

# Fractionation of Marine Microalgae Extract using Supercritical CO<sub>2</sub> with Progressive Addition of Co-Solvent for the Recovering of High-Valuable Compounds

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Microalgae biotechnology is of great interest in the nutraceutical, food, energy, cosmetics, and pharmaceutical industries. The composition of lipids, pigments, and bioactive substances of microalgae depends on the species and especially on the culture conditions. The extraction of these substances of interest is carried out conventionally using organic solvents that are highly toxic and not very selective. The conventional method negatively affects the environment and eventually forces the incorporation of purification steps that make the process more complex and expensive. Supercritical carbon dioxide offers many advantages over organic solvents, especially for its high selectivity for lipophilic substances. Its gaseous state at ambient pressure makes it easy to eliminate the residual solvent and subsequently be reused in other extractions. Although the selectivity for the obtaining of polar substances is limited, the addition of low amounts of co-solvent overcome this limitation. The result is a reduction of solvent amounts compared to conventional extraction techniques. In this way, the progressive addition of the co-solvent offers a new strategy in the fractioning and purification of bioactive compounds of different nature by exhausting the sample, which supposes a high economic impact in the recovery of bioactive compounds from microalgae at an industrial scale for its further use. In this work, a fractionation with consecutive supercritical extractions increasing the percentage of ethanol as cosolvent, from two microalgae species, *Nannochloropsis gaditana* and *Tetraselmis chuii*. The total carotenoid, chlorophyll, and polyphenol content of each fraction as well as their antioxidant activity by DPPH and ABTS methods was evaluated.

## 1. Introduction

Microalgae are an essential part of earth life due to their high photosynthetic production. Their extraordinary adaptive skill grants them the ability to colonize any natural aquatic environment, from fresh or salt waters to extreme habitats such as volcanic waters (Lim & Schenk, 2017). They have a complex chemical composition in lipids, carbohydrates, proteins, pigments and polymers (Greque De Moraes et al., 2015). This wide range of chemical compounds makes microalgae be considered as a potential source of applications, such as food ingredient, environmental use for water treatment, energy application in the production of biodiesel, or for the pharmaceutical sector through the application of their high value compounds (Levasseur et al., 2020).

*Nannochloropsis gaditana* and *Tetraselmis chuii* are among the most cultivated species for aquaculture feed, which has led to a growing interest in their study and valorization (García-Romeral et al., 2017; Di Lena et al., 2020). *Nannochloropsis gaditana* possesses a thick cell wall composed of a cellulose layer and a protective and hydrophobic algaene layer that provide with a strong resistance to rupture (Scholz et al., 2014). In this sense, the recovery of some target compounds as pigments (astaxanthins,  $\beta$ -carotenes, neoxanthins, violaxanthins and zeaxanthins) (Millao & Uquiche, 2016) contained in the chloroplast required techniques with a higher penetration capacity (Macías-Sánchez et al., 2008). The research of *N.gaditana* is focused on the production of

