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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Tommasi, E., Cravotto, G., Galletti, P., Grillo, G., Mazzotti, M., Sacchetti, G., et al. (2017). Enhanced and Selective Lipid Extraction from the Microalga *P. tricornutum* by Dimethyl Carbonate and Supercritical CO₂ Using Deep Eutectic Solvents and Microwaves as Pretreatment. ACS SUSTAINABLE CHEMISTRY & ENGINEERING, 5(9), 8316-8322 [10.1021/acssuschemeng.7b02074].

Availability:

This version is available at: <https://hdl.handle.net/11585/609107> since: 2020-02-21

Published:

DOI: <http://doi.org/10.1021/acssuschemeng.7b02074>

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Tommasi, E., Cravotto, G., Galletti, P., Grillo, G., Mazzotti, M., Sacchetti, G., Samorì, C., Tabasso, S., Tacchini, M., Tagliavini, E. *Enhanced and Selective Lipid Extraction from the Microalga P. tricornutum by Dimethyl Carbonate and Supercritical CO₂ Using Deep Eutectic Solvents and Microwaves as Pretreatment*, *ACS Sustainable Chem. Eng.* 2017, 5, 9, 8316–8322.

The final published version is available online at:

<https://doi.org/10.1021/acssuschemeng.7b02074>

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Enhanced and selective lipid extraction from microalgae by dimethyl carbonate and supercritical CO₂ using Deep Eutectic Solvents and Microwaves as pre-treatment

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Deep Eutectic Solvents, microwaves, microalgae, pre-treatment, dimethyl carbonate, supercritical CO₂, lipid extraction, Phaeodactylum tricornutum.

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.

1. Introduction

In recent years, microalgae have gained attention as a possible solution to some relevant issues such as third generation biofuels or 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 *Phaeodactylum 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 CO₂ (scCO₂), a fluid characterized by low critical temperature and pressure.^{13,14} ScCO₂ 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} ScCO₂ 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 glyco-lipids, chlorophylls, waxes and pigments).^{16,17} For example, when applied to the extraction of the cyanobacterium *Arthrospira platensis*, a combination of scCO₂ and 10% of ethanol proved to be as effective as the benchmark Bligh and Dyer system, while scCO₂ 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.^{1,10,20,21,22,23,24,25,26,27,28} 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). Deep Eutectic Solvents (DES) are a new generation of Ionic Liquids (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.^{29,30} In comparison to many traditional ILs, DES have several benefits such as simple preparation, low cost, low toxicity and high biodegradability.^{30,31} Recently, DES-pre-treatment has been applied for the first time to enhance lipid extraction from freshwater green algae, demonstrating the high potential of this approach.²⁸

Microwaves and ultrasounds are other promising pre-treatment methods to increase extraction efficiency and selectivity.³² The main advantages of these non-conventional techniques are large reductions in extraction time, comparable or higher extract yields and improved selectivity.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 scCO₂). 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 scCO₂.

2. Experimental Section

2.1. Chemicals

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

2.2. Microorganism and culture conditions

P. tricornutum biomass was provided by Micoperi Blue Growth's, 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, in order 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 µE m⁻²s⁻¹. All cultures were mixed through air bubble aeration, and CO₂ was insufflated every morning for inorganic carbon supply and to drop the pH from 9 to 7.5.

2.3. 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.

2.4. Pre-treatments

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

2.4.1. 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 supernatant from biomass. Algal biomass was washed two times more with deionized water (3 mL) and then freeze-dried.

2.4.2. MW pre-treatments

All MW experiments were performed in duplicate on dry biomass, alone or combined with DES pre-treatment.

The microalgae samples (400 mg) were added to the fresh prepared aDES (4g) or simply water, 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 (N₂). A suitable N₂ 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 supernatant from biomass. Algal biomass was washed two times more with deionized water and then freeze-dried.

