegration of the microalgae cell wall has enhanced the recovery of lipid fractions (Cho, Oh, Park, Lee, & Park, 2013). As far as we know, the recovery of *Spirulina* lipids using environmentally friendly pretreatments is quite limited.

Furthermore, the previous recovery of other fractions using mild processing may allow to remove contaminant (but valuable) compounds prior to the lipid extraction, allowing the recovery of a more lipid-concentrated fraction without deleterious effects on the lipidic profile, while simultaneously damaging the algae structure, enabling easier lipid recovery.

In our work, lipids were extracted after aqueous pre-treatment by CE, OH, EAE or OH-EAE. Ethanol 95% (v/v) was used (2nd step), followed by re-extraction with chloroform/methanol for quantification purposes only, and in the 3rd step prolonged extraction (6h) with chloroform/methanol was applied on the residues from the 2nd step. The results of the global extraction yield of *Spirulina* are shown in Table 1 and the fatty acid content in Table 3.

The yield of lipids in the 2nd step of extraction was 6.7 ± 0.3%, 7.4 ± 0.2%, 7.6 ± 0.4% and 8.4 ± 0.5% for CE, EAE, OH and OH-EAE pre-treated biomass, respectively. Comparing these results with total yields (Table 1) we can observe that the fraction resulting from the 2nd extraction step is rich in lipids, presenting about 49-72% of lipids. The lipid extraction yields of the 3rd step correspond to the overall yields shown in Table 1, as this extract was 100 % lipidic. In a previous study, the same *Spirulina* was used and chemically characterized (Ferreira-Santos, Nunes, et al., 2020) and the amount of total lipids present in *Spirulina* was 11.32 ± 0.65%. Therefore, it is possible to state that the combination of technologies (OH-EAE) maximizes lipid extraction.

According to the results of GC-FID analyses, palmitic acid (approx. 45% + 47% in 2nd and 3rd step, respectively), linoleic acid (approx. 19% + 18% in 2nd and 3rd step, respectively) and γ-linolenic (approx. 17% + 15% in 2nd and 3rd step, respectively) were determined as the major fatty acids of lipid extracted fraction of 2nd and 3rd step, the pre-treatment showing no influence. The recovery of lipids in the 3rd step was clearly lower

than in the 2nd step. These results indicate that most of the lipids were extracted using 95% ethanol as extraction solvent. However, it is possible to observe that despite the low amount of lipids recovered in the 3rd step of the sequential extraction, the FAMEs content almost coincided with their content in the whole lipidic fraction (extraction yield similar to FAME content) (see Table 1 and 3).

Several studies have been reporting processes for the recovery of lipids from microalgae, such as *Spirulina*, and their industrial applications (Chew et al., 2017; Yang, Du, Hosokawa, & Miyashita, 2020). Taken together, our results suggest that *S. platensis* may provide beneficial fatty acids in human diets if used as a nutrient source in food products. Furthermore, after a refined purification process, some of the fatty acids can be used for medical applications, such as in the treatment of dermatitis, diabetes, and inflammatory diseases (Yang et al., 2020). On the other hand, our results demonstrate that *Spirulina* lipids can have great relevance for the industrial production of biodiesel.

3.6. Antioxidant activity of extracts

The antioxidant activity results for aqueous and ethanolic extracts of the studied microalgae *S. platensis* are presented in Figure 5. Our results show that the antioxidant activity increased over the 60 min for the first extraction step. These data are directly related to the amount of phycobiliproteins and phenolic compounds present in the aqueous obtained extracts.

The antioxidant activity varied significantly with the type of technology used, as well as the solvent used in each extraction step. Aqueous extracts of *S. platensis* (1st step) yielded maximum DPPH scavenging activity for OH-EAE (87.3 ± 0.09%) and for single OH and EAE (86%), and a lower activity ($p < 0.05$) for extracts obtained by CE

(81.9 ± 0.02%). Similar results were observed with FRAP assay, since the lowest activity was measured for the final aqueous CE extracts (1193.7 ± 21.7 µmol Fe^{2+}/L) compared to extracts obtained by EAE (1444.2 ± 28.9 µmol Fe^{2+}/L), OH (1383.7 ± 20.6 µmol Fe^{2+}/L) and OH-EAE (1580.3 ± 12.7 µmol Fe^{2+}/L), respectively.

For the 2nd extraction step, in which ethanol was used, the results showed that the pre-treatment of the biomass influences the antioxidant activity of the obtained extracts. For example, radical inhibition for the DPPH assay was 9% higher ($p < 0.05$) and antioxidant reduction power (FRAP) was approximately 41% higher ($p < 0.01$) for obtained extracts when the *Spirulina* biomass was pre-treated with OH-EAE compared to CE.

