Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO2Using Deep Eutectic Solvents and Microwaves as Pretreatment

Enhanced and selective lipid extraction from microalgae *P. tricornutum* by dimethyl carbonate and supercritical CO_2 using Deep Eutectic Solvents and Microwaves as pre-treatment

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ABSTRACT: Microalgae are a promising alternative source of several bioactive compounds useful for human applications. However, lipids are traditionally extracted with toxic organic solvents (e.g. mixture of chloroform and methanol or hexane). In this work, we developed a new lipid extraction protocol to obtain a fatty acids rich extract from the diatom *Phaeodactylum tricornutum*. Deep Eutectic Solvents (DES) and microwaves (MW) were investigated as pre-treatments for environmentally friendly solvent extractions using dimethyl carbonate (DMC) and supercritical CO2 (scCO2). Pre-treatments with various DES formed by choline chloride (ChCl) and different hydrogen bond donors (oxalic acid, levulinic acid, urea, ethylene glycol and sorbitol) were tested in combination with DMC extraction. DES formed by ChCl and carboxylic acids gave best results, increasing both selectivity and total fatty acids (TFA) extraction yield of DMC (respectively by 16% and 80%). DES combined with MW heating followed by DMC extraction allows to reach comparable TFA yield and fatty acid profile to traditional Bligh & Dyer extraction method and much better selectivity (88% vs. 35%). This pre-treatment was also demonstrated to improve significantly extraction efficiency of scCO2, increasing by 20 times TFA yield and providing highly purified triglycerides extracts.

Introduction

In recent years, microalgae have gained attention as alternative source of bioactive compounds for human consumption (i.e. omega-3 fatty acids and carotenoids).[1] For example, due to the depletion of fish stocks, it will be increasingly difficult to fulfill the demand for polyunsaturated fatty acids (PUFAs) by fish oil alone; therefore, micro and macroalgae (seaweeds) can become a suitable alternative source of ω-3 fatty acids.[2,3,4]

The marine diatom *Phaedactylum tricornutum* is characterized by a high content of lipids and PUFAs (30–45% of total fatty acids, TFA), among which eicosapentaenoic acid (EPA) is the major one.[5] EPA has an important role in human health: it is involved in the blood lipid equilibrium, has anti-inflammatory properties and can be useful for the prevention of hypertriglyceridemia.[6,7,8]

Traditionally, lipids are extracted from biological matrices using organic solvents as hexane or a combination of chloroform and methanol. The latter procedure, known as the Bligh and Dyer method, originally designed to extract lipids from fish tissue, has been used as a benchmark for comparison of solvent extraction methods.[1,9]

Extraction of lipids from microalgae has been deeply investigated during the last 20 years, especially for biofuels production, and several novel techniques have been developed to achieve better yield and selectivity with respect to traditional solvent extraction methods. Moreover, awareness of the risks related to safety, health, and environment burden associated with the use of organic solvents in food, cosmetics and pharmaceuticals production is constantly increasing both in the scientific community and in the society, fostering the development of new green technologies and solvents suitable to replace traditional extraction methods. These new extraction protocols must be safe, efficient,

time and cost savings, environmentally friendly and scalable.[10]

To this purpose, the use of non-volatile, cheap and non-toxic organic solvents is an excellent choice for the development of safer processes. Among non-VOC solvents, low vapor pressure organic compounds like alkyl carbonates (e.g. dimethyl carbonate DMC) or liquid ion pairs like ionic liquids can represent promising options.[11,12] Another well described extraction methods to recover PUFAs from microalgae is the use of supercritical CO_2 ($scCO_2$), a fluid characterized by low critical temperature and pressure.[13,14] $ScCO_2$ is advantageous from many points of view: i) it provides pure extracts, free of potentially harmful solvent residues (safe for food application); ii) the solvent can be easily recycled; iii) it is suitable for thermally sensitive product.[14,15] $ScCO_2$ is lipophilic and easily solubilizes non polar compounds as neutral lipids (mainly triglycerides) but when combined with polar co-solvents (e.g. ethanol, water, and methanol) in low concentrations (usually 10–15%), it is also able to extract polar lipids (such as phospho and glycolipids, chlorophylls, waxes and pigments).[16,17] For example, when applied to the extraction of the cyanobacterium *Arthrospira platensis*, a combination of $scCO_2$ and 10% of ethanol proved to be as effective as the benchmark Bligh and Dyer system, while $scCO_2$ alone gives highly purified triglyceride extracts but poor overall yields.[17,18,1]

