# Fractionation of the oil extracted from the marine microalgae *Crypthecodinium cohnii* to obtain biofuels and omega-3 compounds (DHA)

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## Abstract

Supercritical carbon dioxide extraction was used in this study, in a biorefinery context, to extract and fractionate the lipids synthesised by the marine microalgae *Crypthecodinium cohnii*. First, the pressure and temperature ranges that maximize the extraction yields of lipids and docosahexaenoic acid were determined through an experimental design, based on a surface response methodology, developed through the spatial distribution of two factors of Doehlert. It was concluded that the pressure and temperature conditions which yield the highest extraction of lipids, 16.89 g/100 $g_{\text{ash-free dry biomass}}$ and of docosahexaenoic acid, 3.51 g/100 $g_{\text{ash-free dry biomass}}$, are 187.5 bar and 41.3ºC. Also, at these pressure and temperature conditions it was possible to fractionate the extracted oil into two distinct fractions - a former rich in saturated and monounsaturated fatty acids and, therefore, suitable for the production of biofuels and a latter that can be purified into docosahexaenoic acid, a high value-added product sought by the pharmaceutical and nutraceutical industries.

Furthermore, it was also possible to extract and quantify the pigments accumulated by *C. cohnii*. The total pigment content of the microalgae, estimated by Soxhlet extraction, is equal to 72.28 µg/$g_{\text{ash-free dry biomass}}$, which is composed mainly of β-carotene and γ-carotene.

**Keywords**: Supercritical extraction; biorefinery; *Crypthecodinium cohnii*; docosahexaenoic acid; pigments.

## 1. Introduction

Over the last few years, with the aggravation of climate change, research into the application of microalgae as a raw material in the production of biofuels has been intensifying. In comparison with 1st and 2nd generation biofuel sources, microalgae present a much higher areal productivity and growth rate and they do not compete with food cultures, arable land and potable water [1]. However, their use in biofuel production is considered by many researchers as not sustainable, due to the high production costs involved [1]. It is therefore crucial to explore approaches that reduce process costs by using low-cost feedstocks and/or co-producing high value-added products in a biorefinery perspective. Microalgae are considered as potential candidates for a biorefinery process, as they are capable of producing multiple products with valuable applications in various industrial sectors, like docosahexaenoic acid.

Docosahexaenoic acid (DHA, 22:6ω-3) is a long chain polyunsaturated fatty acid (LCPUFA) with several health benefits, as it contributes to the prevention of various diseases such as cardiovascular conditions, cancer and neuropsychiatric disorders [2]. DHA accumulates in the membranes of human visual, reproductive and neurological tissues and is also present in breast milk, contributing to the normal development of babies' neurological and visual systems [2].

Microalgae oils are an interesting alternative to the traditional source of DHA, fatty fish oils, as they have a more pleasant taste and odour, are cholesterol-free and contaminants-free (*e.g.* heavy metals) and their composition is more stable and reliable, containing preferably only one type of LCPUFA, which facilitates the purification process [2].

*Crypthecodinium cohnii* is a marine, heterotrophic, colourless, unicellular microalgae capable of accumulating a significant amount of lipids, 20 to 50% of its dry weight, with a high content of DHA (up to 30-50% of total fatty acid content) [2]. Considering that other polyunsaturated fatty acids are present in an amount of less than 1% of the total fatty acid content, the process of separation and concentration of DHA is simpler and less expensive [2]. In addition, the microalgae *C. cohnii* also produces significant amounts of saturated and monounsaturated fatty acids with 12 to 18 carbon atoms, namely lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0) and oleic acid (18:1ω-9), which can be used in biodiesel production [3]. Therefore, this microalgae is a suitable example for the biorefinery concept.

Lipid extraction is one of the main steps involved in the production of biofuels from microalgae, contributing considerably to the overall cost of the process. Traditionally, lipids from microalgae are extracted using organic solvents such as hexane, however, this method has several drawbacks such as long operation times, use of high amounts of toxic solvents and contamination of the residual biomass, restricting its further use [4].

In recent years, supercritical extraction (SCE) emerged as a sustainable method for the recovery of biological compounds synthesised by microalgae, such as lipids for biodiesel production [5] [6] and/or high value-added products - carotenoids and chlorophylls [7], polyunsaturated fatty acids such as DHA [8] and γ-linolenic acid [9], hydrocarbons [10] and polyphenolic

compounds [11]. In comparison with conventional methods, SCE presents several benefits such as: use of non-toxic and cheap fluids; possibility of obtaining high extraction yields; possibility of performing fractionation during decompression of the supercritical solvent; contamination of the final product and residual biomass does not occur and the solvation capacity of the fluid is tuneable by changing the operating conditions of pressure and temperature [12].

This method separates soluble compounds from a solid matrix by contact with a solvent that is above the pressure and temperature that characterize its critical point, presenting in these conditions thermophysical properties intermediate between a gas and a liquid [12]. Various types of compounds can be used in SCE, such as ethane, propane, pentane, ammonia, dimethyl ether and water, but the most commonly used is carbon dioxide, as it is inert, cheap, non-flammable and has a moderate critical pressure and temperature (31.1°C and 72.9 atm), which reduces energy consumption and operating costs and allows the extraction of thermolabile substances without degradation [12]. Furthermore, its separation from bioactive compounds by depressurisation is simple and practically complete, which means that it can be recycled into the process and a good quality end product can be obtained. In the study by Mendes *et al*. (2005) it was found that by adding a small proportion (typically between 1 and 10%) of a cosolvent, such as methanol or ethanol, to supercritical $CO_2$ it is possible to extract more polar compounds [9].

The efficiency of the SCE process is influenced by the pressure, temperature, solvent flow rate and the characteristics of the matrix, such as particle size and shape, porosity, moisture content and the nature of the matrix itself [12].

The application of SCE in the recovery of lipids synthesised by the microalgae *Crypthecodinium cohnii* was previously analysed in the study by Couto *et al*. (2010), in which it was concluded that the highest extraction yield was obtained with the conventional Bligh & Dyer method, however SCE resulted in a lipid fraction richer in DHA (72.3% at 25 MPa and 323 K, as opposed to 49.5% with the Bligh & Dyer method) [8]. The application of a pre-treatment and the optimization of the pressure and temperature conditions could improve the extraction yield obtained.