2.5. 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 total lipid amount (including, TFA, waxes, sterols, hydrocarbons, ketones and pigments) expressed on biomass weight basis (wt%) and selectivity towards TFA (free and bounded, expressed as fatty acids methyl esters (FAME, see 2.5.4) amount on biomass weight basis (wt%).

2.5.1. Bligh & Dyer (B&D) extraction

Biomass (100mg) 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.

2.5.2. 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.

2.5.3. ScCO₂ extraction

ScCO₂ extractions were carried out using an Applied Separations extractor (Allentown, PA, USA) model Spe-ed™ SFE Prime. CO₂ (N4.5 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-ed™ Matrix. After the sample compression a plug of wool (Spe-ed™ Wool) was placed on top and the empty space was filled with the matrix. The extraction CO₂ flow rate was maintained at an average level of 2.5 L/min, 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.

2.5.4. 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 BF₃-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.

2.5.5. 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 fatty acid methyl esters (FAMES). The relative response factors used for the quantitation were obtained by injecting solutions of known amounts of methyl nonadecanoate and commercial FAMES mixture.

3. Results and discussion

3.1. 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,^{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 not 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.*²⁸ 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.²⁸

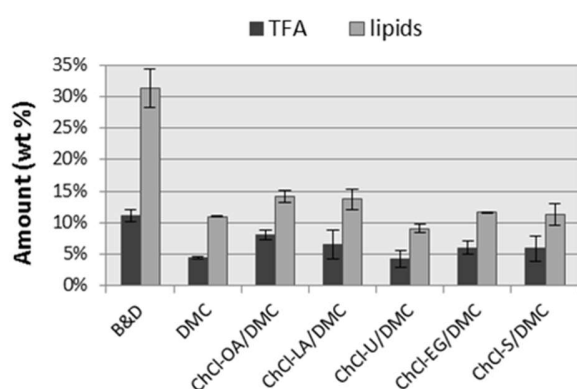


Figure 1 Effect of aDES pre-treatment on total lipids and TFA amount (calculated as FAME), extracted from *P. tricornutum* with DMC in comparison with Bligh & Dyer method (B&D) and DMC without any pre-treatment. Data are expressed on

dry weight basis (wt%), as mean of two replicates \pm 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 through cell walls in some aquatic microorganism.³⁹ 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 (details reported in ESI).

However, 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 (data in ESI), 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} In fact, similarly to conventional ILs, DES can be efficiently coupled with MW irradiation due to their polar nature. In particular, organic acid-based DES show the highest polarity, further increased by water addition. 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.^{43,44}

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.^{45,46} However, when applied to algal biomass, DES-US pre-treatment was not able to increase TFA extraction yield (results and experimental description in ESI), 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.

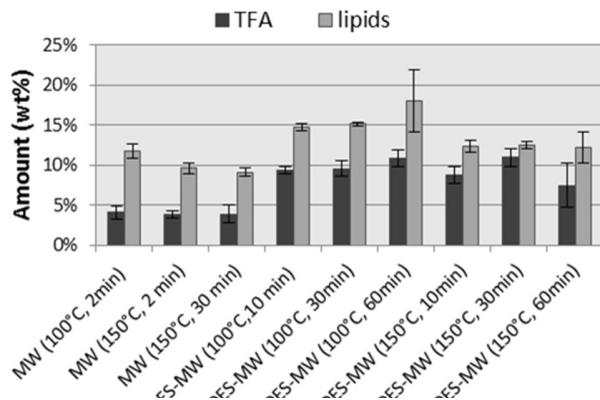


Figure 2 Effect of time and temperature on TFA (determined as FAME) and total lipid amount (wt%) extracted from *P. tricornutum* using DMC, after pre-treatment with MW alone and in combination with ChCl-OA aDES. Data are expressed on dry weight basis (wt%), as mean of two replicates \pm st. dev. (wt%).