Our results also showed that the presence of non-polar compounds such as chlorophylls, carotenoids and lipids, in addition to some phenolic compounds, gives a high reducing antioxidant power (FRAP) activity, showing significant differences between the aqueous (1st step) and ethanolic extracts (2nd step) (see Figure 5A and B). It has been concluded by several authors who worked with different species of microalgae that phycobiliproteins, phenolic compounds, carotenoids and chlorophylls contribute significantly to the antioxidant capacity of these aquatic organisms (Goiris et al., 2012; Matos et al., 2020; Pan-utai & Iamtham, 2019). On the other hand, the obtained extract from the 3rd extraction step did not show any antioxidant activity based on the DPPH and FRAP methods (data not shown).

In a recent study carried out by Matos *et al.* (Matos et al., 2020), *Spirulina* ethanolic extracts also showed greater antioxidant activity assessed by the FRAP method compared to aqueous extracts, but no activity was observed by the DPPH method. In another study, the authors obtained results that clearly showed that both the highest

antiradical and antioxidant activity, determined by the DPPH method, were highly correlated with aqueous extract of *S. platensis* (Shalaby & Shanab, 2013).

3.7. Microscopy analysis

The typical morphology of *Spirulina* is characterized by its regularly coiled trichomes and arrangement in spirals. However, *Spirulina* can occur with abnormal morphologies, such as irregularly curved and even linear shapes, in both laboratory and industry cultures (Wang & Zhao, 2005).

In this work, observation of rehydrated dry *Spirulina* (Figure 6 A) showed green spirals, irregularly curved and even linear shapes. Moreover, when treatments were applied, a clear disintegration and discoloration of the cells was observed (Figure 6 B to G).

Concomitantly, the treatments performed with electric fields and lysozyme showed a higher efficiency in the disintegration and rupture of the biomass cell wall, facilitating the extraction of intracellular compounds (see Figure 6 C, D and E). In general, the application of thermal processes helps to solubilize some proteins of the cell membrane, and the electric current may have destabilized the cyanobacteria macrostructure also at specific more susceptible sites due to the electroporation effects, and not only by non-specific thermal effects (Ferreira-Santos, Nunes, et al., 2020; Vernès et al., 2015). Moreover, lysozyme is suitable for degrading the cyanobacteria's peptidoglycan layer, promoting cell lysis and facilitating the migration of biocompounds (Mehta, Evitt, & Swartz, 2015). These results corroborate the results previously discussed in this work.

In Figure 6 F, we can see that the biomass subjected to pre-treatment (using the example of pre-treated biomass with OH-EAE) and later treated with ethanol underwent cellular dehydration, decreasing the size of the fragments, which indicates the extraction of non-polar compounds soluble in ethanol. This phenomenon was intensified with the

subsequent extraction with chloroform/methanol, leading to cells practically discolored and apparently in smaller fragments (Figure 6 G). These results corroborate the data previously reported for recovery of lipids and pigments. Moreover, this can be explained by the mechanism of extraction by solvents that generally consists in the solvent penetration into microalgal biomass to solvate and separate target product from structural components. Subsequently, solvent in a complex with the bioproduct is transferred outside the cells via diffusion or exocytosis (Miazek et al., 2017).

4. Conclusion

The efficiency of a multi-step extraction procedure for the valorization of *S. platensis* biomass has been investigated. The use of combined physical/thermal (OH) and enzymatic treatments in the extraction process favors the disintegration and rupture of the spirulina cell wall and significantly improves the selectivity and recovery of intracellular compounds (phycobiliproteins) in the first step. Furthermore, the extraction of other fractions (chlorophylls, carotenoids and lipids) in the subsequent steps is also enhanced.

The intensification of the proposed process facilitates the use of alternative and environmentally friendly technologies, and safe and food grade solvents in a multi-stage extraction strategy capable of recovering and isolating different classes of molecules with biofunctional properties, according to the concept of biorefinery. In addition, the safe and inexpensive disposal of all waste products generated during the process can also be considered. Overall, the proposed approach saves energy and time, facilitating scale-up and industrial application to obtain products with high added-value from natural resources such as microalgae.

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Conflict of interest

The authors have no competing interests to declare.

Credit Author Statement

Pedro Ferreira-Santos: Conceptualization; methodology; investigation; formal analysis; data curation; writing–original draft; writing - review and editing;
Sílvia M. Miranda: methodology; investigation; data curation; formal analysis; writing - review and editing;
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Supplementary data

Supplementary material

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Table 1. Extraction yields (%) of different extraction steps from *S. platensis*.

