Article

Sustainable and Selective Extraction of Lipids and Bioactive Compounds from Microalgae

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Abstract:

The procedures for the extraction and separation of lipids and nutraceutics from microalgae using classic solvents have been used many times. However, these production methods usually require expensive and toxic solvents. Based on our studies involving the use of eco-sustainable methodologies and alternative solvents, we select ethanol (EtOH) and cyclopentyl methyl ether (CPME) for extracting bio-oil and lipids from algae. Different percentage of EtOH in CPME favors the production of an oil rich of SFA useful to production biofuel or rich of compounds bioactive.

The proposed method for obtain a rich extract of saturated or unsaturated fatty acids from dry algal biomass is disclosed is eco-friendly and allows a good extraction yield. The method is compared both in extracted oil percentage yield and in extracted fatty acids selectivity to extraction by supercritical carbon dioxide.

Keywords: algal oil, green chemistry, green solvents, extraction, biofuel, bio compound.

1. Introduction

In recent years, the production of algae culture and usage of algal biomass conversion products have received much attention. Microalgae are a potential source of a wide range of high value products for different biotechnological uses [1]. Particularly, the algae have long been considered excellent feedstock to produce oils. The algal oil, in fact, can be used in different sectors in addition to the production of biofuels [2], for example in nutraceutical sector as nutritional supplements and in cosmetics.

The considerable lipids amount contents in microalgae allows the production of alternative renewable cleaner fuels [3].

Biodiesel is a mixture of fatty acid alkyl esters usually obtained by transesterification (ester exchange reaction) of vegetable oils or animal fats [4]. Many research reports and articles described different advantages of using microalgae for biodiesel production in comparison with other available feedstocks [5-12]. The lipid and fatty acid substances of microalgae differ in accordance with culture conditions. In fact, depending on the strain to which the algae belong, it can have between 20-80% of oil by weight of dry mass [13], and it varies also the lipid composition [14].

From a practical point of view, microalgae are easy to cultivate, can grow with little or even no attention, they use water unsuitable for human consumption, and are easy inclined to provide nutrients.

The extracts of microalgae show antimicrobial, antiviral, antibacterial and antifungal properties attributed to the presence of fatty acids [15] and are also used as ingredients of different skin care, sun protection, and hair care formulations. Microalgae are considered in fact as a predominant production sources for polyunsaturated fatty acids (PUFAs) that have to be supplied to the human diet [16,17]. PUFAs have been used in the prevention/treatment of cardiovascular diseases [18-21] and their derivatives, namely g-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), have also been reported for the treatment of type 2 diabetes, inflammatory bowel disorders, skin disorders, and asthma [22-24].

PUFAs play a major role in the treatment of arthritis, obesity, Parkinson's disease, and heart disease [25]. EPA and DHA are the main derivatives of omega-3 fatty acids (PUFA n-3) and play a role in lowering the blood cholesterol and in the fetal brain development, respectively [26]. Carotenoids and pigments are the main constituents of microalgal-based food supplements and possess antioxidant activities to neuroprotective action and protection against chronic diseases [27].

Various extraction methods have been reported in literature for micro-algal lipids. Conventional methods for extracting lipids include hexane extraction and vacuum distillation. The traditional solvent extraction is the most used method thanks to the simplicity in operation and the possibility of use in the industrial field [28]. Usually solvents such as methanol and chloroform, and temperatures between 150°C and 250°C, are used to obtain high extractive yields of microalgae oil [29,30]. The Bligh and Dyer method is the one most used, in the extraction and quantitation of lipids, at the analytical level [29]. The use of flammable or toxic solvents is considered as a very important problem for the adverse health and environmental effects.