Therefore, the best TFA extractions yields are 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 o). 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 the 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%)
o	Bligh & Dyer	31.3 \pm 3.0	11.1 \pm 0.9
1	DMC	11.3 \pm 0.1	4.5 \pm 0.2
2	DES (25°C, 24h) / DMC	14.1 \pm 0.1	8.1 \pm 0.8
3	MW (150°C, 30 min) / DMC	9.2 \pm 0.5	3.9 \pm 0.7
4	DES-MW (150°C,	12.5 \pm 0.4	11.0 \pm 1.1

	30min) / DMC		
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Total lipids and fatty acids extracted from *P. tricornutum* using different pre-treatments and solvents. Data are expressed as mean of two replicates \pm 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.^{15,17,20,16,47,48,49} 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.).^{48,50}

Due to all these variables, comparisons are often meaningless; for this reason 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.⁵¹ Indeed, results in Figure 3 show that all tested pre-treatments improved scCO₂ extraction. Specifically, DES pre-treatment enhance 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-pretreated biomass (7.0 vs 0.3 wt%).

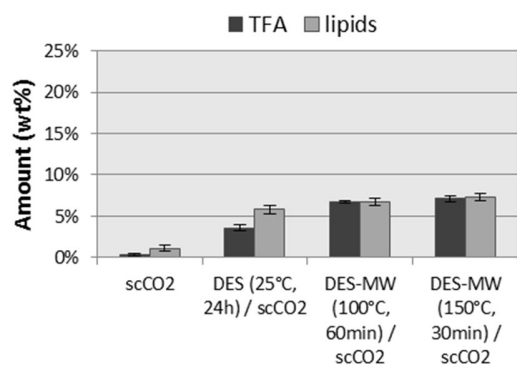


Figure 3 TFA (determined as FAME) and total lipids extracted from *P. tricornutum* using scCO₂ (350 bar, 45°C) with or without pre-treatments with aDES at different conditions of time and temperature. Data are expressed on dry weight basis (wt%) as mean of two replicates \pm st. dev. (wt%).

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 in order 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.

Accordingly 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. ¹H-NMR analysis of the extracts (spectrum reported in ESI) confirmed triglycerides as the main component, proving the high selectivity of scCO₂ as extracting solvent.

3.3. 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 ex-

tracted 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 keeps 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, this lipid extract contains 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 protocol and solvent used.

4. Conclusions

- Among different aDES tested as pre-treatment for DMC extraction, those composed by choline chloride and carboxylic acids (OA and LA) give best results in terms of total lipid yield and selectivity toward fatty acids.

- ChCl-OA pre-treatment at room temperature enhance TFA extraction yield in DMC by 55%
- Pre-treatments with DES-MW at 100°C for 60 and 30 min and at 150°C for 30 min increase DMC selectivity towards fatty acids and give comparable TFA yield in comparison to B&D methodology.
- Synergic effect of DES and microwave was proven to be essential to reduce the time of the pre-treatment and obtain the maximum efficiency and selectivity with DMC as extracting solvent.
- When scCO₂ was used as extracting solvent, DES pre-treatment improved both TFA selectivity and extraction yield. Moreover, a further enhancement was obtained combining MW and DES: DES-MW pre-treatment doubled the percentage of TFA extracted in comparison to DES pre-treatment, and increased by 20 times in comparison to non-pre-treated biomass.
- PUFAs and EPA content in lipid extract depends on pre-treatment and solvent used. DMC extraction after DES-MW gave comparable fatty acids profile with B&D method.

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.

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) ¹H-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.

Funding Sources

We acknowledge the University of Bologna (Ricerca Fondamentale Orientata, RFO) for fundings.