Treatment	1st Step (H_2O)						2nd Step (EtOH 95%)	3rd Step ($CHCl_3$:MeOH)	Ash (Final residue)
Time (min)	10	20	30	40	50	60			
CE	20 ± 2	26 ± 1	28 ± 0	31 ± 2	33 ± 1	35 ± 1[a]	9.6 ± 0.2[a]	1.9 ± 0.2[a]	2.44 ± 0.14[a]
EAE	23 ± 5	27 ± 3	28 ± 0	32 ± 1	35 ± 1	38 ± 2[a]	10.7 ± 0.7[ab]	2.2 ± 0.1[ab]	2.21 ± 0.03[ab]
OH	27 ± 2	29 ± 1	31 ± 0	33 ± 1	37 ± 2	38 ± 0[a]	10.9 ± 0.1[b]	2.6 ± 0.2[bc]	2.22 ± 0.02[ab]
OH-EAE	28 ± 2	29 ± 1	35 ± 2	39 ± 3	43 ± 1	43 ± 2[b]	12.2 ± 0.1[c]	2.8 ± 0.1[c]	2.04 ± 0.05[b]

Values are expressed as mean ± SD of three experiments. Different letters show significant differences ($p < 0.05$) between extraction technologies for the same step.

Table 2. Phenolic compounds identification and quantification from *S. platensis* extracts.

Compounds (µg/g extract)	1st Step (H_2O)				2nd Step (EtOH 95%)			
	CE	**EAE**	**OH**	**OH-EAE**	**CE**	**EAE**	**OH**	**OH-EAE**
ferulic acid	26.8 ± 1.3[a]	25.3 ± 0.2[a]	25.4 ± 1.4[a]	21.3 ± 0.7[a]	n.d.	n.d.	n.d.	n.d.
ellagic acid	502.2 ± 16.2[a]	485.6 ± 4.1[b]	462.3 ± 9.3[b]	424.7 ± 2.9[c]	n.d.	n.d.	n.d.	n.d.
chlorogenic acid	n.d.	n.d.	n.d.	n.d.	148.2 ± 3.0[a]	154.8 ± 1.7[ab]	131.3 ± 0.4[ac]	138.4 ± 3.4[abc]
naringin	24.4 ± 5.4[a]	24.2 ± 4.1[a]	20.2 ± 3.8[a]	18.5 ± 2.7[a]	n.d.	n.d.	n.d.	n.d.
hesperidin	64.1 ± 7.0[a]	75.3 ± 3.4[b]	70.9 ± 6.4[ab]	70.6 ± 0.1[ab]	173.5 ± 0.5[a]	181.8 ± 13.2[a]	172.5 ± 11.4[a]	178.1 ± 1.1[a]
taxifolin	13.3 ± 1.9[a]	15.9 ± 1.5[a]	15.1 ± 2.1[a]	11.0 ± 0.7[a]	n.d.	n.d.	n.d.	n.d.
quercetin	61.9 ± 2.4[a]	54.8 ± 0.4[ab]	54.8 ± 0.0[ab]	49.0 ± 0.0[b]	n.d.	n.d.	n.d.	n.d.
kaempferol	56.4 ± 0.6[a]	54.2 ± 5.2[a]	48.7 ± 0.9[a]	51.0 ± 2.4[a]	308.3 ± 37.6[a]	244.0 ± 2.8[b]	375.6 ± 1.2[c]	320.4 ± 3.2[a]
3,4-hydroxybenzoic acid	153.5 ± 4.7[a]	158.1 ± 1.6[a]	133.1 ± 7.8[b]	130.5 ± 0.2[bc]	52.2 ± 5.2[a]	56.9 ± 2.2[a]	72.1 ± 5.5[a]	63.1 ± 0.1[a]
aloin	152.7 ± 4.1[a]	145.6 ± 8.3[a]	151.7 ± 1.6[a]	120.8 ± 2.0[b]	161.4 ± 8.1[a]	161.5 ± 7.2[ab]	142.9 ± 5.1[abc]	136.3 ± 5.9[c]
TOTAL	1055 ± 44	1009 ± 29	982 ± 33	898 ± 12	844 ± 54	799 ± 27	894 ± 23	836 ± 14

Values are expressed as concentration (µg/g dry extract) mean ± SD of three experiments. n.d.: not detected. Different letters show significant differences ($p < 0.05$) between extraction technologies for the same step and compound.

Table 3. Fatty acid composition of *Spirulina* fractions.