The present work aims to explore the use of supercritical carbon dioxide extraction in the recovery of lipids from the microalgae *C. cohnii*, to implement a simple and environmentally friendly process for the co-production of biodiesel and the value-added product, docosahexaenoic acid. Therefore, this work complements the study of Couto *et al*. (2010) since its main objectives were: (i) compare the supercritical extraction method with the conventional Soxhlet method; (ii) determine the temperature and pressure ranges that maximize the yield in lipids, in DHA and in fatty acids that can be used in biodiesel production, through an experimental design, based on a surface response methodology, according to the distribution for two factors of Doehlert; (iii) study the relative impact of pressure and temperature on the extraction yields of the process; (iv) perform the fractionation of the oil extracted from *C. cohnii* into two distinct fractions in a biorefinery perspective - a first fraction rich in saturated and monounsaturated fatty acids that can be directed towards biodiesel production and another fraction with a higher abundance of the value-added product, the DHA – and (v) analyse, identify and quantify the pigments present in the extracted oil by means of high performance liquid chromatography and spectrophotometry.

## 2. Methods

### 2.1. Materials

Liquid $CO_2$ with a purity of 99.998% was purchased from Air Liquide. The chemical reagents n-hexane (p.a 95%, Fisher Chemical), methanol (p.a, 99.9%, Merck), sodium sulphate anhydrous (99%, Merck), acetyl chloride (98.5%, Panreac), n-heptane (p.a, 99.93%, Fisher Scientific), acetone (≥99.5%, Sigma Aldrich), ethanol (≥99.9%, Carlo Erba), methanol (HPLC grade, ≥99.9%, Honeywell) and acetonitrile (HPLC grade, ≥99.9%, Honeywell) were used. The β-carotene standard with a purity of ≥97% was purchased from CalBiochem.

### 2.2. Microalgae

*Crypthecodinium cohnii* (ATCC 30772 from American Type Culture Collection) was grown in a 7 L bench scale bioreactor containing a culture medium with crude glycerol as carbon source (23.94 g/L crude glycerol provided by IBEROL, 25 g/L sea salt, 0.5 g/L yeast extract, 4.59 g/L corn steep liquor provided by COPAM and 1 mL/L of the mixture of 3 antibiotics - chloramphenicol (5 mg/L), penicillin G (62 mg/L) and streptomycin (100 mg/L)). The pH of the medium was adjusted to a fixed value of 6.5.

The fed-batch fermentation process was carried out with an air inlet flow rate of 1 g/L.h and at a temperature of 27ºC. The stirring speed was adjusted between 100 and 275 rpm, to ensure a dissolved oxygen level above 30%.

The culture was grown for 185 hours (approximately 8 days) to ensure high cell density and a high DHA content in the biomass.

The biomass was harvested by centrifugation and lyophilised at -55ºC for 24 hours.

### 2.3. Lipid extraction

Microalgal lipids from freeze-dried *C. cohnii* were extracted by supercritical carbon dioxide and by the conventional extraction method of Soxhlet. The amount of lipids in each extract was determined gravimetrically.

Previously to extractions, the freeze-dried biomass was ground using a ball mill. Half a gram of freeze-dried

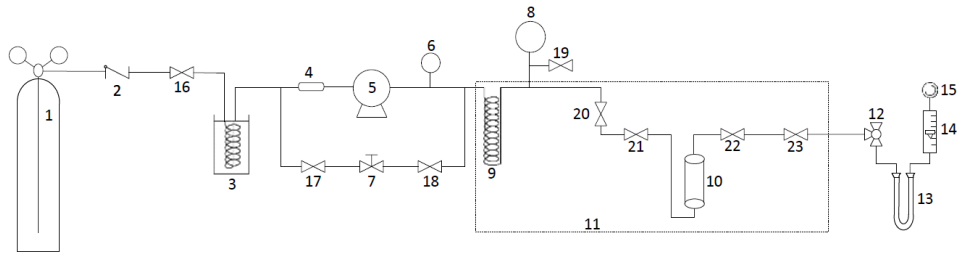


*Figure 1. Schematic diagram of the supercritical extraction apparatus. 1 - Gas cylinder with a plunger tube; 2 - check valve; 3 - ice cooled coil; 4 - high pressure filter; 5 – mini-pump; 6, 8 - pressure gauges; 7 – back-pressure regulator; 9 - heat exchanger; 10 - extraction cell; 11 - water bath; 12 - three-way valve; 13 - glass U-tube; 14 - rotameter; 15 – wet test meter; 16-23 - valves.*

biomass was milled for 3.5 min with a frequency of 25 Hz.

All experiments were performed in duplicate or triplicate, to statistically validate the results obtained.

#### 2.3.1. Soxhlet extraction

The Soxhlet extraction with hexane was carried out using half a gram of freeze-dried and ground microalgae biomass, which was packed in a cellulose cartridge. The extraction lasted 6 hours and a volume of n-hexane equal to 150 mL was used. Once the extraction was complete, the extract was vacuum filtered and the solvent was separated from the lipids in a rotary vacuum evaporator, to concentrate the extracted compounds and allow their quantification gravimetrically.

#### 2.3.2. Supercritical extraction

The supercritical $CO_2$ extraction studies were carried out in the flow apparatus represented in Figure 1, which was described in detail by Mendes *et al*. (1995) [13]. Liquid $CO_2$ was pumped to the system by a mini-pump, 5, being cooled beforehand in a coil immersed in an ice bath, 3, to ensure $CO_2$ was liquified at the entering of the pump. The pressure was controlled by a back-pressure regulator and accurately measured with a Bourdon-type Heise gauge, 8. The target temperature was achieved by the flow of the fluid through a heat exchanger, 9, before it entered the extraction cell, 10, being both immersed in a controlled temperature water bath. The extraction cell (5 $cm^3$) was filled with about 1.2 grams of freeze-dried and ground microalgae biomass mixed with glass beads (3 mm Ø), put between two layers of cotton wool. After leaving the extraction cell, the supercritical fluid was expanded to atmospheric pressure in a three-way valve, 12, and the extract precipitated in a cooled glass U-tube filled with cotton wool, 13. The three-way valve was heated to the working temperature. The U-tube, as well as the adjacent tubing, were washed with hexane to collect all precipitated material and the solvent was separated from the extract in a rotary vacuum evaporator, to quantify gravimetrically the lipids.