ACKNOWLEDGMENT

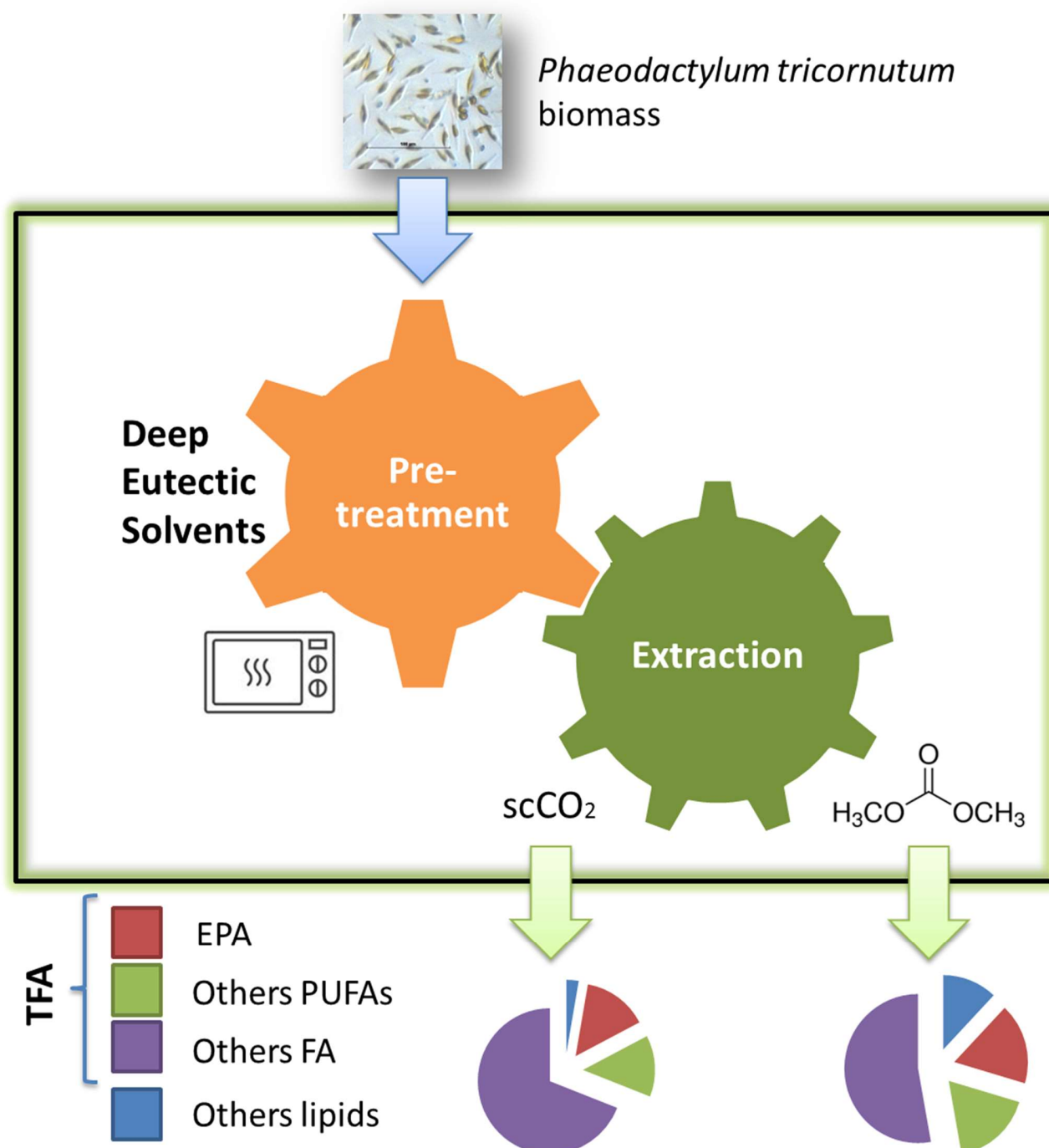
Micoperi Blue Growth srl is gratefully acknowledged for providing algal biomass and scientific support for algae cultivation.