Fatty acid (mg/g $_{extract}$)		Palmitic (C16:0)	Palmitoleic (C16:1)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	γ-Linolenic (C18:3)	TOTAL
CE	2nd step	325.3 ± 1.1[a]	35.8 ± 0.6[a]	15.0 ± 0.3[a]	81.3 ± 0.5[a]	137.1 ± 2.2[a]	128.4 ± 4.2[a]	720.5 ± 8.9
	3rd step	507.8 ± 26.5[ac]	57.0 ± 1.3[a]	32.4 ± 3.7[a]	129.7 ± 5.3[a]	203.1 ± 11.6[a]	153.9 ± 8.7[a]	1083.9 ± 57.1
EAE	2nd step	283.5 ± 16.8[b]	25.5 ± 6.1[ab]	13.0 ± 0.5[a]	58.7 ± 3.5[b]	97.6 ± [illegible][b]	133.2 ± 9.15[a]	611.5 ± 42.4
	3rd step	514.7 ± 1.5[b]	50.3 ± 8.3[a]	60.0 ± 15.4[ab]	106.1 ± 28.1[a]	181.1 ± 28.1[a]	177.9 ± 0.8[a]	1090.1 ± 82.5
OH	2nd step	196.0 ± 4.4[c]	21.6 ± 3.9[b]	10.2 ± 0.9[a]	49.0 ± 1.4[b]	83.7 ± 2.8[bc]	130.0 ± 12.5[a]	490.5 ± 25.9
	3rd step	506.6 ± 22.2[ac]	56.2 ± 4.9[a]	70.4 ± 4.1[b]	135.6 ± [illegible]	204.6 ± 16.6[a]	158.3 ± 10.3[a]	1131.9 ± 62.4
OH-EAE	2nd step	215.8 ± 1.3[d]	23.1 ± 1.7[b]	13.7 ± 0.0[a]	54.8 ± 0.9[b]	91.2 ± 0.3[bc]	127.5 ± 3.7[a]	525.9 ± 7.9
	3rd step	484.5 ± 0.1[a]	50.7 ± 2.8[a]	63.2 ± 10.6[b]	131.7 ± 16.5[a]	180.3 ± 8.4[a]	153.2 ± 1.3[a]	1063.1 ± 39.5

Values are expressed as concentration (mg/g dry extract) mean ± SD of three experiments. Different letters show significant differences ($p < 0.05$) between extraction technologies for the same step and compound.

Figure 1. Schematic presentation of sequential multi-stage extraction procedures.

Figure 2. Phycobiliproteins recovery (mg/$g_{dw\ Spirulina}$) for the 1st step extraction at different exposure times (10 to 60 min). C-phycocyanin (**A**), allophycocyanin (**B**) and phycoerythrin (**C**). Error bars represent mean ± SD of three experiments. Different letters show significant differences ($p < 0.05$) between extraction technologies.

Figure 3. Total phenolic compounds (TPC, mg GAE/$g_{dw\ Spirulina}$) recovery for 1st step extraction (**A**) and 2nd step extraction (**B**). Error bars represent mean ± SD of three experiments. Different letters show significant differences ($p < 0.05$) between extraction technologies.

Figure 4. Chlorophyll and carotenoids recovery (mg/$g_{dw\ Spirulina}$) (**A**) and concentration (µg/$g_{dw\ extract}$) (**B**) for 2nd step extraction. Error bars represent mean ± SD of three experiments.

Figure 5. Antioxidant activity measurement by FRAP (**A**,**B**) and DPPH (**C**,**D**) in extracts obtained over time (10-60 min) from 1st step (**A**,**C**) and for 2nd step extraction (**B**,**D**). Error bars represent mean ± SD of three experiments. Different letters show significant differences ($p < 0.05$) between extraction technologies.

Figure 6. Microscopic images of *S. platensis* biomass (100X). Image **A** represents untreated cells, images from **B** to **E** correspond to aqueous treated cells of the 1st extraction step (CE (**B**), EAE (**C**), OH (**D**) and OH-EAE (**E**)), image **F** represents the cells after ethanolic extraction (2nd step) and image **G** represents the cells after extraction with chloroform/methanol (3rd step). Scale bar of 100 µm applies to all images.