Biomass pre-treatments aim to increase solvent diffusion by disrupting or damaging aggregates and cell wall; therefore, they can be a very important step for the extraction of biomolecules from natural sources allowing the use of milder and more sustainable solvents and improving extraction yield.[19,10] Finding an effective and inexpensive pre-treatment is especially important for microalgae characterized by a thick and robust cell wall, as diatoms (*P. tricornutum*) whose cell wall is mainly composed by silica. Different algal pre-treatments have been reported in the literature; many of them are able to increase extraction rate and yields reducing overall costs and time if compared to extraction processes without pre-treatments.[1,10,20,21,22,23,24,25,26] Cell disruption methods are classified in: mechanical (e.g. bead milling), thermal (e.g. microwaves), physical (e.g. osmotic shock and ultrasounds), enzymatic and chemical (e.g. acid, basic, Ionic Liquids or Deep Eutectic Solvents treatment). Specifically, Ionic Liquids (ILs) mode of action has been claimed to involve an interaction between specific functional groups on algal cell wall/membrane surface (e.g. negatively charged silica-associated organic components in diatoms) and cations and anions of the ion pair.[27] Consequently, mixtures of organic solvents and ILs have been used to dissolve biomass and extract with high efficiency lipids from *Chlorella vulgaris*.[24] Deep Eutectic Solvents (DES) are a new generation of ILs composed of an organic salt (such as choline chloride) and a hydrogen-bond donor (HBD) (such as amides, amines, alcohols, and carboxylic acids) that self-associate through hydrogen bonds to form an eutectic mixture with a melting point lower than that of each individual component.[28,29] In comparison to many traditional ILs, DES have several benefits such as simple preparation, low cost, low toxicity and high biodegradability.[30] For these reasons, DES-pre-treatment has been recently applied for the first time to enhance lipid extraction from freshwater green algae, demonstrating the high potential of this approach.[26]

Microwaves and ultrasounds are other promising pre-treatment methods able to increase extraction efficiency and selectivity.[31,32] These innovative green techniques typically involve less time, energy and amount of solvents than conventional thermal heating, being an excellent choice to reduce environmental impact of the process.[33]

The aim of the present study is to develop new extraction methods to safely, selectively and efficiently obtain a lipid extract enriched in fatty acids from the diatom *P. tricornutum*. In order to reach this goal, we investigated the effects of different pre-treatments coupled with green solvents (DMC and $scCO_2$) extraction. As pre-treatment, we investigated the efficacy of different aqueous DES (aDES) singularly and in combination with microwaves or ultrasounds at different time and temperatures, optimizing pre-treatment protocol in terms of total fatty acids (TFA) content and extraction yield of DMC and $scCO_2$.

Experimental Section

Chemicals

All chemicals and reagents were purchased from Sigma Aldrich and used without any further purifications.

Microorganism and culture conditions

P. tricornutum biomass was provided by Micoperi Blue Growth srl, an Italian start-up company (Ravenna, Italy). Semicontinuous cultivation was performed with the synthetic marine medium f/2 modified with five-fold nutrient concentration and 2/3 decreased in nitrogen content (cultivation under nitrogen starvation), low salinity (15 ‰), and at pH 7.5, through two bubble column photobioreactors of 120 L each. [34] During the six months of cultivation, every two weeks the culture was monitored and harvested with a continuous flow centrifuge (MAC FUGE), once the algae growth regime reached half of the stationary phase. The nutrients supply was performed occasionally, to restore the optimum concentration in the medium.

The cultures were maintained at temperature of 18-20 °C, with a photoperiod of 12:12 h light-dark cycle and an artificial light intensity of 100-110 $\mu E\ m^{-2}s^{-1}$. All cultures were mixed through air bubble aeration, and CO_2 was insufflated every morning for inorganic carbon supply and to drop the pH from 9 to 7.5.