REFERENCES

1. Mercer, P., Armenta, R. E. Developments in oil extraction from microalgae. *Eur. J. Lipid Sci. Tech.* **2011**, 113 (5), 539-547.
2. Radmer, R. J.; Omega-3 fatty acids from algae. In *Omega-3 Fatty Acids in Health and Diseases*; Lees, R. S., Karel, M. Eds.; Marcel Dekker: New York, **1990**.
3. Yongmanitchai, W., Ward, O. P. Omega-3 fatty acids: alternative sources of production. *Process biochem.*, **1989**, 24 (4), 117-125.
4. Cheung, P. C. Temperature and pressure effects on supercritical carbon dioxide extraction of n-3 fatty acids from red seaweed. *Food Chem.* **1999**, 65 (3), 399-403.
5. Fajardo, A. R., Cerdan, L. E., Medina, A. R., Fernández, F. G. A., Moreno, P. A. G., Grima, E. M. Lipid extraction from the microalga *Phaeodactylum tricornutum*. *Eur. J. Lipid Sci. Tech.* **2007**, 109 (2), 120-126.
6. Cartens, M., Grima, E. M., Medina, A. R., Giménez, A. G., Gonzalez, J. I. Eicosapentaenoic acid (20: 5n-3) from the marine microalga *Phaeodactylum tricornutum*. *J. Am. Oil Chem. Soc.* **1996**, 73 (8), 1025-1031.
7. Simopoulos, A. P. Omega-3 fatty acids in health and disease and in growth and development. *Am. J. Clin. Nutr.* **1991**, 54 (3), 438-463.
8. Rambjør, G. S., Windsor, S. L., Harris, W. S. Eicosapentaenoic acid is primarily responsible for hypotriglyceridemic effect of fish oil in humans. *Lipids* **1996**, 31 (1), S45-S49.
9. Bligh, E. G., Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, 37(8), 911-917.
10. Michalak, I., Chojnacka, K. Algal extracts: Technology and advances. *Eng. Life Sci.* **2014**, 14 (6), 581-591.
11. Lee, O. K., Kim, Y. H., Na, J. G., Oh, Y. K., Lee, E. Y. Highly efficient extraction and lipase-catalyzed transesterification of triglycerides from *Chlorella* sp. KR-1 for production of biodiesel. *Bioresour. Technol.* **2013**, 147, 240-245.
12. Kumar, S. J., Kumar, G. V., Dash, A., Scholz, P., Banerjee, R. Sustainable green solvents and techniques for lipid extraction from microalgae: A review. *Algal Res.* **2017**, 21, 138-147.
13. Andrich, G., Nesti, U., Venturi, F., Zinnai, A., Fiorentini, R. Supercritical fluid extraction of bioactive lipids from the microalga *Nannochloropsis* sp. *Eur. J. Lipid Sci. Tech.* **2005**, 107 (6), 381-386.
14. Sahena, F., Zaidul, I. S. M., Jinap, S., Karim, A. A., Abbas, K. A., Norulaini, N. A. N., Omar, A. K. M. Application of supercritical CO₂ in lipid extraction—a review. *J. Food Eng.* **2009**, 95 (2), 240-253.
15. Mendiola, J. A., Jaime, L., Santoyo, S., Reglero, G., Cifuentes, A., Ibanez, E., Senorans, F. J. Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*. *Food Chem.* **2007**, 102 (4), 1357-1367.