Highlights

- A sequential extraction strategy is presented for the integrated valorization of *Spirulina*
- Ohmic heating (OH) and enzymatic treatment (EAE) improves phycobiliproteins extraction
- Combined OH and EAE enhances the extraction efficiency and process sustainability
- Combined OH and EAE pretreatment maximizes lipid extraction
- Disintegration and rupture of the *Spirulina* cell wall was observed

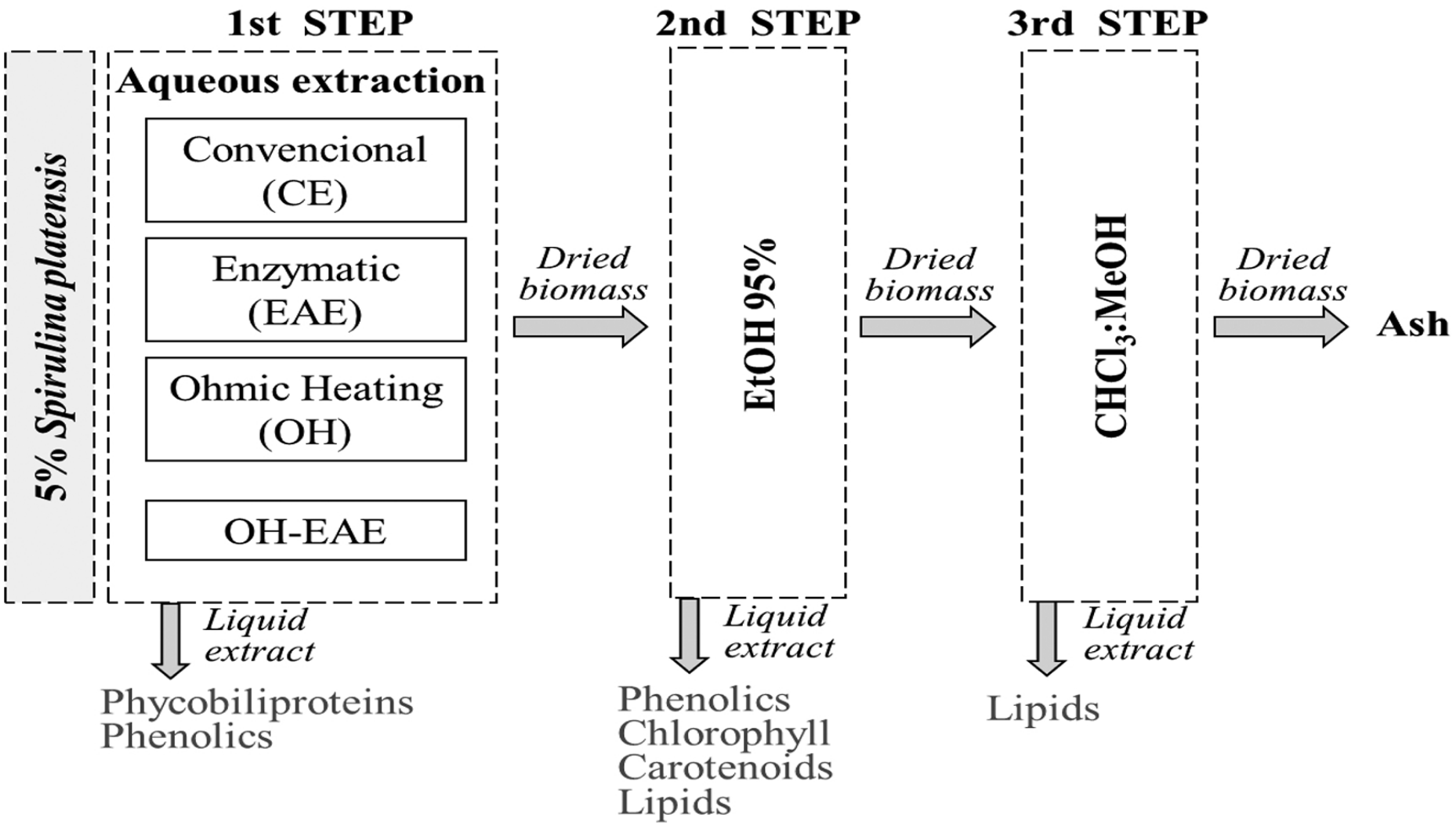
1st STEP
2nd STEP
3rd STEP
5% Spirulina platensis
Aqueous extraction
Convencional (CE)
Enzymatic (EAE)
Ohmic Heating (OH)
OH-EAE
Dried biomass
EtOH 95%
Dried biomass
CHCl3:MeOH
Dried biomass
Ash
Liquid extract
Phycobiliproteins
Phenolics
Liquid extract
Phenolics
Chlorophyll
Carotenoids
Lipids
Liquid extract
Lipids