Over the years, several research groups have determined the profile of triglycerides in the oil extracted from microalgae, which is relevant for the production of biofuels [9,30-33]. New algae oil extraction techniques are being developed, such as enzyme-assisted extraction [34], microwave-assisted extraction [35], ultrasound-assisted extraction [36], pressurized liquid extraction [37] and supercritical fluid extraction [38,39]. Recent studies have shown that total lipids/bio-oil extraction from algae can be through using supercritical carbon-dioxide (SC-CO_2) assisted with azeotropic co-solvents as hexane and ethanol 1/1 at reaction pressure of 340 bar, temperature of 80 °C in 60 min, obtaining a total algal lipid yield of 31.37% based on dry basis and a percentage eicosapentanoic acid (EPA) in the range of 20-32 % [40]. This procedure increase the total lipid yield and the selectivity but the usage of hexane as a solvent lead to numerous consequences such as air pollution and toxicity.

Our research group has done extensive work on the identification and molecular characterization of food compounds [41-43]. We have developed environmentally friendly methods for the extraction and next chemical manipulation of natural bioactive molecules [44-52] reducing or eliminating the use and generation of harmful substances and solvents such as the Green chemistry encouraging [53]. In this study, according to the previous studies based on the use of non-toxic solvent [54-64], we have focused our attention on the development of a selective extractive processes rich in PUFA rather than saturated fatty acids and vice versa, using green solvent such as Cyclopentyl Methyl Ether (CPME) and ethanol (EtOH).

CPME is an unconventional and an ethereal environmental solvent very stable to peroxide formation and with low volatility and low water solubility [65], that has increased interest as an industrial solvent [66]. It is no genotoxity or mutagenicity [66], and is produced by the addition of methanol to cyclopentene, with a 100% atom economy for its synthesis. It has also been studied in many important chemical processes including furfural synthesis [67] and extraction of natural products [68]. Previous workes studied the liquid–liquid equilibria for ternary systems of water/CPME/alcohol (methanol, ethanol, 1-propanol, or 2-propanol) to test greener solvent systems that substitute and simulate the Bligh and Dyer method to extraction of oil [69]. Thus, the objective of this work was to evaluate the use of an environmental binary system CPME/EtOH for the

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selective extraction of lipids and bioactive compounds from microalgae. The various lipid components within each fraction has been characterized and quantificated using GC-MS and LC-MS [70-72], following standard procedure of transmethylation [73] available to our goal. The information on complete lipid characterization of extracts is been essential for successful selection of the process extraction useful for production of biofuels or for develop of potential nutraceuticals.

2. Results and Discussion

The biodiesel or saturated fatty acids production process involves different steps including lipid extraction and purification of fatty acids. The classic extraction processes often involve the use of toxic substances. Furthermore, the selective extraction processes that lead to obtain oils rich in PUFA or saturated fatty acids, and the separation of individual fatty acids, are difficult for production of highly concentrated w-3 components. Docosahexaenoic acid (DHA, 22:6w3) is considered to be a crucial nutrients for fetal and infant development [74,75] and only recently researchers have developed the urea complexation to concentrate DHA from Crypthecodinium cohnii CCMP 316 biomass [76] and the production of enriched algal oil of w-3-PUFA [77,78] useful for human health.

The use of SC-CO_2 is a most environmentally sustainable extraction methods [39], but to have a higher extraction yield, it has been seen that it is necessary to use co-solvents such as hexane [40], invaliding the sustainability of the method itself. Moreover, this method is not always easily applicable at an industrial level.

In the present work, according to the studies based on the use of non-toxic solvent [79,80], extracted lipid rich of PUFA or saturated fatty acids by dried microalgae were obtained, using CPME and ethanol as green solvents at different percentage. The optimization of the method was carried out comparing the oil extraction yield obtained using different percentage of ethanol in CPME with the oil extraction yield obtained by carbon-dioxide supercritical extraction (SC-CO_2) [38]. As can be seen in Figure 1, the extraction yield increases considerably with the use of 80% of ethanol in CPME (39,4 %) .