16. Mouahid, A., Crampon, C., Toudji, S. A. A., Badens, E. Supercritical CO₂ extraction of neutral lipids from microalgae: Experiments and modelling. *J. Supercrit. Fluids* **2013**, 77, 7-16.
17. Mendes, R. L., Reis, A. D., Palavra, A. F. Supercritical CO₂ extraction of γ -linolenic acid and other lipids from *Arthrospira* (Spirulina) maxima: comparison with organic solvent extraction. *Food Chem.* **2006**, 99 (1), 57-63.
18. Andrich, G., Zinnai, A., Nesti, U., Venturi, F. Supercritical fluid extraction of oil from microalga *Spirulina* (*Arthrospira*) platensis. *Acta Alim.* **2006**, 35 (2), 195-203.
19. Soh, L., Zimmerman, J. Biodiesel production: the potential of algal lipids extracted with supercritical carbon dioxide. *Green Chem.* **2011**, 13 (6), 1422-1429.
20. Dejoye, C., Vian, M. A., Lumia, G., Bouscarle, C., Charton, F., & Chemat, F. Combined extraction processes of lipid from *Chlorella vulgaris* microalgae: microwave prior to supercritical carbon dioxide extraction. *Int. J. Mol. Sci.* **2011**, 12 (12), 9332-9341.
21. Bermúdez Menéndez, J. M., Arenillas, A., Menéndez Díaz, J. Á., Boffa, L., Mantegna, S., Binello, A., Cravotto, G. Optimization of microalgae oil extraction under ultrasound and microwave irradiation. *J. Chem. Technol. Biot.* **2014**, 89 (11), 1779-1784.
22. Prabakaran, P., Ravindran, A. D. A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Lett. Appl. Microbiol.* **2011**, 53 (2), 150-154.
23. Lee, H., Shin, W. S., Jung, J. Y., Kim, C. W., Lee, J. W., Kwon, J. H., Yang, J. W. Optimization of variables affecting the direct transesterification of wet biomass from *Nannochloropsis oceanica* using ionic liquid as a co-solvent. *Bioprocess Biosyst. Eng.* **2015**, 38 (5), 981-987.
24. Kim, Y. H., Choi, Y. K., Park, J., Lee, S., Yang, Y. H., Kim, H. J., Lee, S. H. Ionic liquid-mediated extraction of lipids from algal biomass. *Bioresource technol.* **2012**, 109, 312-315.
25. Ranjith Kumar, R., Hanumantha Rao, P., Arumugam, M. Lipid extraction methods from microalgae: a comprehensive review. *Front. Energy Res.* **2015**, 2, 61.
26. Halim, R., Danquah, M. K., Webley, P. A. Extraction of oil from microalgae for biodiesel production: A review. *Bio-technol. Adv.* **2012**, 30 (3), 709-732.
27. Biller, P., Friedman, C., Ross, A. B. Hydrothermal microwave processing of microalgae as a pre-treatment and extraction technique for bio-fuels and bio-products. *Biore-source technol.* **2013**, 136, 188-195.
28. Lu, W., Alam, M. A., Pan, Y., Wu, J., Wang, Z., Yuan, Z. A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production. *Bioresource Technol.* **2016**, 218, 123-128.
29. Abbott, A. P., Boothby, D., Capper, G., Davies, D. L., Rasheed, R. K. Deep eutectic solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids. *J. Am. Chem. Soc.* **2004**, 126 (29), 9142-9147.
30. Zhang, Q., Vigier, K. D. O., Royer, S., Jérôme, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **2012**, 41 (21), 7108-7146.
31. Radošević, K., Bubalo, M. C., Srček, V. G., Grgas, D., Dragičević, T. L., Redovniković, I. R. Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotox. Environ. Safe.* **2015**, 112, 46-53.
32. Chemat, F., Vian, M. A., Cravotto, G. Green extraction of natural products: concept and principles. *Int. J. Mol. Sci.* **2012**, 13 (7), 8615-8627.
33. Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., Cintas, P. Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrason. Sonochem.* **2008**, 15 (5), 898-902.
34. Guillard, R. R., Ryther, J. H. Studies of marine planktonic diatoms: I. *Cyclotella* Nana Hustedt, and *Detonula* Confervacea (CLEVE) Gran. *Can. J. Microbiol.* **1962**, 8 (2), 229-239.