Microalgae biomass was finally harvested and freeze-dried.

aDES preparation

All aDES were prepared by mixing appropriate stoichiometric ratio of choline chloride and different HBD: oxalic

acid (1:2), levulinic acid (1:2), urea (1:2), ethylene glycol (1:2), and sorbitol (1:1). 40% by weight of deionized water was then added. The mixture was heated at 70°C and magnetically stirred until a uniform colourless liquid was obtained.

Pre-treatments

All pre-treatments were performed in duplicate on a sample of dry biomass of *P. tricornutum* cultivated under nitrogen starvation.

aDES pre-treatments

aDES (1 mL) was added to algal biomass (100 mg) and the mixture was magnetically stirred for 24 hours at RT. The algal suspension was diluted with deionized water (3 mL) and then centrifuged to separate the surnatant from biomass. Algal biomass was washed two times more with deionized water (3 mL) and then freeze-dried.

MW pre-treatments

The microalgae samples (400 mg) were added to the fresh prepared aDES (4 mL) or simply water (4 mL), using a 20-mL borosilicate glass vial. The vial was inserted in a multimodal MW autoclave reactor (SynthWAVE, Milestone s.r.l.) which allows fast heating, fast cooling and inert atmosphere (N_2). A suitable N_2 pressure avoided any boiling (2 bar at 100°C and 5 bar at 150°C).

The selected screening parameters were temperature (100°C, 150°C), process time (2, 10, 30 and 60 min) and solvent type (DES and water).

At the end of each treatment, the algal suspension was diluted with deionized water (5 mL) and centrifuged to separate the surnatant from biomass. Algal biomass was washed two times more with deionized water and then freeze-dried.

Lipid extraction and analysis

All lipid extractions were performed in duplicate on dry biomass, pre-treated or not (Bligh & Dyer was applied only on not pre-treated biomass). Each extraction protocol was evaluated in terms of:

- extracted total lipid amount (including, TFA, waxes, sterols, hydrocarbons, ketones and pigments) expressed as percentage respect to biomass dry weight (wt%);

- TFA amount among lipids (free and bounded) expressed as percentage respect to biomass dry weight (wt%). TFA were determined as their corresponding fatty acids methyl esters (FAME) through GC-MS analysis.

Bligh & Dyer (B&D) extraction

Biomass (100 mg) was extracted with a mixture of methanol (1 mL) and chloroform (2 mL) for 2 hours at 50 °C under magnetic stirring. Sample was centrifuged and organic phase was withdrawn; these conditions were repeated three times and the organic phases collected before drying under nitrogen.

DMC extraction

Biomass (100 mg) was extracted with DMC (3 ml) for 2 hours at 50 °C under magnetic stirring. Sample was centrifuged and organic phase was withdrawn; these conditions were repeated three times and the organic phases collected before drying under nitrogen.

ScCO2 extraction

ScCO2 extractions were carried out using an Applied Separations extractor (Allentown, PA, USA) model Spe-edTM SFE Prime. CO_2 ($N_4.5$ purity grade, 99.995 %) was supplied by SOL S.p.a. A 10 mL extraction vessel was then loaded with 400 mg of powdered sample mixed with an equal amount of Spe-edTM Matrix. After the sample compression, a plug of wool (Spe-edTM Wool) was place on top and the empty space was filled with the matrix. The extraction CO_2 flow rate was maintained at an average level of 2.5 L min^{-1}, and the condition of pressure and temperature of the process were the following: 350 bar and 45°C, 25 min of static extraction, followed by 100 min of dynamic extraction.

TFA derivatization procedure into FAME

Lipid samples (about 2 mg) were dissolved in DMC (0.4 mL). 2,2-dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL) were then added; the samples were placed in an incubator at 90°C for 30 min. After cooling for 5 min to room temperature, 1.3 M BF_3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (0.02 mg) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-DMC layer, containing FAMEs, was transferred to vials for GC-MS analysis. Each analysis was repeated in duplicate.

GC-MS analysis

GC-MS analyses were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12-600 m/z range. The temperature of the column was increased from 50°C up to 180°C at 50°C min^{-1}, then from 180°C up to 300°C at 5°C min^{-1}. Methyl nonadecanoate was utilized as internal standard for quantification of free and bounded fatty acids converted into FAME. The relative response factors used for the quantitation were obtained by injecting solutions of known amounts of methyl nonadecanoate and commercial FAME mixture.

