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Original Paper

Extraction of carotenoids and chlorophyll from microalgae with supercritical carbon dioxide and ethanol as cosolvent

The extraction of carotenoids and chlorophylls using carbon dioxide modified with ethanol as a cosolvent is an alternative to solvent extraction because it provides a high-speed extraction process. In the study described here, carotenoid and chlorophyll extraction with supercritical CO_2 + ethanol was explored using freeze-dried powders of three microalgae (*Nannochloropsis gaditana*, *Synechococcus* sp. and *Dunaliella salina*) as the raw materials. The operation conditions were as follows: pressures of 200, 300, 400 and 500 bar, temperatures of 40, 50 and 60°C. Analysis of the extracts was performed by measuring the absorbance and by using empirical correlations. The results demonstrate that it is necessary to work at a temperature of 50 – 60°C and a pressure range of 300 – 500 bar, depending on the type of microalgae, in order to obtain the highest yield of pigments. The best carotenoid/chlorophyll ratios were obtained by using supercritical fluid extraction + cosolvent instead of using conventional extraction. The higher selectivity of the former process should facilitate the separation and purification of the two extracted pigments.

Keywords: Carotenoids / Chlorophyll / Cosolvent / Microalgae / Supercritical fluid extraction

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1 Introduction

Seaweeds have been used as foodstuffs and in agricultural applications for a long time. At present, many commercial applications of seaweeds are known and these are due to the chemical constitution of these plants [1, 2]. For example, seaweeds are used to increase the nutritional content of animal and human foodstuffs, in aquaculture [3] and in the cosmetics industry [4].

Furthermore, seaweeds have been exploited for about a century as a source of colloids, which find use as thickeners, gelling agents and stabilisers in the foodstuffs industry. Nevertheless, in the last thirty years seaweeds have been recognised as sources of high added-value compounds. This is the situation with polyunsaturated fatty acids, which are added to children's foodstuffs and nutritional supplements and are also a component of pigments used as natural colourants [5, 6].

Carotenoids or tetraterpenoids are terpenoid pigments that contain forty carbon atoms. These materials constitute an important group of natural pigments due to their structural diversity and numerous functions. These compounds play an essential role in photosynthesis and for oxygen-consuming life. In microalgae they function as secondary pigments in photosystems, where they work as photo-protectors and as a constituent part of the systems involved in light capture [7 – 9].

Carotenoids are used as colourants in foodstuffs, *e.g.* they are used as suspensions to give colour to drinks (for instance, β-carotene is used in orange drinks). This colourant is unaffected by ascorbic acid, heating or freezing and is active at very low concentrations (1 ppm).

Chlorophylls are not used as additives but as protectors of natural chlorophylls already present in foodstuffs that are vegetal in origin. Natural chlorophylls are highly unstable and are affected by light, acids or oxygen and their degradation is associated with ageing [10]. These compounds also find use in oils, chewing gums, ice creams, drinks, ready-made soups and in cheeses and yoghurts [11, 12]. Colourant-grade chlorophylls do not have a dose limit for their use, as they are essentially not absorbed by the digestive tract. During technological processes such as scalding or canning of vegetables, heating leads to the disappearance of magnesium from chlorophylls. This reaction transforms the chlorophylls into olive green/brown compounds (pheophytins), whose col-

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our is less appealing than the characteristic green of the parent compounds. Long-term storage leads to the destruction of natural chlorophylls.

Conventional extraction techniques for the aforementioned pigments for use in the foodstuffs industry involve the use of organic solvents, and this is a practice that is currently being phased out for environmental, health and safety reasons.

Supercritical extraction with CO_2 is an advanced technology that has a low environmental impact due to the undisputed advantages of CO_2 as a solvent, *i.e.* low toxicity, low cost, easy separation from extracts [13]. In addition, the use of CO_2 gives an added advantage in terms of quality, as extracts do not suffer excessive heating, which would destroy thermally unstable compounds.

Previous studies have shown that the supercritical CO_2 extraction of carotenoids from carrots [14], cabbage [15] or microalgae [16 – 19] proceeds with high yields. On the other hand, very few studies have been carried out on the supercritical CO_2 extraction of chlorophylls.

In this work, we report a supercritical CO_2 extraction procedure for pigments from microalgae *Nannochloropsis gaditana*, *Synechococcus* sp. and *Dunaliella salina*, which belong to different classes of algae. Each of these algae has a different cellular structure.

N. gaditana, of the chromophyta group, has a thick cellular wall and its pigments are located in the chloroplast. This species is found in the Bay of Cadiz and it is very well adapted to the local conditions.

The pigments in *Synechococcus* sp., which belong to the cyanophyta group or blue-green algae, are not located in an organelle but can be found near to the cell wall. Previous studies show that this prokaryotic organism is a favourable candidate for the commercial production of carotenoids.