bio-oils and bio-fuels due to its high lipid content (it can even achieve a 70% in dry weight) of diverse origin, such as triacylglycerides, palmitoleic acid, palmitic acid, palmitic acid, PUFAs, and EPA, among others (Sánchez-Camargo et al., 2018). On the other hand, *Tetraselmis chuii* has great potential as a source of functional foods given its high protein content (Nunes et al., 2020), essential fatty acids, esterols and other organic acids (Goiris et al., 2012) Haga clic o pulse aquí para escribir texto.. In fact, the freeze-dried form of this microalgae has been included in the list of novel foods for human consumption by EFSA (Regulation EU 2017/2470), being a food with a high nutritional value. This complex composition provide to both algae with a high bioactivity and content of compounds of interest (Monteiro et al., 2020) that place them as potential candidates to use in the food, pharmaceutical and cosmetic industries, either for direct consumption or for the recovery of target compounds for the development of food supplements or functional constituents (Marino et al., 2021). However, their application in these fields involves a previous extraction step, the recovery rate of which is strongly related to the method employed. Traditionally, maceration in organic solvents has been employed (Maadane et al., 2015) although their long waiting times and toxicity have positioned non-conventional methods, such as supercritical fluid extraction, in better choices considering the promising results obtained (Sánchez-Camargo et al., 2018). The supercritical CO<sub>2</sub> (scCO<sub>2</sub>) is a lipophilic solvent that has a high selectivity for non-polar compounds (Díaz-Reinoso et al., 2006) such as pigments and lipids, avoiding further purification processes. However, this selectivity is an obstacle when extracting more polar compounds, such as neutral lipids, proteins or sugars. In this sense, the addition of a polar co-solvent leading to an enhanced extraction (ESE) or even a pressurized fluid extraction (PLE), allows the depletion of the raw material of other compounds of interest while reducing the processing time compared to conventional or Soxhlet maceration, and minimizing the amount of solvent used. Consequently, the industrial interest in the development of faster and less toxic techniques has made PLE popular, especially in the pharmaceutical and food industry field. Grierson et al. (2012) obtained better extraction yields of oils derived from microalgae by PLE with ethanol (15% w/w) than SFE. Sanchez-Camargo et al. (2018) extracted hydrates and sugars with PLE from polar solvents such as water from *N. gaditana*, and Cha et al. (2010) hydrophilic compounds by PLE with ethanol from *Chlorella vulgaris*. Fractionated extraction is the ideal method for screening or valorization of biomass, since it is capable of combining different extraction methods to provide extracts enriched in target compounds, which avoids subsequent purification processes. Fractionation with microalgae has been found to be efficient when using *Spirulina platensis* (J. A. Mendiola et al., 2007) and *N. gaditana* (Sánchez-Camargo et al., 2018), so the success of the fractioning can be studied in different micro and macroalgae. In the present study is performed a fractionation process with two marine microalgae, *N. gaditana* and *T. chuii*, in order to obtain extracts rich in pigments and polyphenols with different bioactivity and purity enough for its further application in other industrial processes, while exhausting the raw material. The fractioning consists of consecutive extractions where the composition of the solvent system, and therefore the polarity of the solvent used, will be modified. After extraction, all fractions will be analyzed chemically and functionally to evaluate the suitability of this type of fractionation in the purification and concentration of products from this microalgae.

## 2. Material and Methods

### 2.1. Raw materials

Spray dried powder from the microalga *Tetraselmis chuii* was purchased from Allmicroalgae-Natural Products, S.A (Pataias, Portugal). Aluminum oxide (1:1 w/w) (Alumina A-50, Sigma, St Louis, MO, USA) was used for the cell disruption of the microalgal biomass for 3 h. On the other hand, *N. gaditana* was supplied freeze-dried by Fitoplancton Marino (El Puerto de Santa María, Spain). All biomass was stored in the dark at 10 °C until extraction was carried out.

### 2.2. Extraction conditions

The fractioning of each of the algae used in this experiment was developed in lab-scale high-pressure equipment provided by Thar Technologies (Pittsburgh, PA, USA), which includes a condenser, two P50 high-pressure pumps, a pre-heater, a 100 mL thermostated vessel, and a back-pressure regulator (BPR). All units are monitored by a controller. ~37 mg of raw material was introduced in a paper cartridge and introduced into the vessel. The extractions were carried out in semicontinuous mode with a total flow of 20 g/min during 2 h, conducting an extraction of a total time of 10 h following the conditions shown in Table 1. Each fractionation will start with an SFE step using scCO<sub>2</sub> as solvent, followed by three ESEs with 5%, 20% and 50% ethanol as solvent, and finally a PLE using ethanol as solvent. The extracts were stored at 4 °C in darkness until analysis.

Table 1. Extraction conditions of the fractioning of each microalgae specie.