The volume of $CO_2$ used in each measure was determined by a wet test meter, 15, and the $CO_2$ flow rate, which was fixed at approximately 0.24 $L^{PTN}$/min (0.47 g/min), was monitored with a rotameter, 14.

In the experimental design tests, four extracts were collected at different times throughout the operation period - after 30 minutes, 1 hour, 1.5 hours and after 3 hours.

### 2.4. Surface response methodology

A surface response methodology (SRM), based on the Doehlert distribution for two factors [14], was used to determine the pressure and temperature ranges that maximize the extraction yields and study the relative impact of pressure and temperature on the yield. In this experimental design (ED), the variables or factors studied and the respective experimental domains tested were: pressure ($X_1$: 150-300 bar) and temperature ($X_2$: 40-60ºC). Fourteen experiments (seven conditions in duplicate) were carried out and the responses studied ($Y_i$) were: yield in lipids ($g_{lipids}/100g_{ash\text{-}free\ dry\ biomass}$), yield in DHA ($g_{DHA}/100g_{ash\text{-}free\ dry\ biomass}$) and yield in fatty acids for biodiesel ($g_{fatty\ acids\ for\ biodiesel}/100g_{ash\text{-}free\ dry\ biomass}$). The model used to express the responses was a second-order polynomial model: $Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$, where: $Y_i$ – response from experiment; β – parameters of the polynomial model and $X$ – experimental factor level.

The yield in lipids was calculated as the ratio between the total mass of lipids extracted in the process and the amount of microalgae biomass, in a dry and ash-free basis, placed inside the extractor.

The yields in DHA and in fatty acids for biodiesel were calculated as the ratio between the total mass of DHA and fatty acids for biodiesel extracted in the process, respectively, and the amount of microalgae biomass, in a dry and ash-free basis, placed inside the extractor. The fatty acids considered for biodiesel production were: capric acid (10:0), lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1), palmitic acid (16:0), palmitoleic acid (16:1ω-9), stearic acid (18:0), oleic acid (18:1ω-9), arachidic acid (20:0) and behenic acid (22:0).

### 2.5. Fatty acid analysis

Oil extracts (approximately 20 mg) were transmethylated with 2 mL of methanol:acetyl chloride (19:1 v/v) and 0.2 mL of heptadecanoic acid (5 mg/mL) as internal standard. The mixture was sealed in a light protected test tube under nitrogen atmosphere and heated at 80ºC for 1h. The test tube content was then cooled, diluted with 1 mL of distilled water and the methyl esters were extracted with 2 mL of n-heptane. The

organic phase was dried using sodium sulphate anhydrous and placed in a vial adequate for gas chromatography analysis.

The methyl esters were then analysed by gas-liquid chromatography, on a Bruker Scion 436-GC equipped with a flame ionisation detector. Separation was carried out on a 0.32x30 m fused silica capillary column (Supelcowax 10) with helium as carrier gas at a flow rate of 3.5 mL/min. The column pressure was 13.5 psi and its temperature was programmed at an initial value of 200ºC for 8 min, then increased at 4ºC/min to 240ºC and was held there for 16 min. Injector and detector temperatures were 250 and 280ºC, respectively, and the split ratio was 1:50 for 5 min and then 1:10 for the remaining time. Peak identification and response factor calculation were carried out using known standards (GLC 459, GLC 85 and GLC 75, Nu-chek-Prep). The quantities of individual fatty acids were calculated by dividing the peak area corresponding to the fatty acid by the peak area of the internal standard, heptadecanoic acid, and multiplying this ratio by the response factor of the respective fatty acid. The relative content of each fatty acid is the result of dividing the mass of a specific fatty acid by the sum of the masses of all the fatty acids identified in the chromatogram. Each sample was prepared in duplicate and injected twice.

### 2.6. Pigment analysis and quantification

To quantify and analyse the pigments contained in the microalgae *C. cohnii* the conventional Soxhlet method with hexane was used to determine the total pigments present in the biomass. The total volume of extracts obtained was measured.

The pigment content of the extracts was quantified by UV/Visible spectrophotometry. Spectra were run between 380 and 700 nm and total pigment concentration was determined using Beer-Lambert's law in equivalents of β-carotene. The value used for the specific optical coefficient was 259.2 ($L.g^{-1}.cm^{-1}$) [15] in hexane.

The pigments present in the extracts were also analysed by high performance liquid chromatography (HPLC). The HPLC system consisted of a liquid chromatograph, Hewlett Packard 1100 series, with UV/Vis detector adjusted to 457 nm (maximum absorption wavelength of the extracts). A reverse phase column, C18-Vydac 201 TP54 (250x4.6 mm) was used. The elution applied was of the isocratic type, the eluent being a mixture of acetonitrile and methanol (10:90 v/v, respectively) pumped at a flow rate of 1 mL/min. The β-carotene in the extracts was identified by comparison with the retention time of the CalBiochem standard compound. Of the remaining pigments detected in the chromatograms, it was possible to identify the presence of γ-carotene, taking into consideration existing data in the literature for carotenoids present in this microalgae [16], as well as by comparison with literature data for this carotenoid in chromatographic analyses [17].

## 3. Results and Discussion

### 3.1. Supercritical extraction

#### 3.1.1. Surface response methodology

Aiming to define the pressure and temperature ranges that maximize the yield of the supercritical extraction of lipids synthesised by the microalgae *C. cohnii*, an experimental design, based on the SRM according to the Doehlert distribution, was performed to evaluate the influence of two factors - pressure (factor 1) and temperature (factor 2) - on the yield in lipids, in DHA and in fatty acids that can be used in the production of biodiesel. Table 1 summarises all the experimental tests performed within the ED and the responses obtained.

Regarding the lipid extraction yield, it is observed that for the fixed temperature at the centre of the experimental domain (50ºC) (tests 1-6), the increase of the pressure from 150 to 225 bar results in an increase of the yield in 49.4%. On the other hand, the increase of the pressure from 225 to 300 bar does not result in a relevant difference in the yield in lipids. On tests 7-10 the effect of varying the pressure from 187.5 to 262.5 bar, at a constant temperature of 41.3°C, produces an average reduction of 15.4% in lipid yield. In tests 11 to 14, at a constant temperature of 58.7°C, the same pressure variation results in an average 34% increase in lipid yield.