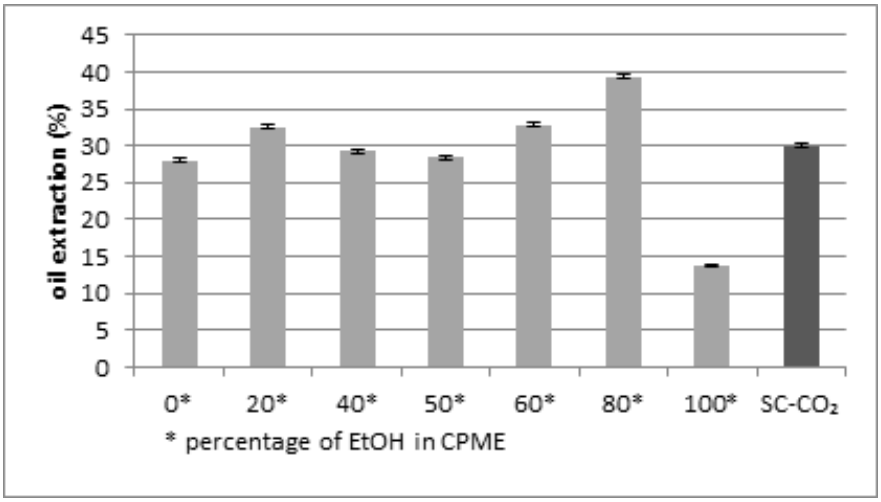

Figure 1. Oil extraction yields (g for 100 g of dry matter) using different volume percentage of ethanol in CPME, compared to the yield obtained by SC-CO_2. $p < 0.05$

The determination of fatty acid composition using GC-MS quantitative analysis in the extracted samples (see supporting information) was performed. The GC-MS analysis of the methyl transesterified [81] algal oil extracted using SC-CO_2 (see supporting information, S1) revealed the presence of nine fatty acids (Table 3). At time retention of 13.35 min peak of internal standard (methyl tricosanoate, 23:0).

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Peak identification of the saturated fatty acids (SFA) and unsaturated fatty acids (PUFAs and MUFA) in the analyzed microalgae oil samples were carried out by the comparison with retention time and mass spectra of known standard (Table 1). Samples were analyzed in triplicate.

Table 1. Fatty acids composition of algal oil by GC-MS analysis.

S/N	RT (Min)	Name of Compound	Mol. Formula	Classification
1	1.5	Myristic acid (14:0)	$C_{14}H_{28}O_2$	SFA
2	1.7	Pentadecylic acid (15:0)	$C_{15}H_{30}O_2$	SFA
3	2.1	Palmitic acid (16:0)	$C_{16}H_{32}O_2$	SFA
4	2.7	Heptadecanoic acid (17:0)	$C_{17}H_{34}O_2$	SFA
5	3.1	Linoleic acid (18:2 ω-6)	$C_{18}H_{32}O_2$	PUFA
6	3.2	γ-Linolenic acid (18:3 ω-6)	$C_{18}H_{30}O_2$	PUFA
7	3.4	Oleic acid (18:1 ω-9)	$C_{18}H_{34}O_2$	MUFA
8	3.5	Stearic acid (18:0)	$C_{18}H_{36}O_2$	SFA
9	5.4	Eicosapentaenoic acid, EPA (20:5 ω-3)	$C_{20}H_{30}O_2$	PUFA
10	5.5	Eicosatrienoic acid (20:3 ω-6)	$C_{20}H_{34}O_2$	PUFA
11	5.6	Eicosatetraenoic acid (20:4 ω-6)	$C_{20}H_{32}O_2$	PUFA
12	6.2	Eicosanoic acid (20:0)	$C_{20}H_{40}O_2$	PSFA
13	9.1	Docosapentaenoic acid, DPA, (22:5 ω-3)	$C_{22}H_{34}O_2$	PUFA
14	9.4	Docohexanoic acid, DHA, (22:6 ω-3).	$C_{22}H_{32}O_2$	PUFA

From the GC-MS analysis was found that the most abundant fatty acids methyl ester (FAME) present in the various extracts are above all myristic and palmitic acid (two saturated fatty acids), and EPA and DHA (two ω-3 polyunsaturated fatty acids) derivative. These were considered to evaluate the performance the mixture EtOH/CPME comparing with SC-CO_2 in the extraction process (Figure 2).