Fraction	Regime	% scCO <sub>2</sub>	% Ethanol	P (bar)	T (°C)
1	SFE	100	0	400	55
2	ESE	95	5	100	55
3	ESE	80	20	100	55
4	ESE	50	50	100	55
5	PLE	0	100	100	55

### 2.3. Antioxidant capacity of the fractions

The antioxidant activity of the extracts was quantified by two methodologies, comparing the results obtained with the reaction with the 2,2-Diphenyl-1-picrylhydrazyl reagent (DPPH) and the 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) one. For the DPPH reaction, 100 µL of extracted oil were introduced in 3.9 mL of a ethanolic solution of 6·10<sup>-5</sup> M DPPH. The reaction was measured at 515 nm. For the ABTS reaction, ABTS 7 mM y K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> 2,45 mM in distilled water and left 12-16 h in the dark to allow oxidation of the ABTS to occur. After the formation of the ABTS<sup>+</sup> radical, the solution was diluted with ethanol as the original extract, to an absorbance of approximately 0.7 (±0.1) at 734 nm. Samples were then prepared with 0.04 ml of extract and 4 ml of the dilution prepared with ABTS<sup>+</sup>, and the reaction was performed for 10 min in the dark. Then the decrease of the absorbance at 734 nm was measured with a 1-cm cuvette in a UV-vis spectrophotometer. In both cases, the antioxidant activity was expressed as efficient concentration (EC50). All analyses were done in duplicate.

### 2.4. Total polyphenol content (TPC) of the fractions

Total polyphenol content (TPC) was calculated according to the Folin-Ciocalteu (FC) method (Sánchez-Camargo et al., 2018) with some modifications. 40 µl of each extract was prepared with 3160 µl of Milli-Q water and 200 µl of FC reagent. The mixture was shaken and then the alkaline medium (600 µl of Na<sub>2</sub>CO<sub>3</sub> 20%) was added to avoid oxidation of phenols. The sample was left 2 h in dark to the reaction take place and absorbance was measured spectrophotometrically at 760 nm. A calibration line was developed using gallic acid at different final concentrations [0-500ppm]. The PTC was expressed as mg GAE/g extract and/or mg GAE/g fraction (equation 2).

$$\text{Abs } 760 = 0.0009 \text{ mg GAE/g} + 0.0209; R^2 = 0.990 \quad (2)$$

## 3. Results and discussion

### 3.2. Composition of the fractions on pigments and total phenolic compounds

The overall concentration of the fractions obtained was slightly higher in *N. gaditana* than *T. chunii* (Figure 1), in the first fractions, where the non-polar compounds are mainly extracted, and are most abundant of *N. gaditana* (García-Romeral et al., 2017; www.seaweed.es). The fractioning was also more effective, since the technique was able to separate one fraction pure in lipophilic carotenoids from the rest, obtaining an extract with an orange colour (Figure 1b). On the contrary, the fractioning of *T. chunii* is less efficient, showing a mixture of both pigments in all fractions, as can be seen in figure 1b. The incorporation of co-solvent generates extracts with richer composition in other components decreasing its selectivity, being the fraction of 5% (*N. gaditana*) and 20% (*T. chunii*) the most complex ones. The enhanced recovery when using a low percentage of co-solvent agrees with other authors observed in the extraction of astaxanthin from *H. pluvialis*, obtaining the 82% of recovery using scCO<sub>2</sub> + 13% ethanol (Reyes et al. 2014) due to the increase in its solubility. From this fraction on, the proportion of the studied compounds decreases progressively, and only traces are obtained at 100% ethanol, so the raw material can be considered depleted of those compounds at the end of the extraction. The compounds recovered in *N. gaditana* in each fraction fits with the data collected in figure 1A, but is not the case of *T. chunii*. It should be kept in mind that these microalgae are very rich in other non-polar compounds (PUFAs, TAG) that can be also extracted in the first fractions (Sánchez-Camargo et al., 2018). It is noticeable that *N. gaditana* responds efficiently to the extraction using supercritical techniques despite its rough structure, considering that unlike *T. chunii*, it has not been pretreated, and even somewhat better results are obtained, so the algaen layer did not hindered the extraction process using scCO<sub>2</sub>.