By analysing the three groups of tests mentioned, one can infer that the increase in pressure presents two effects at constant temperature, as found in the study of Couto *et al.* (2010) [8]. At high temperatures (50 and 58.7ºC), the increase in pressure results in the increment of the lipid extraction yield, since an increase in the solvent density is induced and, consequently, the solubility of the solute increases. However, at low temperatures (41.3ºC), the increase in pressure results in the reduction of the extraction yield, since the dominant effect is the reduction in the diffusivity of carbon dioxide, which means that the capacity of the supercritical solvent to diffuse inside the pores of the solid matrix is reduced.

It is important to note that tests 7-14 can also be analysed from the perspective in which the pressure is fixed. When the pressure is equal to 187.5 bar, the change in temperature from 41.3 to 58.7ºC results in a 37.9% reduction in lipid yield (tests 7, 8, 11 and 12). However, at a fixed pressure of 262.5 bar, the same temperature variation produces a slight increase (10%) in lipid yield (tests 9, 10, 13 and 14).

Therefore, it may be concluded that the temperature increase, at constant pressure, also exerts two distinct thermodynamic effects. At the highest pressure of 262.5 bar, the increase in temperature induces an increase in the solute's vapour pressure, since under these conditions the density of supercritical $CO_2$ decreases moderately with increasing temperature. For the lower pressure of 187.5 bar, the predominant effect, resulting from the increase in temperature, consists in the reduction of the density of the supercritical solvent. Accordingly, it may be said that the crossover pressure

*Table 1. Experimental design according to the Doehlert distribution for two factors - pressure and temperature - and the evaluated responses (yield in lipids, in DHA and in fatty acids for biodiesel). The respective standard deviation (SD) of each response is also presented.*

| Test | Pressure (bar) | Temperature (ºC) | Yield in lipids ($g/100g_{ash\ free\ dry\ biomass}$) | SD | Yield in DHA ($g/100g_{ash\ free\ dry\ biomass}$) | SD | Yield in fatty acids for biodiesel ($g/100g_{ash\ free\ dry\ biomass}$) | SD |
|---|---|---|---|---|---|---|---|---|
| **1** | 150.0 | 50.0 | 7.52 | 0.27 | 0.82 | 0.29 | 1.84 | 0.39 |
| **2** | 150.0 | 50.0 | 8.06 | | 1.41 | | 2.63 | |
| **3** | 225.0 | 50.0 | 15.20 | 0.20 | 3.09 | 0.06 | 5.25 | 0.12 |
| **4** | 225.0 | 50.0 | 15.60 | | 3.21 | | 5.01 | |
| **5** | 300.0 | 50.0 | 14.95 | 0.01 | 2.74 | 0.06 | 4.68 | 0.13 |
| **6** | 300.0 | 50.0 | 14.93 | | 2.86 | | 4.94 | |
| **7** | 187.5 | 41.3 | 17.19 | 0.30 | 3.77 | 0.26 | 4.59 | 0.15 |
| **8** | 187.5 | 41.3 | 16.59 | | 3.25 | | 4.90 | |
| **9** | 262.5 | 41.3 | 14.01 | 0.28 | 2.35 | 0.18 | 3.99 | 0.40 |
| **10** | 262.5 | 41.3 | 14.58 | | 2.72 | | 4.78 | |
| **11** | 187.5 | 58.7 | 11.47 | 1.03 | 2.11 | 0.31 | 3.92 | 0.45 |
| **12** | 187.5 | 58.7 | 9.42 | | 1.48 | | 3.02 | |
| **13** | 262.5 | 58.7 | 16.14 | 0.26 | 2.54 | 0.15 | 3.85 | 0.30 |
| **14** | 262.5 | 58.7 | 15.63 | | 2.84 | | 4.45 | |

for this system will be between 187.5 and 262.5 bar, so that above 262.5 bar there will be an increase in yield with the increase in temperature for each value of pressure and below 187.5 bar there will be a decrease in yield with the increase in temperature, at each value of pressure.

Regarding the yield in DHA and in fatty acids for biodiesel presented in Table 1, it is observed that these present the same trends and variations verified for the yield in lipids in the conditions of pressure and temperature analysed previously. Only the following exceptions are noticed: for the fixed temperature of 41.3ºC, the increase of the pressure from 187.5 to 262.5 bar doesn't result in a relevant difference in the yield of fatty acids for biodiesel, registering a very slight decrease (in 7.6%) of this yield, however the reduction is more accentuated for the other two responses evaluated (15.3% for lipids and 27.6% for DHA). At 262.5 bar, the temperature increase from 41.3 to 58.7ºC resulted in a very slight decrease (by 5.5%) of the yield in fatty acids for biodiesel, while the same temperature variation induced slight increases in the yields in lipids and in DHA (10 and 5.6%, respectively).

Despite the existence of these two exceptions for the yield in fatty acids for biodiesel, it can be stated that the two main conclusions presented for the influence of pressure and temperature are valid for the three responses analysed in this experimental design.

Figure 2 shows the response surfaces obtained for the yield in lipids (a), in DHA (b) and in fatty acids for biodiesel (c) within the limits of the experimental domain.

In Figure 2 (a) the general tendency of the distribution of the isoresponse curves takes the form of lines with a slight inclination relative to vertical lines, which indicates that the variation of the lipid yield is more accentuated along the abscissae than the ordinate, i.e. the increase in temperature does not have such a considerable effect on the lipid yield when compared to the increase in pressure. In this figure, as well as in Figure 2 (b), it can also be seen that for low pressures, below 250 bar, the lipid extraction yield decreases with increasing temperature, but for higher pressures the opposite effect is observed. This observation is in agreement with the findings of the analysis of the experimental results and with the conclusion of the two thermodynamic effects exerted by the temperature increase at constant pressure.

The isoresponse surfaces corresponding to the maximum value of the lipid yield, 16 $g/100g_{ash\text{-}free\ dry\ biomass}$, are obtained in two different areas of the graph: the first is registered for pressure values between 187.5 and 240 bar and temperatures between 40 and 46ºC and the second area is comprised of pressures between 250 and 300 bar and temperatures between 52 and 60ºC. Given the existence of two ranges of pressure and temperature that maximize the lipid yield, the choice of the working conditions must include the analysis of other factors, namely economic, energetic and environmental. Thus, from an energetic, economic and environmental point of view, it is preferable to operate at lower pressure and temperature conditions.