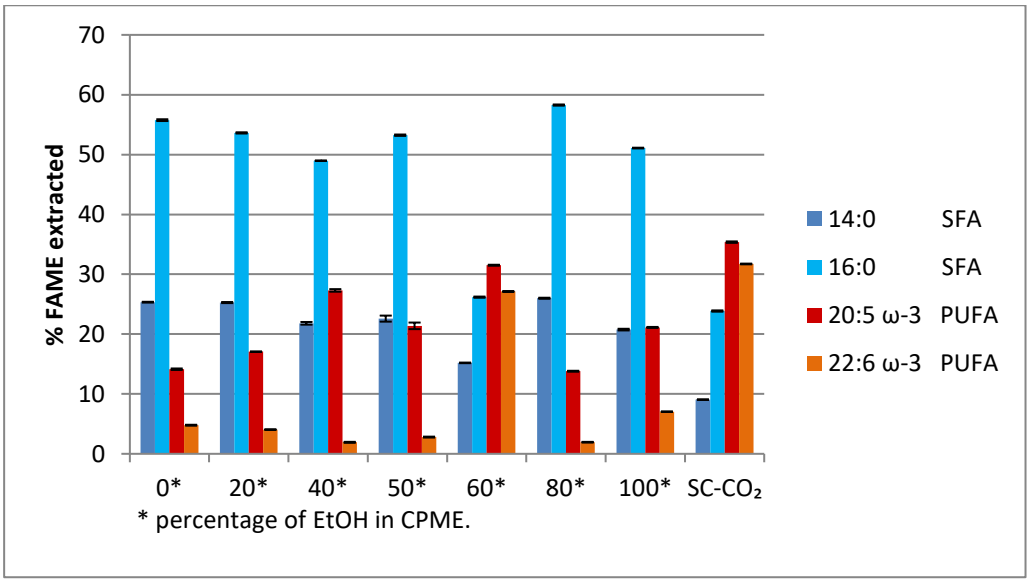

Figure 2. % of myristic acid, palmitic acid (satured fatty acids, SFAs), EPA and DHA (polyunsaturated fatty acids, PUFAs) extracted with a mixture EtOH /CPME and SC-CO_2. $p < 0.05$

It is evident that an oil more rich in saturated fatty acids (Figure 2) has been obtained using a mixture of EtOH / CPME 8/2, the same solution solvent useful for obtaining a higher yield of algal oil extraction.

On the contrary, an oil rich in EPA and DHA was obtained using a mixture of EtOH / CPME 6/4 (Figure 2), the same mixture of solvents which showed a yield of the extracted oil equal to 32.8% against 30.0 % yield obtained with SC-CO_2.

From results of quantitative analysis of all fatty acids methyl esters (see Supplementary Material, S2) the yield percentage of all saturated fatty acids and all unsaturated fatty acids was calculated. Table 2 illustrates how the percentage of total SFA and total UFA (polyunsaturated and monounsaturated fatty acids) concentrations varies according to the percentage of EtOH in CPME (Table 2, entries 1-7) and using SC-CO_2 (Table 2, entry 8).

Table 2. SFA (saturated fatty acids) and UFA (unsatured fatty acids) percentage concentration variation as a function of solvent/solvent mixture used.

Entry	Extraction method	% SFA	% UFA
1	0*	78,25 ± 0,16	21,75± 0,16
2	20*	73,34 ± 0,04	26,66± 0,06
3	40*	65,34 ± 0,09	34,66± 0,07
4	50*	69,80 ± 0,15	30,20± 0,10
5	60*	38,59 ± 0,03	61,41± 0,05
6	80*	82,83 ± 0,13	17,17± 0,09
7	100*	71,46 ± 0,01	28,54± 0,07
8	SC-CO_2**	33,06 ± 0,18	66,93± 0,09

*Percentage of EtOH in CPME. Solvent mixture used for Shoxlet system. **Supercritical carbon-dioxide extraction method. The values of percentages are in mean ± SD (n = 3).