Results and discussion

Lipid extraction by DMC

A first set of experiment was carried out to understand if DMC, previously reported as good solvent for lipid extraction from microalgae due to its suitable polarity and high stability,[11,35,36] could provide comparable fatty acids extraction yield to B&D benchmark protocol. Even if DMC is a non-volatile, cheap, noncorrosive, non-toxic, and eco-friendly solvent, it is nor efficient or selective as B&D method, as testified by the lower lipid yield (11.0 vs 31.3 wt%) and TFA amount (4.5 vs 11.1 wt%) (Figure 1).[37] Therefore, to improve the performance of DMC and enhance its permeation into cells, the application of a pre-treatment is mandatory.

Recently, W. Lu et al. described the application of various DES pre-treatments to enhance lipid extraction from *Chlorella sp.*.[26] A biomass pre-treatment with a choline chloride and oxalic acid aDES increased lipid recovery from 50 to 80% (of total lipid content) by using a mixture of ethanol and ethyl acetate for the extraction and a biphasic purification system with hexane and water. Unfortunately, the effect of the pre-treatment on the TFA content and composition in lipid extracts was not reported.

We thus tested various aDES based on choline chloride (ChCl) to enhance DMC efficiency in terms of total lipid extraction yield and selectivity toward fatty acids. Different H-bond donors (HBD) were used: levulinic acid (LA), oxalic acid (OA), urea (U), sorbitol (S), and ethylene glycol (EG). Data in Figure 1 clearly show that composition of various aDES influences their ability to interact with cellular membranes and enhance cell wall permeability. When the HBD is a polyol (S and EG) or urea (U), the pre-treatment does not significantly affect the TFA selectivity or lipid extraction, even if these aDES are reported to give stronger interaction with cell wall polysaccharides.[38] On the contrary, both aDES formed by ChCl and carboxylic acids (LA and OA) were able to increase the total lipids extraction by DMC. Pre-treatment with ChCl-LA was poorly reproducible (high standard deviation) and, on average, provided lipid extracts with lower TFA content than ChCl-OA (47% vs 57% of TFA in lipid extract). ChCl-OA pre-treatment proved to be the best system: in fact, TFA extraction yield with DMC increases by 80% in comparison to DMC-extraction without any pre-treatment, reaching a value of 8.1 wt%. This result confirms what observed by Lu et al. on *Chlorella sp.* and demonstrates the suitability of ChCl-OA for the pre-treatment of algal biomass.[26]

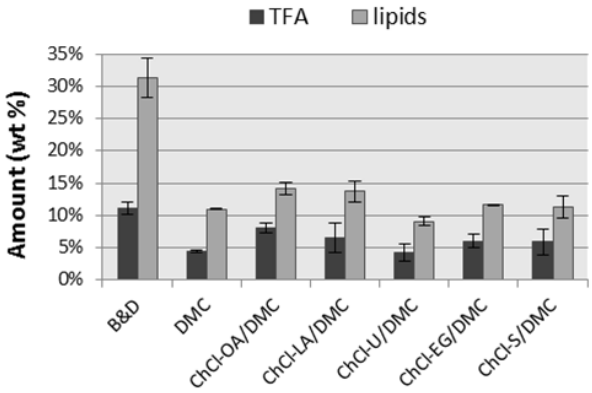

Figure 1 Effect of aDES pre-treatment on total lipids and TFA amount (calculated as FAME), extracted from *P. tricornutum* with DMC in comparison with Bligh & Dyer method (B&D) and DMC without any pre-treatment. Data are expressed on dry weight basis (wt%), as mean of two replicates ± st. dev. (wt%).

A possible explanation could be that, as reported in literature, a decrease in pH due to the presence of carboxylic acids improves the permeability of lipophilic solutes trough cell walls in some aquatic microorganism.[39] However, when a pre-treatment with oxalic acid alone was performed, lower yield and selectivity was obtained, demonstrating the importance of the synergy between ChCl and OA in this step (Figure S1).

Nevertheless, ChCl-OA pre-treatment followed by DMC extraction still provides lower lipid and TFA yield compared to B&D method; even when pre-treatment parameters (DES/water ratio and aDES/biomass ratio) and temperature (50 and 70°C) were varied (Figure S2), no improvement was observed. For this reason, DES pre-treatment was further studied in combination with promising novel technologies in the field of algae treatment as microwaves (MW) and ultrasounds (US).