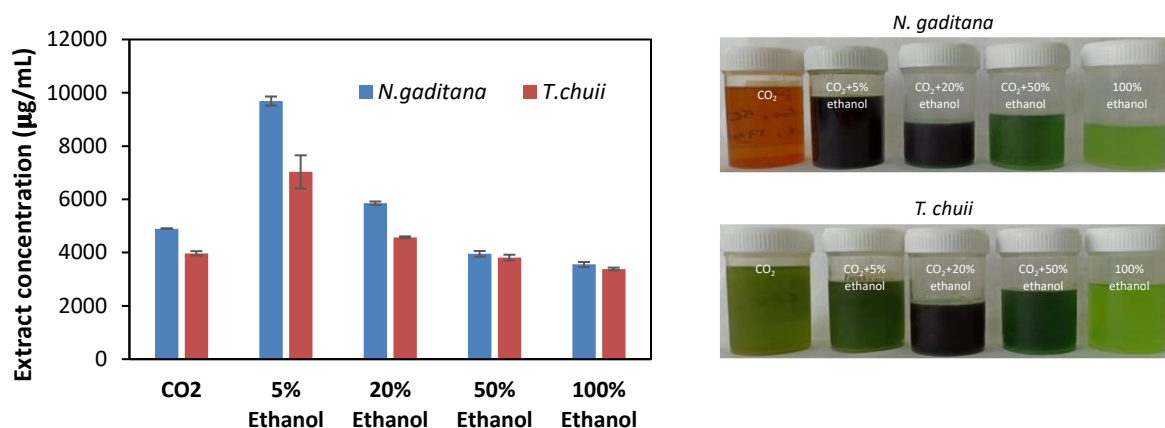


Figure 1. a) Concentration of each fraction and b) Appearance of the fractions.

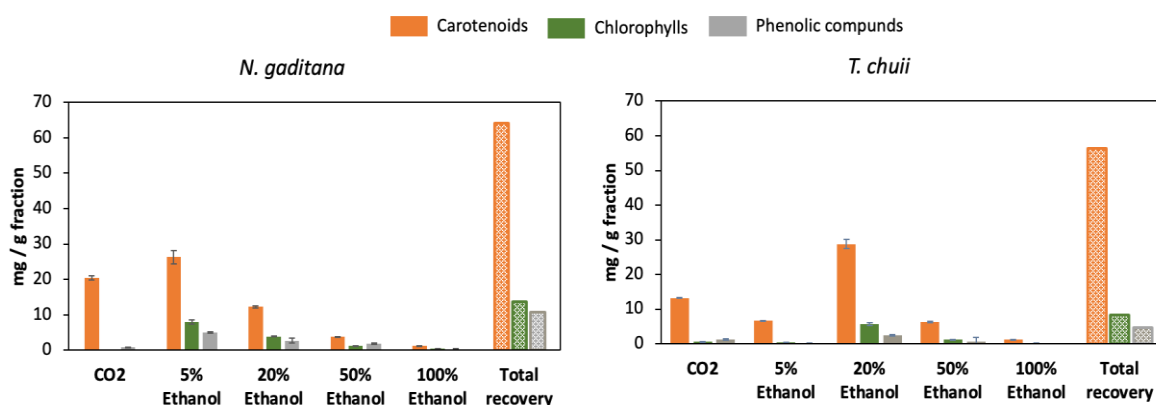


Figure 2. Composition of carotenoids, chlorophylls and phenolic compounds

### 3.3. Antioxidant activity

The antioxidant bioactivity is tightly related to the phenolic abundance, since in all cases the fraction more enriched in phenolic compounds are those with lower EC<sub>50</sub> levels. Those compounds have a higher antioxidant activity than the pigments and promote the bioactivity of the extracts. In general terms, the EC<sub>50</sub> of *N. gaditana* is lower than that of *T. chuii*, providing significantly higher antioxidant activity. *N. gaditana* showed in general better antioxidant activity when using intermediate fractions of co-solvent, showing the best result at 5% ethanol by both the DPPH and ABTS methods ( $230.54 \pm 12.20$  and  $111.67 \pm 9.95$  ppm, respectively). On the other hand, the use of co-solvent in the extraction of *T. chuii* did not influence in the recovering of antioxidant fractions, showing similar values both in the CO<sub>2</sub> fraction and the 20% ethanol one. Possibly, the abundance of carotenoids above the other compounds in *N. gaditana* hinders bioactivity, since the CO<sub>2</sub> fraction is the purest in carotenoids but shows less bioactivity. This conclusion can be extrapolated also to *T. chuii*. Again, in this case, the fraction of 20% ethanol, despite of achieving the higher phenolic concentration, did it also in carotenoids, decreasing its bioactivity to similar levels to the previous fractions. The great amount of non-polar compounds of algae make the use of PLE using ethanol at the end of the process not necessary, since if did not offer substantial recovery of compounds of interest, both on amount and bioactivity, in the conditions studied. Regarding the methodologies, the EC<sub>50</sub> obtained is not equal for the two methods used, although both methods have been widely used in microalgae. In all cases, the ABTS method provides better results than DPPH. According to Monteiro et al. (2020), ABTS reacts with any hydroxylated aromatic compound, regardless of its antioxidant potential, resulting in better EC<sub>50</sub> values. Furthermore, the same authors state that an important difference between both methods is solubility. ABTS is soluble for aqueous and organic solvents, which makes it a suitable method for determining antioxidant activity with hydrophilic and lipophilic components. However, DPPH is soluble only in an organic medium, especially alcohols, which limits the interpretation of hydrophilic antioxidants.