Electron ionization (EI) MS coupled to Gas chromatography (GC) to analyze fatty acids [82] has been usually applied but in addition to GC-MS, liquid chromatography (LC)-MS is also a useful method for the accurate analysis of profiling of FFAs [83-85] and for qualitative determination of nonvolatile compounds in food [86]. The use of electrospray ionization (ESI) MS, a soft ionization technique, provides the information of molecular ions. Therefore, tandem MS (MS/MS) is applied for a most sensitive and selective analysis of FFAs.

To provide the identification of components in the extracted samples, liquid-chromatography mass spectrometry (LC/MS) was employed. ESI-MS/MS analysis were performed for all the ions present in the full scan chromatogram for each algae extract.

The analytical technique has thus confirmed in a more detailed way the presence of the extracted and quantified fatty acids previously. (see Supplementary Material, Method Validation).

Based on this result it is possible to choose the type of solvent / solvent mixture to be used depending on the type of extract to be obtained (richer than SFA or UFA). Both analytical techniques were useful and necessary to determine the type of fatty acid present in the extracted samples. Thus, it was possible to identify an environmentally sustainable extraction method for saturated or unsaturated fatty acids with excellent extractive yield and significant selectivity.

3. Materials and Methods

3.1 Chemicals

Solvents, reagents and thimbles purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO). Dry algal biomass provided by Aquafauna Biomarine inc. (P.O. Box 5, Hawthorne, California, 90250 USA).

3.2 Supercritical Fluid Extraction

Fractionation of algal oil for SC-CO_2 from algal biomass was carried out in a continuous supercritical fluid using a Applied Separations Speed SFE model 7070 apparatus (Applied Separations Inc., Allentown, PA). About 5 g of sample and glass beads (size about 3mm), was weighed and added to a vessel (1.27 cm i.d. x 25.4 cm long), sealed with polypropylene wool at the top and the bottom of the extraction vessel. The oven temperature used is 80 °C and the vessel MPa; room temperature, 25°C). The extracted oils were collected with 10 mL vials. Each extraction was replicated three time.

3.3 Soxhlet Extraction

Soxhlet Extraction was carried out using 100 mL of solvent on 5 g of dried alga sample by Soxhlet apparatus. The extraction lasted for 1 h. The extract was filtered to remove possible solid particles. Organic solutions were then concentrated by rotary evaporation and the traces of solvent in residual oil were removed by nitrogen flushing. Yield was calculated based on weight of extracted oil and weight of start sample. The same process was repeated with different solvent mixtures; CPME 100%, CPME /EtOH (80:20, 60:40, 50:50, 40:60, 20:80) and EtOH 100%. Each extraction was replicated three time.

3.3 Preparation of Methyl Esters of constituent Fatty Acids

Fatty acids compositions were determined by their conversion to methyl esters. 15 mg of each oil was added to the internal standard (250 ng/ 100 µl chloroform, methyl tricosanoate, 23:0). The oil was subjected to transmethylation by treating 15 mg of the each oil with 6 mL of methanolic solution 0.2 M of sulfuric acid following standard procedure and 15 mg of hydroquinone [64]. The mixture was incubated for 12 hour at 60°C and subsequently cooled. 1 mL of distilled water was added to each vial and extracted 3 times with 1.5 mL of heptane. The organic phase containing the fatty acid methyl esters (FAMEs) was separated and evaporated under a stream of nitrogen The FAMEs obtained was dried over anhydrous magnesium sulfate and kept for Gas Chromatography-Mass Spectrometry (GCMS) analysis.