Figure 1

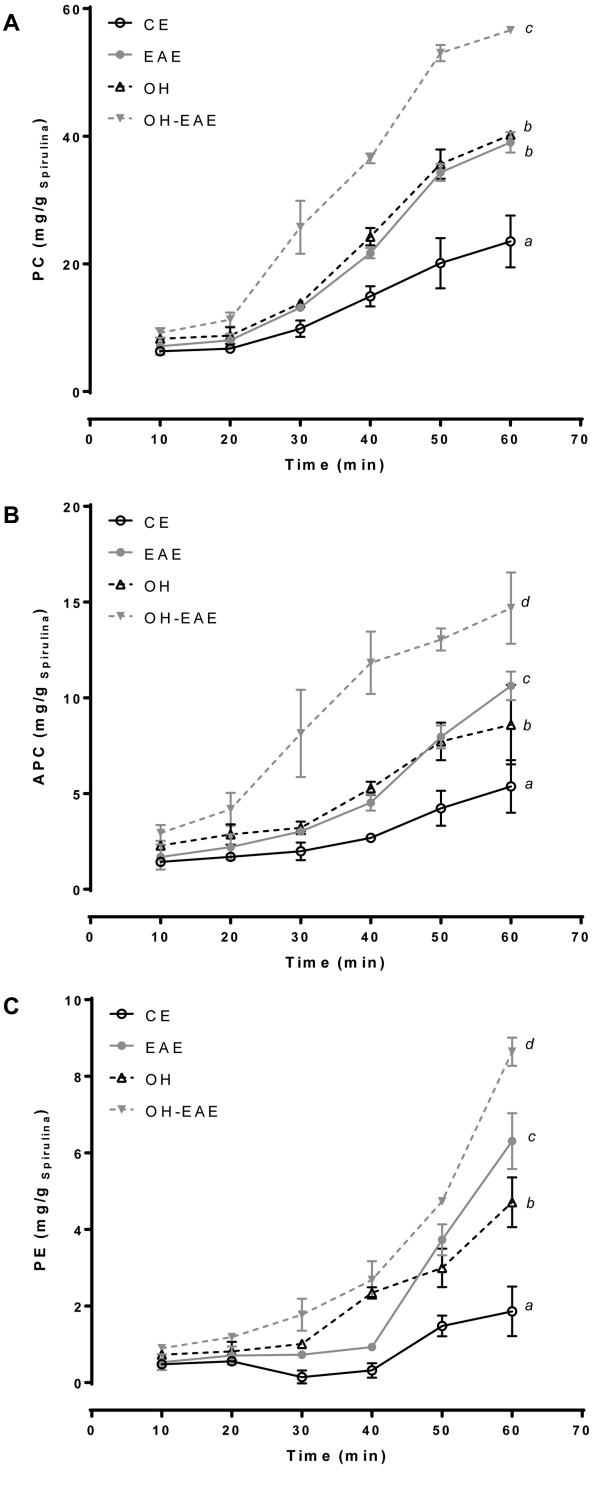
A
CE
EAE
OH
OH-EAE
PC (mg/g Spirulina)
Time (min)
a
b
b
c
B
APC (mg/g Spirulina)
Time (min)
a
b
c
d
C
PE (mg/g Spirulina)
Time (min)
a
b
c
d

Figure 2

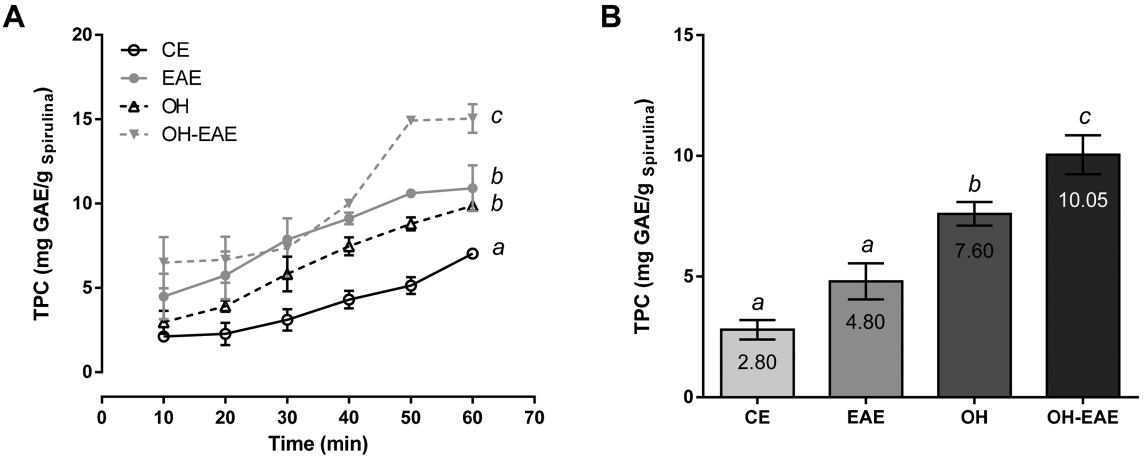
A
CE
EAE
OH
OH-EAE
TPC (mg GAE/g spirulina)
Time (min)
a
b
b
c
B
TPC (mg GAE/g spirulina)
2.80
4.80
7.60
10.05
CE
EAE
OH
OH-EAE