In Figure 2 (b), the general trend of the distribution of the isoresponse curves takes the form of diagonal lines, which indicates that both factors have a similar influence on the DHA yield. For higher pressure values, starting from 250 bar, the inclination of the isoresponse curves decreases, presenting a tendency of verticalization. This observation indicates that at these pressure conditions, the temperature increase does not produce such a significant effect on the DHA yield in comparison with the pressure variation. The isoresponse surface corresponding to the maximum value of the yield in DHA, 3.2 $g/100g_{ash\text{-}free\ dry\ biomass}$, is obtained in the lower quadrants for intermediate pressure values (190-235 bar), and low temperatures (40-47ºC).

Figure 2 (c) shows that in the lower quadrants the general trend of the distribution of the isoresponse curves takes the form of vertical lines, which indicates that the increase in temperature does not induce a considerable effect on the yield in fatty acids for

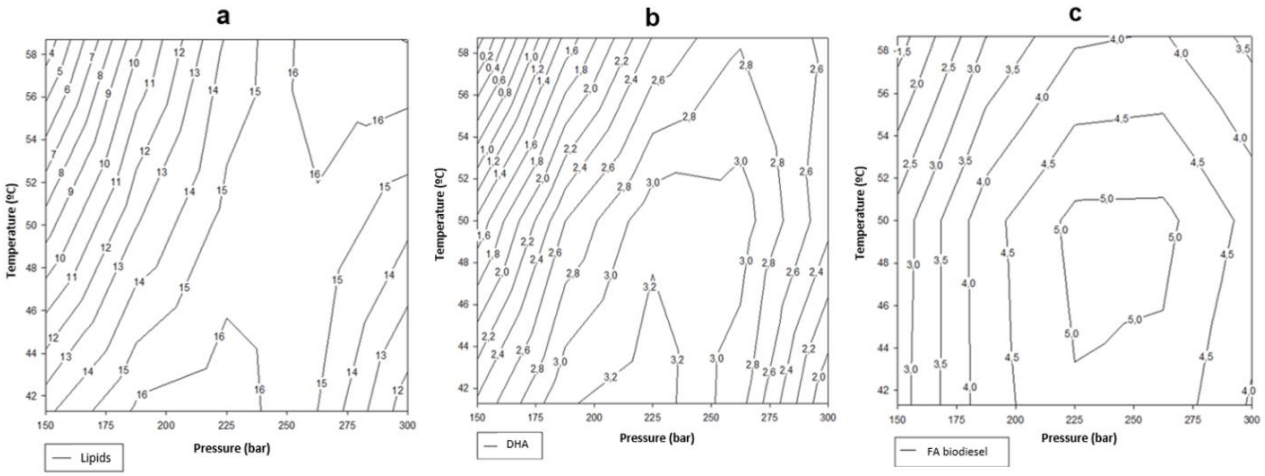


*Figure 2. Response surfaces for the yield in lipids (g/100g$_{ash\text{-}free\ dry\ biomass}$) (a); yield in DHA (g/100g$_{ash\text{-}free\ dry\ biomass}$) (b) and yield in fatty acids for biodiesel (g/100g$_{ash\text{-}free\ dry\ biomass}$) (c), obtained in the ED for the factors pressure (150 to 300 bar) and temperature (40 to 60ºC).*

biodiesel. However, in the upper quadrants, for elevated temperature values, it is found that the isoresponse curves present a slight inclination, indicating that the influence of both factors on the response is more similar. The isoresponse surface corresponding to the maximum value of the yield in fatty acids for biodiesel, 5.0 g/100g $_{ash\text{-}free\ dry\ biomass}$, is obtained in the lower right quadrant, i.e. for low temperature values (43-51ºC) and high pressures (225-270 bar).

**Analysis of experimental design parameters**

The data obtained from the experimental design were further used for a regression analysis and the polynomial model derived parameters ($\beta_0$–$\beta_{22}$) are presented in Table 2.

The β parameters of the polynomial models used to estimate the responses have the following meanings: $\beta_0$ represents the response at the centre of the experimental domain; $\beta_1$ and $\beta_2$ indicate the relative importance of the factors pressure and temperature, respectively, on the responses; the interaction parameter, $\beta_{12}$, indicates how simultaneous variation of both factors affect the response and, finally, the values of $\beta_{11}$ and $\beta_{22}$ determine how the response surface folds downward (negative values) or upward (positive values) quadratically, more or less rapidly in accordance with the magnitude of the absolute value.

By analysing the coefficients of the polynomial model related to the yield in lipids it is verified that the value of $\beta_1$ is significantly higher than that of $\beta_2$, indicating that pressure presents a much higher influence than temperature. This trend is not observed so distinctly in the distribution of the response surfaces, Figure 2 (a), since these curves do not take the form of perfectly vertical lines, but rather present a slight inclination.

Regarding the polynomial model representing the yield in DHA, the values of $\beta_1$ and $\beta_2$ are similar compared to the value of $\beta_0$, which means that both factors have a similar influence on the response in question, as can be seen in the analysis of Figure 2 (b). However, these values do not demonstrate the verticalization trend presented by the isoresponse surfaces for pressure values higher than 250 bar.

In the polynomial model representing the yield in fatty acids for biodiesel, the value of $\beta_1$ is higher than the value of $\beta_2$, indicating that pressure presents a more considerable influence compared to temperature in this response. This observation is in conformity with what was found in the lower quadrants of the graph in Figure 2 (c) but does not justify the slight inclination that the isoresponse surface lines present in the upper quadrants.

All responses present a slightly negative value of $\beta_2$, which indicates that temperature may present a negative influence on the responses.

Examining the interaction term, $\beta_{12}$, of the polynomial models for the yield in lipids and in DHA it is verified that this parameter is higher than the β coefficients of each experimental factor, therefore the interaction between pressure and temperature is significant and these two parameters should be controlled simultaneously during the extraction process. In the case of the polynomial model representing the yield in fatty acids for biodiesel, the interaction term is slightly lower than the β coefficients of each factor, which indicates that the interaction between pressure and temperature may not present as a significant influence on the response as the individual variation of each factor. However, the interaction between the factors should not be disregarded and therefore both pressure and temperature should be controlled simultaneously to ensure efficient recovery of fatty acids for biodiesel.