The high EC<sub>50</sub> values obtained for the DPPH method in *T. chuii* (see Fig.16B), which in most cases reach a difference of at least 1000 units with respect to the ABTS method, suggest the high presence of other more polar compounds, capable of reacting preferable with ABTS.

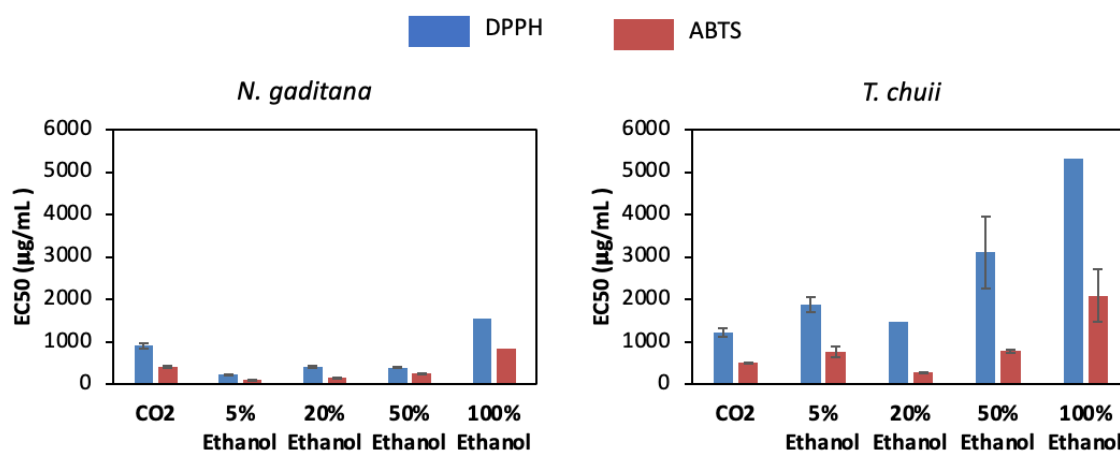


Figure 3. Antioxidant activity of the fraction extracted

#### 4. Conclusions

The consecutive extraction by supercritical techniques allows a high raw material depletion, achieving a high extraction in target compounds such as chlorophylls, phenolic compounds and carotenoids. Especially, the methodology allows the fractionation and purification of carotenoids mainly in the first stages, where the polarity of the solvent is higher. The existence of different types of carotenoids seems evident in the different microalgae fractions given that the fractions with the highest concentration of carotenoids do not match in the both microalgae species, being scCO<sub>2</sub> + 5% ethanol in the case of *N.gaditana* and scCO<sub>2</sub> + 20% ethanol in the case of *T.chuii*, which could be attributed to a different solubility of these carotenoids. All the fractions obtained in both microalgae showed antioxidant activity, being the fractions with the highest AA the ones with the highest phenolic content. *N.gaditana* seem to shows better results than *T.chuii* and, considering its morphology, supercritical techniques are a good candidate for the recovery of its compounds, considering that any pretreatment was employed before the extraction. The different composition of each fraction, which are enriched with higher bioactive compounds, offer a good variety of products more in accordance to potential subsequent applications depending on the polarity required, all from the same initial raw material, which contribute to low even more environmental impact of using this technique. The first fraction of *N. gaditana* possesses a high carotenoids purity and not even required any further treatment. However, further studies should be developed to increase even more the purity of the other fractions.

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