Previous studies reported the application of DES-MW/US for the extraction of polyphenols, flavonoids and alkaloids from various vegetable or waste biomasses, confirming the positive role of a combined chemical and thermal approach.[40,41,42,43] In fact, similarly to conventional ILs, DES can be efficiently coupled with MW irradiation due to their polar nature. Especially organic acid-based DES, which show the highest polarity. The coupling of the solvent dipoles with the electromagnetic field induces a strong in-core heating effect, ensuring a homogeneous temperature profile, completely focused on the sample.[44,45]

The application of US in conjunction with DES, conversely, offers the chance to overcome issues due to the moderate viscosity of these solvents. Collapsing cavitation bubbles enhance at the same time mass transport and cell walls disruption.[46,47] However, when applied to algal biomass, DES-US pre-treatment was not able to increase TFA extraction

yield (Figure S3), probably due to excessive biomass disaggregation that hampered separation of biomass from water and solvent phase.

On the other hand, DES-MW effectiveness depended on time and temperature (Figure 2):

• When only MWs are applied, extraction with DMC is scarcely effective and selective, with no significant effect of time or temperature on TFA and lipid yield;

• MW heating at 100°C combined with ChCl-OA pre-treatment at increasing time (10, 30 and 60 min) is always much more effective for lipid and TFA extraction than MW alone, but longer times lead to a slight decrease in selectivity;

• Combination of MW heating at 150°C with DES significantly increases the amount of TFA extracted in comparison to MW. On the other hand, total lipid extraction is less effective than DES-MW at 100°C, resulting in an increased selectivity towards TFA. This was especially true for the 30 min treatment.

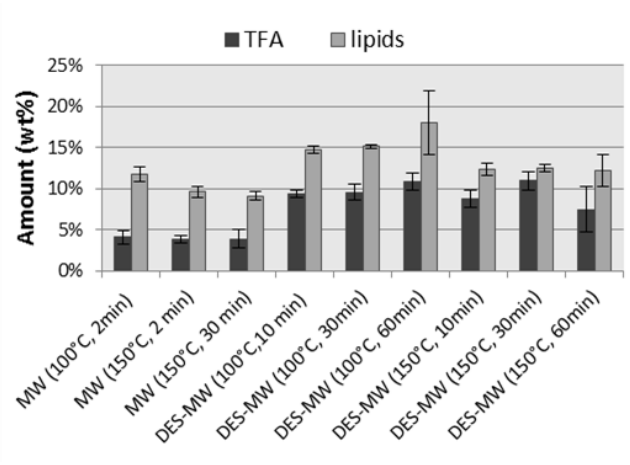

Figure 2 Effect of time and temperature on TFA (determined as FAME) and total lipid amount (wt%) extracted from *P. tricornutum* using DMC, after pre-treatment with MW alone and in combination with ChCl-OA aDES. Data are expressed on dry weight basis (wt%), as mean of two replicates ± st. dev. (wt%).

Best TFA extractions yields are thus obtained after pre-treatment with DES-MW at 100°C for 30 and 60 min or 150°C for 30 min. However, the latter conditions would be preferable in order to maximize also the purity of final lipid extract (increased selectivity towards TFA).

Results summarized in Table 1 demonstrate that the combination of DES and MW (entry 4) can effectively enhance the efficiency and selectivity of fatty acids extraction by DMC. This condition in fact, is the only one able to provide comparable TFA yield to B&D method (entry 0). Additionally, DES-MW allows to obtain a lipid extract composed by 88% of fatty acids, being significantly more selective than B&D (35% of TFA in lipid extract). DMC alone (entry 1), DMC after DES pre-treatment at RT (entry 2) and MW alone followed by DMC (entry 3), prove that a synergic effect of DES and MW is essential to: i) reduce pre-treatment time (from 24h to 30 min); ii) obtain the maximum efficiency and selectivity with DMC as extracting solvent.

Table 1 Effect of different pre-treatments on lipid and TFA extraction yield.