For the validation of the polynomial models as to their representation of the data sets, two statistical tests based on Fisher's test of variance ratio were performed: the test for the effectiveness of the coefficients and the test for the lack of fit of the model. The first statistical test assesses the level of significance of the amount of variance in the data that has been accounted for by the β coefficients of the model and the second test was

*Table 2. Parameters of the polynomial models representing the studied responses in the experimental design.*

| | Model | Yield in lipids | Yield in DHA | Yield in fatty acids for biodiesel |
|---|---|---|---|---|
| Model parameters | $\beta_0$ | 15.4 | 3.15 | 5.13 |
| | $\beta_1$ | 2.86 | 0.51 | 0.82 |
| | $\beta_2$ | -1.40 | -0.52 | -0.6 |
| | $\beta_{12}$ | 4.64 | 0.94 | 0.27 |
| | $\beta_{11}$ | -4.03 | -1.19 | -1.61 |
| | $\beta_{22}$ | -0.02 | -0.37 | -0.91 |
| Model validation | Effectiveness of parameters | 22.16 | 5.39 | 3.97 |
| | Significance level (α), F(5, 8) | 0.01 | 0.02 | 0.04 |
| | Lack of fit | 15.38 | 15.55 | 14.60 |
| | Significance level (α), F(1, 7) | 0.006 | 0.006 | 0.007 |
| $R^2$ | Coefficient of multiple determination | 0.93 | 0.77 | 0.71 |

performed to determine whether the origin of the variance was due to the experimental error.

Table 2 presents the values of $F_{calculated}$ and $F_{critical}$ corresponding to the tests of effectiveness of the coefficients and validation of the models, with the degrees of freedom according to Deming and Morgan, (1987) [18]. In this table it is also observed the significance levels (α) determined with the tool available in [19]. The first statistical test shows that the coefficients of the models represent the effect of the factors analysed in the experimental design with a significance level between 0.01 and 0.04 for the responses evaluated. The second test shows that the source of the variance contained in the residuals was explained by experimental error at the significance level below 0.007.

Therefore, under these validation conditions, the models were used to study the relative impact of the factors in the responses, their interaction and to produce the response surfaces within the limits of the experimental domain.

### 3.1.2. Comparison with Soxhlet extraction

Soxhlet extraction with hexane was applied in order to compare this process with supercritical extraction.

The average yields in lipids, in DHA and in fatty acids for biodiesel obtained for the Soxhlet method were 16.84, 2.49 and 4.52 g/100$g_{ash\text{-}free\ dry\ biomass}$, respectively. With the average value of the yield in lipids of this method and the mass of lipids extracted in supercritical extraction, it was determined the recovery of lipids for each condition experimentally tested, Table 3.

*Table 3. Recovery of lipids for each condition tested in the ED.*

| Pressure (bar) | Temperature (°C) | Recovery of lipids (%) |
|---|---|---|
| 150.0 | 50.0 | 46.3 |
| 225.0 | 50.0 | 91.4 |
| 300.0 | 50.0 | 88.4 |
| 187.5 | 41.3 | 100.3 |
| 262.5 | 41.3 | 84.9 |
| 187.5 | 58.7 | 62.0 |
| 262.5 | 58.7 | 94.3 |

Table 3 shows that lipid recovery depends considerably on the pressure and temperature conditions applied in the supercritical extraction process, since their values vary between 46 and 100%. Most of the conditions tested allow the recovery of more than 80% of the lipids. In fact, at 41.3ºC and 187.5 bar, it is possible to recover a slightly higher amount of lipids by SCE than with the Soxhlet method.

These high results were expected considering that hexane does not have the viscosity and diffusivity that supercritical $CO_2$ has in certain conditions of pressure and temperature, despite having a higher solvent power than $CO_2$. This proves that, under certain conditions of pressure and temperature, supercritical extraction achieves a higher yield of lipid extraction than the conventional Soxhlet method, which is favourable considering that the former is a more environmentally sustainable technique.

## 3.2. Fatty acid composition of lipids extracts

Figure 3 presents the fatty acid profile of the extracts collected at 41.3°C and 187.5 bar and of the lipid extract obtained by Soxhlet method. The other SCE conditions tested are not represented since the trends are similar between the extraction curves. Only the most important fatty acids identified in the chromatograms are represented in this figure.

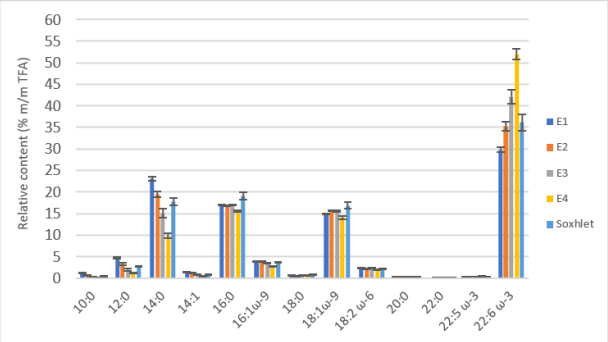


*Figure 3. Fatty acid composition of oil extracts obtained by SCE and Soxhlet method.*

Upon examination of the relative fatty acid contents for the four extracts collected (E1 to E4) it is concluded that the most abundant fatty acid is DHA, showing a relative content between 30 and 52% (m/m TFA). The other major fatty acids are myristic acid (14:0), palmitic acid (16:0) and oleic acid (18:1 ω-9) with a relative content of more than 10% (m/m TFA). The remaining

fatty acids can be considered as minor since they present relative contents lower than 5% (m/m TFA). These observations are in agreement with the analysis of the fatty acids profile of the oil extracted by the conventional Soxhlet method.

When comparing the relative contents of the extracts collected, it is verified that the content of some saturated and monounsaturated fatty acids - 10:0, 12:0, 14:0, 14:1, 16:0 and 18:1ω-9 - is higher in extracts E1 and E2, while extracts E3 and E4 are richer in DHA. This difference can be justified by the fact that docosahexaenoic acid, as well as other polyunsaturated fatty acids from *C. cohnii*, are mostly associated with phospholipids of the cell membrane. Therefore, in the initial phase of the extraction process, the supercritical solvent extracts preferentially the fatty acids that are dispersed in the cytoplasm of the cell, whereas the extraction of membrane associated fatty acids, such as DHA, starts afterwards and so their relative content increases along the extraction time.