Figure 3

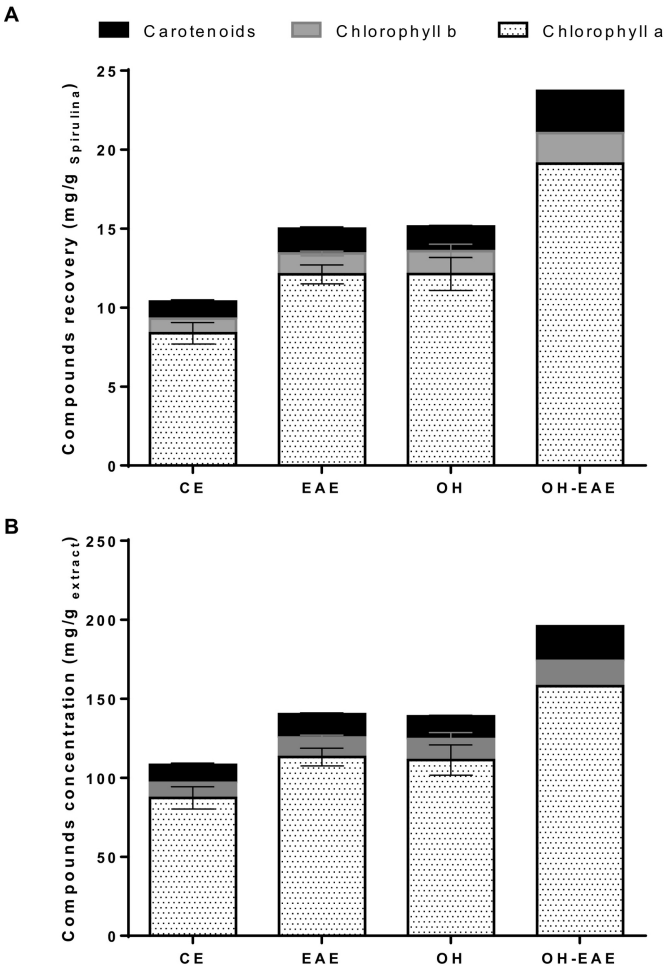
A
Carotenoids
Chlorophyll b
Chlorophyll a
Compounds recovery (mg/g Spirulina)
25
20
15
10
5
0
CE
EAE
OH
OH-EAE
B
Compounds concentration (mg/g extract)
250
200
150
100
50
0
CE
EAE
OH
OH-EAE