Entry	Pre-treatment /extraction	Lipid amount (wt%)	TFA amount (wt%)
0	Bligh & Dyer	31.3 ±3.0	11.1 ±0.9
1	DMC	11.3 ±0.1	4.5 ±0.2
2	DES (25°C, 24h) / DMC	14.1 ±0.1	8.1 ±0.8
3	MW (150°C, 30 min) / DMC	9.2 ±0.5	3.9 ±0.7
4	DES-MW (150°C, 30min) / DMC	12.5 ±0.4	11.0 ±1.1

Total lipids and fatty acids extracted from *P. tricornutum* using different pre-treatments and solvents. Data are expressed as mean of two replicates ± st. dev. (wt%).

3.2. Lipid extraction by scCO2

The combined synergic effect of DES and MW was also tested by using scCO2 as extracting solvent. Therefore, the best pre-treatment conditions found for DMC extraction were applied and compared with the results achieved without any pre-treatment. ScCO2 extraction of lipids from microalgae has been widely studied and many papers report optimized parameters for temperature, pressure and time to maximize lipid and TFA yield.[15,17,20,16,48,49,50] However, these results are often variable and sometimes even discordant. This is probably due to several factors that can influence the extraction process, such as type of extractor, algal biomass composition, strain, and cultivation, amount of water (moisture) in the sample, and biomass pre-treatment (bead milling, freeze drying etc.).[49,51]

Due to all these variables, comparisons are often meaningless; we decided to keep scCO2 parameters fixed while focusing our attention on changes in relative yield and selectivity, in order to preliminary identify the best pre-treatment conditions.

The presence of 10-15% of a co-solvent such as ethanol is often reported to be beneficial for increasing total lipid yield. However, neutral lipids represent just a fraction of this total extract composed mainly by glycolipids.[17] In this study, to maximize the selectivity towards neutral lipids (and more specifically triglycerides) we did not add any co-solvent.

As expected from previous literature, scCO2 alone is scarcely effective on non-pre-treated microalgal biomass (extracted TFA amount of 0.3 wt%, Figure 3).[17] This could

be explained by the thick silica wall of diatom *P. tricornutum* that can prevent an effective permeation of the solvent inside the cells.[52] Indeed, results in Figure 3 show that all tested pre-treatments improved scCO2 extraction. Specifically, DES pre-treatment enhances both selectivity (from 27% to 67%) and TFA extraction yield (from 0.3 to 3.5 wt%) of scCO2. On the other hand, DES-MW pre-treatment, show a more significant extraction enhancement than DES, doubling the percentage of TFA extracted (from 3.5 to 7.0 wt%). This pre-treatment is therefore able to increase by 20 times TFA yield in comparison to non-pre-treated biomass (7.0 vs 0.3 wt%).

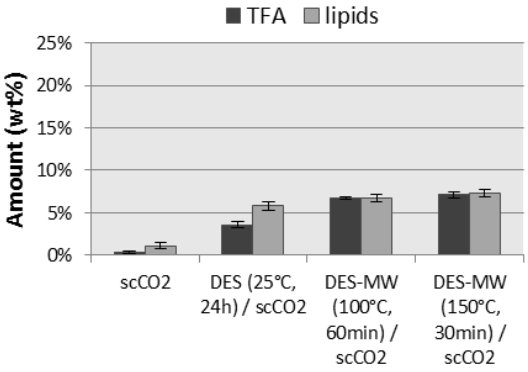

Figure 3 TFA (determined as FAME) and total lipids extracted from *P. tricornutum* using scCO2 (350 bar, 45°C) with or without pre-treatments with aDES at different conditions of time and temperature. Data are expressed on dry weight basis (wt%) as mean of two replicates ± st. dev. (wt%).

Both MW heating conditions tested (100 °C for 60 min and 150°C for 30 min) give comparable results for what concerns efficiency and selectivity. Consequently, since a less energy demanding process is preferable to minimize costs, a preliminary energy consumption evaluation was performed (Table 2). MW heating at 100°C for 60 minutes saves 27% of energy in comparison with consumption of heating at 150°C for 30 min. Thus, longer DES pre-treatment at lower temperature appears to be the most appropriate condition for extraction of TFA with scCO2.

Table 2 Energy consumption of MW pre-treatments

MW parameters	Energy consumption (W)
100°C, 60min	310
150°C, 30 min	424

Estimated energy consumption (expressed in Watt) for MW heating at different conditions of time and temperature.