Therefore, the analysis of Figure 3 enables the conclusion that the oil from the first two extracts is suitable to be applied in biodiesel production and the oil corresponding to the last two extracts can be purified in DHA and used by the pharmaceutical and nutraceutical industries.

### 3.3. Fractionation of the extracted oil

Analysing the response surfaces obtained in the ED and the fatty acid profile of the extracts collected throughout the process, Figure 3, it is concluded that to fractionate the SCE oil into two distinct fractions - one rich in fatty acids suitable for biodiesel production and the other with a higher abundance of DHA - it is necessary to consider the influence of time, pressure and temperature on the composition of the lipid extract.

Therefore, the yields in lipids, DHA and fatty acids for biodiesel obtained for each extract collected from the different conditions tested in the ED were analysed, to elaborate a fractionation protocol that would allow obtaining the two desired fractions, but that did not require changes in temperature and/or pressure between fractions, thus ensuring its technical viability on an industrial scale. Only one protocol met these criteria: extraction carried out at 41.3ºC and 187.5 bar, with the first fraction collected after 30 minutes and the second after 2.5 hours. The results obtained for the yields and recoveries of lipids, DHA and fatty acids for biodiesel, as well as the fatty acids profile, can be observed in Table 4 and Figure 4. The recoveries presented were calculated as the ratio of the yield obtained for the respective fraction by the maximum yield recorded in the ED.

*Table 4. Results obtained in the fractionation of the SCE oil.*

| Fraction | Yield in lipids ($g/100g_{ash\ free\ dry\ biomass}$) | Recovery of lipids (%) | Yield in DHA ($g/100g_{ash\ free\ dry\ biomass}$) | Recovery of DHA (%) | Yield in FA for biodiesel ($g/100g_{ash\ free\ dry\ biomass}$) | Recovery of FA for biodiesel (%) |
|---|---|---|---|---|---|---|
| **1** | 3.62 | 21 | 0.44 | 13 | 1.39 | 27 |
| **2** | 10.69 | 63 | 2.25 | 64 | 3.33 | 65 |
| **Total** | 14.31 | 84 | 2.69 | 77 | 4.72 | 92 |

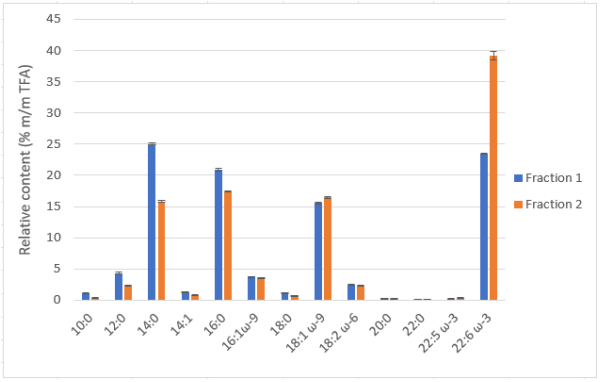


*Figure 4. Fatty acid composition of the fractions obtained in the fractionation protocol.*

In Table 4 it is verified that the first fraction presents a yield in total lipids of 3.62 $g/100g_{ash\text{-}free\ dry\ biomass}$, i.e., 21% of the lipids present in the biomass were recovered. This fraction presents a concentration of fatty acids for biodiesel of 38% (m/m), having been recovered 27% of these fatty acids. On the other hand, the DHA concentration in the lipids is 12% (m/m).

When analysing the fatty acid profile of the first fraction, Figure 4, it can be seen that, although the most abundant fatty acid does not correspond to DHA, its relative content is high and therefore this fatty acid should be removed before considering the use of the remaining oil in the production of a biofuel. This remaining oil, without DHA, has a high content of saturated fatty acids, in particular myristic acid, which indicates that direct transesterification of this oil may result in a biodiesel whose properties do not comply with the specifications established in the international biodiesel standard for vehicles (EN14214), namely regarding cold flow properties.

Therefore, a more viable alternative consists in the addition of the remaining oil (in proportions to be studied) to oils with a reduced content of saturated fatty acids, such as soybean oil or used cooking oil, resulting in a blend with more favourable characteristics to be used in biodiesel production. However, it may be even more viable to apply the remaining oil (without DHA) in the production of biofuels substitutes for diesel or gasoline by catalytic hydrogenation.

The second fraction presents a yield in total lipids of 10.69 $g/100g_{ash\text{-}free\ dry\ biomass}$ and a yield in DHA of 2.25 $g/100g_{ash\text{-}free\ dry\ biomass}$, which corresponds to a recovery of 64% of the DHA present in the biomass. Hence, the concentration of DHA in the lipids that constitute the second fraction is 21% (m/m).

With the results obtained, it can be inferred that the fractionation protocol carried out resulted in a first fraction with a reduced concentration of DHA and a second fraction that maintains a higher abundance of DHA in relation to the other fatty acids, as shown in the analysis of Figure 4. Therefore, the favourability of the application of SCE in a biorefinery has been proven, since it allows the fractionation of the oil recovered from the microalgae *C. cohnii* by means of a protocol without

great complexity, enabling the production of a biofuel and the obtainment of a high value-added product, which can make the process more sustainable economically.

### 3.4. Analysis of the pigments of *C. cohnii*

The total pigment content of *C. cohnii*, expressed in β-carotene equivalents, was determined using the Soxhlet extraction method with hexane applied to freeze-dried and ground microalgae biomass. The average yield in total pigments obtained is equal to 72.28 μg/$g_{ash\text{-}free\ dry\ biomass}$, which is close to the value reported by Withers and Tuttle (1979) [16] and by Zhang *et al*. (2020) [20] for other *C. cohnii* strains grown on visible light, as can be seen in Figure 5. On the other hand, the yield obtained in this study is considerably higher than the values reported for biomass resulting from a fermentation in the dark, which may indicate that the other culture conditions applied in the fermentation enhance pigment accumulation in the *C. cohnii* strain studied.