35. Fabbri, D., Baravelli, V., Chiavari, G., Prati, S. Dimethyl carbonate as a novel methylating reagent for fatty acids in analytical pyrolysis. *J. Chromatogr. A* **2005**, 1065 (2), 257-264.
36. Ono, Y. Dimethyl carbonate for environmentally benign reactions. *Catal. Today* **1997**, 35 (1-2), 15-25.
37. Kim, S. C., Kim, Y. H., Lee, H., Song, B. K. Lipase-catalyzed synthesis of glycerol carbonate from renewable glycerol and dimethyl carbonate through transesterification. *J. Mol. Catal. B: Enzym.* **2007**, 49 (1), 75-78.
38. Das, A. K., Sharma, M., Mondal, D., Prasad, K. Deep eutectic solvents as efficient solvent system for the extraction of κ -carrageenan from *Kappaphycus alvarezii*. *Carbohydr. Polym.* **2016**, 136, 930-935.
39. Vigneault, B., Percot, A., Lafleur, M., Campbell, P. G. Permeability changes in model and phytoplankton membranes in the presence of aquatic humic substances. *Env. Sci Technol.* **2000**, 34 (18), 3907-3913.
40. Bubalo, M. C., Čurko, N., Tomašević, M., Ganić, K. K., Redovniković, I. R. Green extraction of grape skin phenolics by using deep eutectic solvents. *Food Chem.* **2016**, 200, 159-166.
41. Bakirtzi, C., Triantafyllidou, K., Makris, D. P. Novel lactic acid-based natural deep eutectic solvents: Efficiency in the ultrasound-assisted extraction of antioxidant polyphenols from common native Greek medicinal plants. *J. Appl. Res. Med. J Appl Aromat. Plants* **2016**, 3 (3), 120-127.
42. Dai, Y., Witkamp, G. J., Verpoorte, R., Choi, Y. H. Natural deep eutectic solvents as a new extraction media for phenolic metabolites in *Carthamus tinctorius* L. *Anal. Chem.* **2013**, 85 (13), 6272-6278.
43. Ghanemi, K., Navidi, M. A., Fallah-Mehrjardi, M., Dadollahi-Sohrab, A. Ultra-fast microwave-assisted digestion in choline chloride-oxalic acid deep eutectic solvent for determining Cu, Fe, Ni and Zn in marine biological samples. *Anal. Methods* **2014**, 6 (6), 1774-1781.
44. Hoffmann, J., Nüchter, M., Ondruschka, B., Wasserscheid, P. Ionic liquids and their heating behaviour during microwave irradiation—a state of the art report and challenge to assessment. *Green Chem.* **2003**, 5 (3), 296-299.
45. Mason, T. J. *Practical sonochemistry user's guide to application in chemistry and chemical engineering*; Ellis Horwood: UK, 1991.
46. Singh, B. S., Lobo, H. R., Pinjari, D. V., Jarag, K. J., Pandit, A. B., Shankarling, G. S. Ultrasound and deep eutectic solvent (DES): a novel blend of techniques for rapid and energy efficient synthesis of oxazoles. *Ultrason. Sonochem.* **2013**, 20 (1), 287-293.
47. Cheng, C. H., Du, T. B., Pi, H. C., Jang, S. M., Lin, Y. H., Lee, H. T. Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO₂. *Biore-source Technol.* **2011**, 102 (21), 10151-10153.

48. Balasubramanian, R. K., Doan, T. T. Y., Obbard, J. P. Factors affecting cellular lipid extraction from marine microalgae. *Chem. Eng. J.* **2013**, 215, 929-936.
49. Mubarak, M., Shaija, A., & Suchithra, T. V. A review on the extraction of lipid from microalgae for biodiesel production. *Algal Res.* **2015**, 7, 117-123.
50. Crampon, C., Mouahid, A., Toudji, S. A. A., Lépine, O., Badens, E. Influence of pretreatment on supercritical CO₂ extraction from *Nannochloropsis oculata*. *J. Supercrit. Fluids* **2013**, 79, 337-344.
51. Borowitzka, M. A., Volcani, B. E. The Polymorphic Diatom *Phaeodactylum tricornutum*: Ultrastructure of Its Morphotypes. *J. Phycol.* **1978**, 14 (1), 10-21.



Schematic representation of the developed protocol for biocompatible lipid extraction from microalgae. Combination of pre-treatment with Deep Eutectic Solvents and microwaves with supercritical CO₂ (scCO₂) or dimethyl carbonate extraction to obtain lipid extracts enriched in total fatty acids (TFA), Eicosapentenoic Acid (EPA) and polyunsaturated fatty acids (PUFAs).