According to our results, DES-MW pre-treatment followed by scCO2 extraction is a promising protocol that provides highly purified and biocompatible microalgal lipid extracts, composed almost completely by fatty acids. ^{1}H-NMR analysis of the extracts (Figure S4) confirmed triglycerides as the main component, proving the high selectivity of scCO2 as extracting solvent.

Comparison in PUFAs and EPA content in lipid extracts

PUFAs, and especially EPA, are the most valuable bioactive compounds contained in the lipid fraction of *P. tricornutum*. To understand if the developed protocols are suitable for PUFAs recovery from algal biomass, it was important to determine and compare their content in the TFA fraction with the benchmark method. For this reason, relative percentages of EPA and PUFAs in TFA extracted with all methods mentioned above are reported in Table 3. No PUFAs or EPA were detected in lipid extract obtained with scCO2 without any pre-treatment. A general trend is observed for both DMC and scCO2 protocols: the relative percentage of PUFAs and EPA decreases by increasing TFA extraction yield. As for the absolute amount, the two extraction systems behave differently: using DMC the absolute amount of EPA and PUFAs increases with TFA yield, reaching comparable value to B&D method in case of DES-MW pre-treatment (2.2 vs 2.0 wt% of EPA and 4.4 vs 4.4 wt% of PUFAs). On the contrary, no significance difference in total EPA and PUFAs amount was detected comparing DES and DES-MW scCO2 extraction. Comparing these two results it also appears that even if DES/scCO2 provides a lower amount of TFA than DES-MW/scCO2, gave the highest relative percentage of EPA (35% of TFA) among all the extracts. This result could indicate that MW enhances extraction of lipids that does not contain EPA.

Table 3 EPA and PUFAs content in lipid extracts

Pre-treatment /extraction	TFA amount (wt%)	EPA, % in TFA (wt%)	PUFAs, % in TFA (wt%)
Bligh & Dyer	11.1	18 (2.0)	40 (4.4)
DMC	4.5	24 (1.1)	58 (2.6)
DES/DMC	7.3	22 (1.6)	49 (3.6)
DES-MW (150°C, 30 min)/DMC	11.0	20 (2.2)	40 (4.4)
DES-MW (100°C, 60 min)/DMC	10.9	20 (2.2)	42 (4.6)
CO_2	0.3	n.d.	n.d.
DES/CO_2	2.8	35 (1.0)	54 (1.5)
DES-MW (150°C, 30 min)/CO_2	7.1	15 (1.1)	29 (2.1)
DES-MW (100°C, 60 min)/CO_2	6.7	15 (1.0)	30 (2.0)

TFA content (wt%) and relative composition in EPA and PUFAs (% in TFA) in lipid extract obtained from *P. tricornutum* using different pre-treatments and solvents.

In summary, we can affirm that PUFAs and EPA are not degraded by DES or DES-MW pre-treatment and can be extracted effectively by using both DMC and scCO2 protocols. However, relative percentages of PUFAs and EPA in

TFA are significantly influenced by the extraction method and solvent used.

Conclusions

In conclusion, DES-MW demonstrated to be an effective and suitable pre-treatment for the enhancement of TFA extraction selectivity from *P. tricornutum* biomass. Combination of this pre-treatment with environmentally friendly solvents as DMC and scCO2 allowed to obtain highly purified lipid extracts and comparable TFA yield with respect to the benchmark B&D, avoiding toxic and dangerous solvents. Moreover, these protocols allowed to reduce extraction of non-desirable lipids such as sterols, waxes and hydrocarbons that are extracted with B&D. This protocol is therefore very promising, especially for potential human applications, and will be further investigated to i) deepen the effect of scCO2 parameters (temperature, pressure and time) on selectivity and extraction yield; ii) evaluate the possibility of scale up and recovery of DES; iii) compare feasibility and sustainability of this multi-step process that employs green solvents to chloroform-methanol single step extraction, by performing a complete and detailed LCA study.