3.4 GC-MS Analysis

The FAMEs obtained from the different algal oils were analyzed on Shimadzu GC/MS-QP 2010 gas chromatography instrument with autosampler AOC-20i (Shimadzu) equipped with a 30 m-QUADREX 007-5MS capillary column, operating in the "split" mode, 1 mL min-1 flow of He as carrier gas. The injector was operated at 250 °C and while the detector was operated at 380 °C. The oven temperature was programmed to rise from 70 to 135 °C at a heating rate of 2 °C/min, from 135 to 220 °C at a heating rate of 4°C/min, from 220 to 270 °C at a heating rate of 3.5 °C/min. The injector and detector temperature were 280 °C and 290 °C respectively. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range 50-500 m/z. The FAMEs were identified based on the authentic samples previously injected in combination with the examination with individual molecular weight, mass spectra and comparison of fragmentation pattern in the mass spectrum with that of the National Institute of Science and Technology, NIST library.

3.5 Data analysis

Results were analyzed by using a one-way ANOVA (GraphPad Software Inc., San Diego, CA, USA) and data are presented as the mean ± standard error of mean (SEM) except otherwise indicated. All experiments were performed in triplicate. Values at $p < 0.05$ were taken as significant.

3.6 Flow injection analysis/mass spectrometry (FIA/MS)

The mass spectrometer system used for the qualitative analysis of the algal oil extracts was a Q-Trap API 4000 (MSD Sciex Applied Biosystem). The analysis were performed by flow injection

analysis (FIA) in both negative and positive ion modes. In general, the intensity of ions observed as positive ions was lower than those observed as negative ions. Therefore, the experiments were conducted using the negative mode. The most important point of this experiment is to confirm that ions generated from target compounds are observed, rather than that merely any sort of ions are observed. Therefore, it is necessary to attribute observed ions to specific compounds. Fundamentally, in the attribution process, assuming that positive ions are protonated molecules $[M+H]^+$ and negative ions are deprotonated molecules $[M-H]^-$, it is verified whether they are consistent with the molecular mass of target compounds. Structural assignment was, therefore, based on the accurate mass of the pseudo-molecular ion $[M-H]^-$, present in the negative ESI-MS chromatogram, and on the corresponding fragments ions detected by collision-induced dissociation (CID) under nitrogen (25% normalized collision energy) in the ion trap. The instrument parameters were set as follow: ion spray voltage (IS) -4600 V; curtain gas 10 psi; ion source gas 12 psi; collision gas thickness medium; entrance potential 10 eV, declustering potential 70 eV, collision energy (CE) between 15 and 30 eV and collision exit potential (CXP) between 5 and 9 eV.

4. Conclusions

In this work a new eco-friendly and effective technique for extracting total lipids/bio-oil from algae was showed. The maximum total algal lipid yield (39.4% based on dry basis) was obtained using a solution of EtOH/CPME (8:2) at temperature of 80 ° C and reaction time of 60 min. This extraction condition is advantageous to obtain an oil rich of SFA, useful to production biofuel. An oily extract rich in bio-compounds that contribute to human well-being such as eicosapentanoic acid, EPA (C20: 5, ω-3) and docosahexaenoic acid, DHA (C22: 5, ω-3) was obtained using an EtOH / CPME, 6: 4, at the same temperature an reaction time. In these extraction conditions the oil extraction yield is equal to 32.8% against 30.0% if SC-CO_2 is used.

This method offers many advantages over the conventional extraction technologies such as increased total lipid yield, increased selectivity and preserved thermo-labile compounds. It is a alternative method for a sustainable production of algal biofuel and development of high value co-products.

Supplementary Materials: The following are available online, GC-MS and MS(EI) spectra of products, quantitative analysis of all fatty acids methyl esters , negative ESI full scan mass spectrum of algal oil sample, negative ESI full scan mass spectrum of standard solution of DHA, Stearic acid and butyric acid, negative ESI full scan mass spectrum of standard solution of oleic alcohol and palmitic acid

Author Contributions: Conceptualization, M.N. and I.S.; formal analysis, C.B. and P.C.; project administration, G.S. and G.G.; validation, G.S. and G.G.; Supervision, A.P.

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