Figure 4

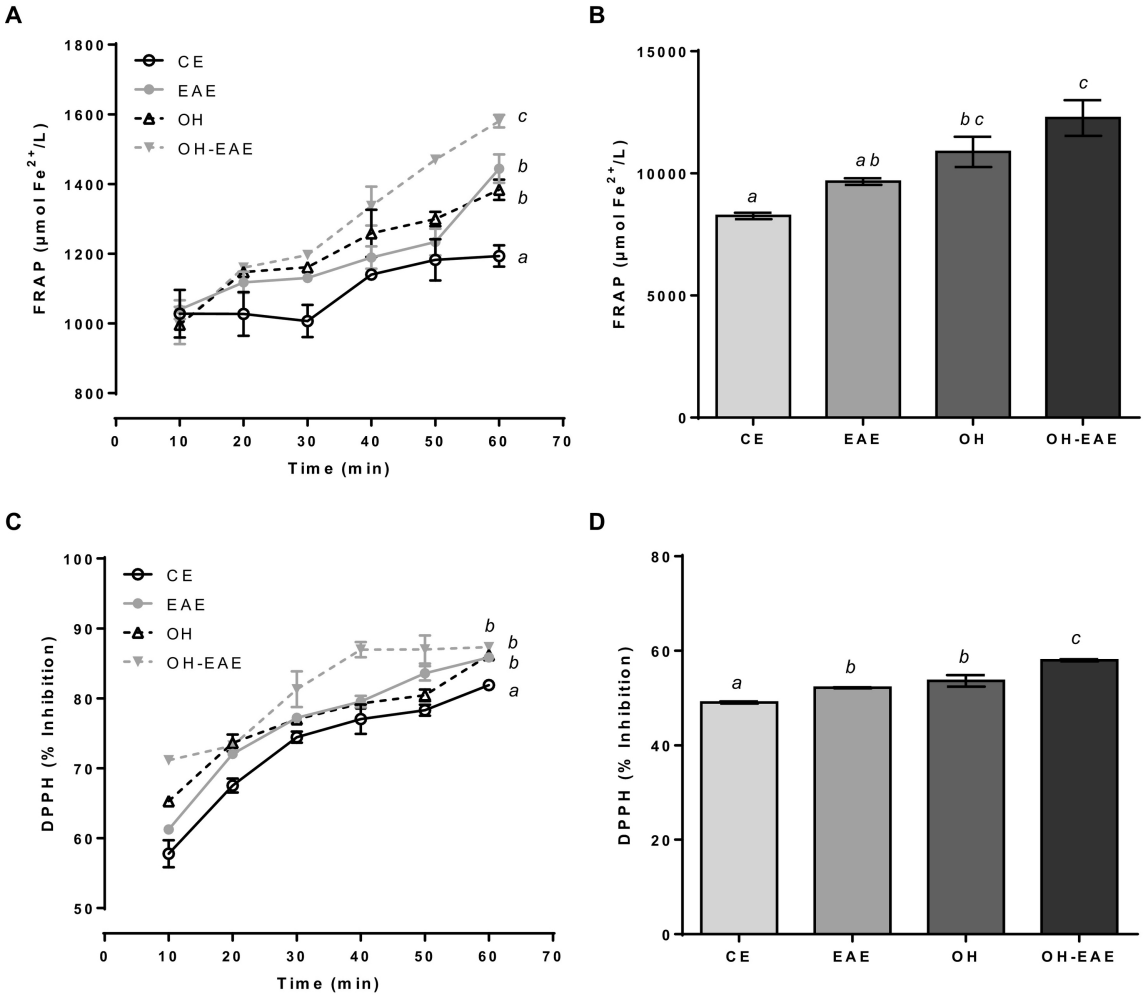
A
B
C
D
CE
EAE
OH
OH-EAE
FRAP (µmol Fe2+/L)
Time (min)
DPPH (% Inhibition)
a
b
c
a b
b c

Figure 5

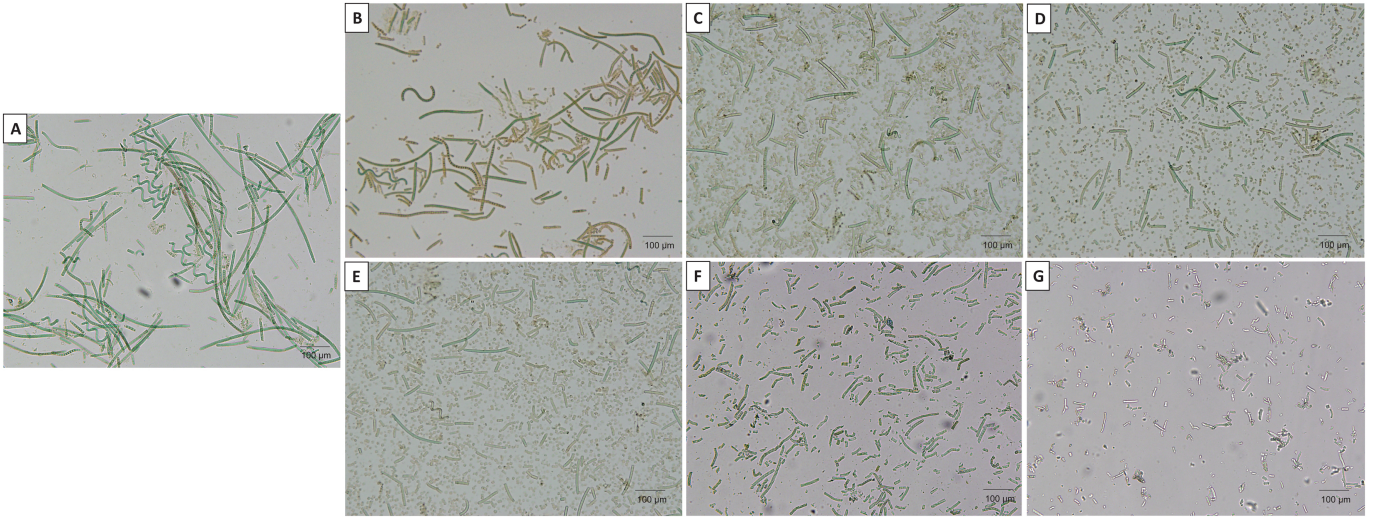

Figure 6