ASSOCIATED CONTENT

Supporting Information available: 1) study of DES pre-treatment parameters (DES-water ratio, aDES-biomass ratio and temperature); 2) single DES components pre-treatment; 3) DES-US pre-treatment; 4) fatty acids characterization; 5) ^{1}H-NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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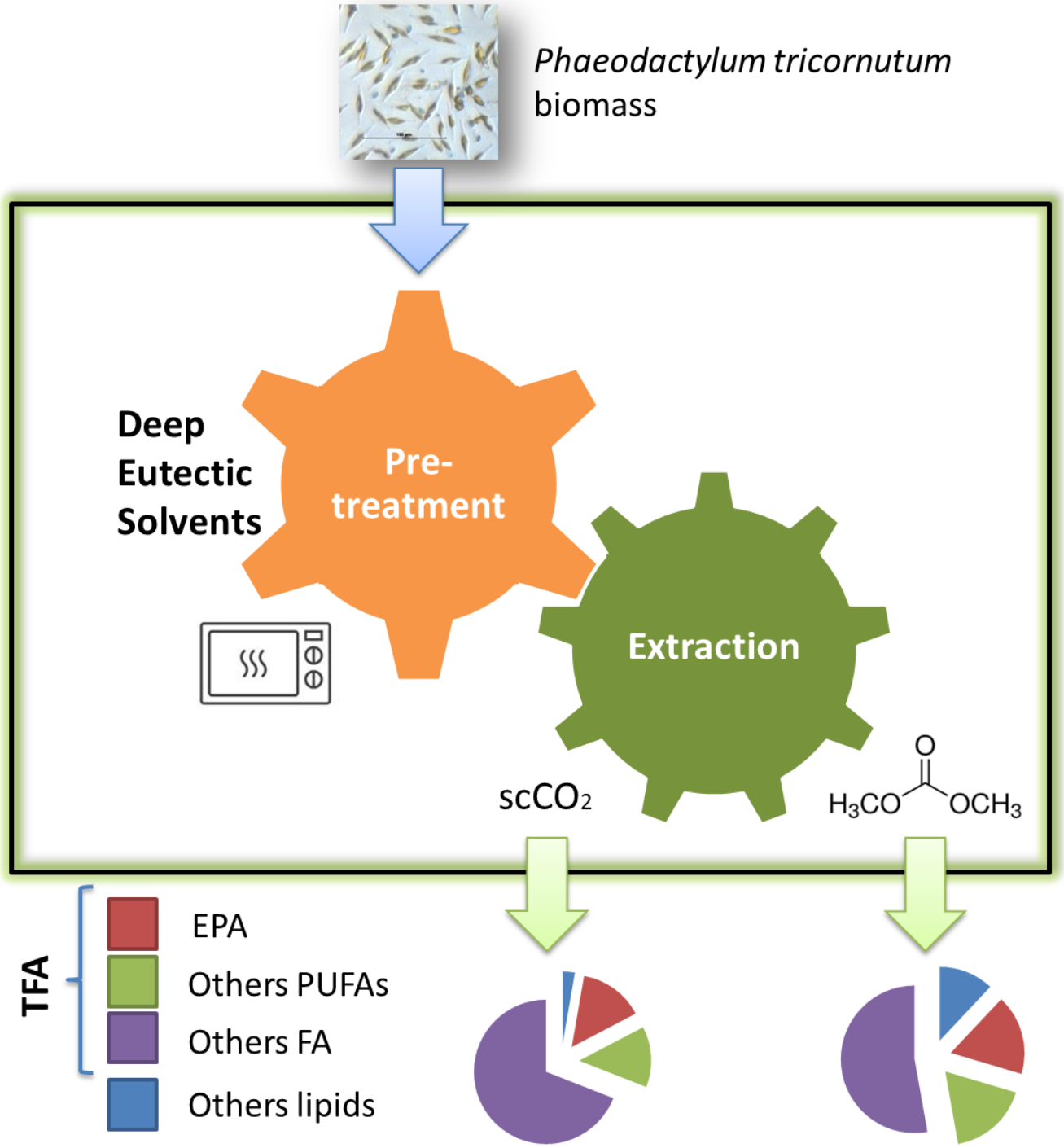

Schematic representation of the developed protocol for biocompatible lipid extraction from microalgae. Combination of pre-treatment with Deep Eutectic Solvents and microwaves with supercritical CO_2 ($scCO_2$) or dimethyl carbonate extraction to obtain lipid extracts enriched in total fatty acids (TFA), Eicosapentenoic Acid (EPA) and polyunsaturated fatty acids (PUFAs).