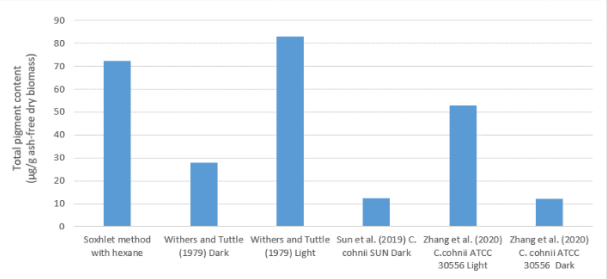


*Figure 5. Total pigment content of C. cohnii obtained in this study and reported by other authors.*

The Soxhlet lipid extract was also analysed by HPLC to identify the carotenoids present in the extracted oil. Table 5 shows the contents of carotenoids registered in the chromatograms in relation to total pigments and total carotenes.

*Table 5. Relative content of carotenoids and carotenes identified in the chromatograms of the Soxhlet lipid extracts.*

| Carotenoids | % (m/m total pigments) | % (m/m carotenes) |
|---|---|---|
| **Other** | 5.23 | - |
| **β-carotene** | 31.4 | 33.1 |
| **γ-carotene** | 31.3 | 33.0 |
| **Other carotene** | 19.9 | 21.0 |
| ***Minor* carotenes** | 12.2 | 12.9 |

Table 5 shows that β-carotene and γ–carotene are the major pigments present in the Soxhlet extract, with a relative content of 31% (m/m total pigments). The chromatograms also recorded the presence of a more polar carotenoid and other peaks that could not be identified, but it is considered that these correspond to carotenes based on the information available in the literature [16]. Within these other peaks the "other carotene" is highlighted, as it has a relative content close to those of the most abundant pigments. The remaining peaks were grouped and categorized as "minor carotenes" since individually they have a very small contribution to the total pigments.

Therefore, it can be stated that the pigments present in the Soxhlet extract are mainly carotenes, with a content of 95% relative to the total pigments, and that the preferentially accumulated carotenes are β-carotene and γ-carotene, since these correspond to 66% of the total carotenes.

In this study β-carotene and γ-carotene are accumulated in the same proportion by *C. cohnii* cells, i.e., the ratio β-carotene/γ-carotene is equal to 1, which is at odds with the results reported in other studies. Withers and Tuttle (1979) concluded that *C. cohnii* cells resulting from a fermentation in the dark accumulate more significant amounts of γ-carotene than of β-carotene, as the ratio β-carotene/γ-carotene is equal to 0.67 [16]. On the other hand, Sun *et al*. (2019) found that the *C. cohnii* SUN strain grown in the dark preferentially accumulates β-carotene as opposed to γ-carotene, since they obtained a ratio between these pigments equal to 3 [21]. These observations, in conjunction with the analysis of Figure 5, allow the conclusion that the content of total pigments and the type of carotenoids accumulated by *C. cohnii* depend considerably on the strain under study and the culture conditions applied in the microalgae production step.

#### 3.4.1. Pigments extracted by supercritical extraction

The lipid extracts collected during the SCE were also analysed by UV/Visible spectrophotometry, presenting in Table 6 the yields in total pigments obtained in each pressure/temperature condition tested in the ED.

*Table 6. Yield in total pigments obtained for each pressure-temperature condition tested in the supercritical extraction method.*

| Pressure (bar) | Temperature (°C) | Yield in total pigments (μg/$g_{ash\text{-}free\ dry\ biomass}$) |
|---|---|---|
| 150.0 | 50.0 | 28.53 |
| 225.0 | 50.0 | 49.55 |
| 300.0 | 50.0 | 43.87 |
| 187.5 | 41.3 | 49.40 |
| 262.5 | 41.3 | 50.01 |
| 187.5 | 58.7 | 18.82 |
| 262.5 | 58.7 | 34.89 |

The analysis of Table 6 indicates that the total pigment yield obtained in the SCE depends considerably on the pressure and temperature conditions applied during the process, since its values vary between 18.82 and 50.01 μg/$g_{ash\text{-}free\ dry\ biomass}$. However, for most of the experimentally tested conditions it is possible to obtain a pigment yield comprised in the range 40 to 50 μg/$g_{ash\text{-}free\ dry\ biomass}$.

By observing Table 6 it is also noted that the temperature and pressure conditions that maximize the total pigment extraction yield are 41.3°C and 262.5 bar, achieving a yield equal to 50.01 μg/$g_{ash\text{-}free\ dry\ biomass}$. Comparing this value with the total pigment content of the biomass estimated by Soxhlet extraction, 72.28 μg/$g_{ash\text{-}free\ dry\ biomass}$, it is concluded that in the best conditions of pressure and temperature, supercritical extraction allows the recovery of approximately 69% of

the pigments present in the microalgae. Therefore, it is possible to conclude that the pressure and temperature conditions experimentally tested during the supercritical extraction process do not present a high efficiency in the recovery of the pigments accumulated by the cells of the microalgae *C. cohnii*.

## 4. Conclusions

This study highlighted the favourability of applying supercritical carbon dioxide extraction in the biorefinery of the marine microalgae *C. cohnii*, since it was demonstrated that it is possible to perform the fractionation of the extracted oil into two distinct fractions - a former rich in saturated and monounsaturated fatty acids and, therefore, suitable for the production of a biofuel and a latter with a greater abundance of the value-added ω-3 compound coveted by the pharmaceutical and nutraceutical industries, the DHA.

In the experimental design, based on the surface response methodology, it was concluded that under conditions of reduced to intermediate pressure and temperature, i.e. between 187.5 and 250 bar and between 40 and 50ºC, it is possible to maximise the extraction yields of lipids, DHA and fatty acids for biodiesel. The experimental design also led to the conclusion that, overall, pressure variation has a more significant influence than temperature variation on the extraction yields, however both factors should be controlled simultaneously during supercritical extraction to ensure the efficiency of the process.

Furthermore, it was concluded that in certain pressure and temperature conditions, supercritical extraction achieves a higher yield in lipids than that attained with the conventional Soxhlet method, which is extremely important considering that supercritical extraction can be considered as a more environmentally sustainable process.

Finally, it was found that in the best conditions of pressure and temperature, supercritical extraction allows the recovery of 69% of the pigments accumulated by *C. cohnii*, which are mainly composed of β-carotene and γ-carotene.

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