D. salina is a eukaryotic micro-organism that lacks a proper cell wall. This organism, which belongs to the chlorophyta group has been commercially exploited as a source of β-carotene.

An experimental program based on a multilevel factorial design was carried out to assess the influence of the temperature and pressure on the yield of the extraction of carotenoids and chlorophylls from the aforementioned algae with a gaseous mixture of supercritical CO_2 and ethanol as cosolvent. An empirical equation was developed (STATGRAPHICS Plus 5.1 software) which is able to predict the yields of carotenoids and chlorophylls in the extraction processes. Finally, a comparison was made, for each of the three algae, between the results obtained by using the supercritical procedure presented here and the conventional methodology in which methanol is used as a solvent.

2 Experimental

2.1 Raw materials

The raw materials employed in the experiments were obtained from the Marine Microalgae Culture Collection of the Instituto de Ciencias Marinas de Andalucia (CSIC, Spain). The biomass was freeze-dried after being cultivated in seawater enriched with f/2 medium [20] at 20°C and aerated with atmospheric air. Once the sample had been obtained, it was stored under vacuum in darkness until the extraction procedure was carried out. *D. salina* was freeze dried in glycerol in order to protect the cellular structure of the microalga.

2.2 Chemicals

The chemicals and their characteristics, purity and company of origin are shown in Table 1.

The choice of ethanol was based on ref. [21 – 23], in which ethanol is considered to be a very effective cosolvent for the supercritical extraction of hydroxycarotenoids from different matrixes. The presence of ethanol (in traces) in the final extracts does not compromise the use of the products in nutraceutical or pharmaceutical applications [24].

Although one reference considered that the use of 15% of ethanol led to the best extraction yields [21], previous experiments with *Synechococcus* sp. showed that the appropriate percentage of cosolvent was 5%. For the other microalgae studied the same percentage of cosol-

Table 1. Characteristics of the chemicals used in the present work

Chemical	Purity	Company	Use
Carbon dioxide	99.995%	Carburos Metálicos	Supercritical extraction solvent
Methanol	HPLC grade	Panreac	Storage solvent for the extracts Conventional extraction solvent
Ethanol	Instrumental analysis grade	Panreac	Collection solvent for the extracts Used as cosolvent
Acetone	Industrial	PQS	Cleaning of supercritical extraction equipment
Nitrogen	99.99%	Carburos Metálicos	Evaporation of solvents
DMF	Instrumental analysis grade	Panreac	Conventional extraction solvent

vent was used in order to make the yields directly comparable.

Methanol was selected as a conventional solvent instead of acetone due to the results obtained in previous experiments with freeze-dried *N. gaditana* [18]. These results indicated that the use of acetone gave quite low yields. For this reason, methanol was selected as the solvent as the yields obtained were higher.

In the same way, the reason for using DMF as a conventional solvent was that it allowed the extraction of the maximum amounts of pigments from the total contained in the raw material studied.

2.3 Supercritical fluid extraction

The aim of this study was to ascertain the influence of both pressure and temperature, when using a mixture of supercritical carbon dioxide with a molar concentration of 5% of ethanol, for the extraction of carotenoids and chlorophyll from freeze dried powders of three different microalgae, were obtained by carrying out a number of experimental runs at 200 – 500 bar and 40 – 60°C.

To this purpose a microscale supercritical extraction apparatus obtained from Isco (Nebraska, model SFX 220) was used in order to carry out the experimental development. The equipment consisted of an extractor, an SFX 200 controller, a restrictor and two syringe pumps (models 260D and 100DX) to introduce carbon dioxide and ethanol, respectively (Fig. 1). A more detailed description of the apparatus is provided in a previous publication [19].

The operating methodology involved loading the extraction cartridge with approximately 0.200 g of *N. gaditana*, 0.100 g in the case of *Synechococcus* sp. and 0.105 g for *D. salina* (0.025 g in dry weight). These raw materials had previously been homogenised to maintain a constant apparent density in all experiments. The cartridge was then introduced into the extractor and left for 15 min to reach the operating temperature.

The flow was regulated with a micrometric valve, which was thermostated at 50°C until a constant flow of 4.5 mmol/min was achieved. An extraction was then carried out for a total of 3 h. An extraction time of 3 h was chosen because the majority of carotenoids and chlorophyll extracted with supercritical carbon dioxide had been obtained after this period.

The extracts were collected in glass tubes containing ethanol. After the extraction process was complete, the solvent was removed by passing a nitrogen stream over the product at a temperature of 40°C. The extracted product was dissolved in methanol (5 mL) and stored at 4°C with the exclusion of light until subsequent analysis. Finally, the extractor and the pipes were cleaned with acetone. A diagram showing the flow in the extraction process is shown in Fig. 1.

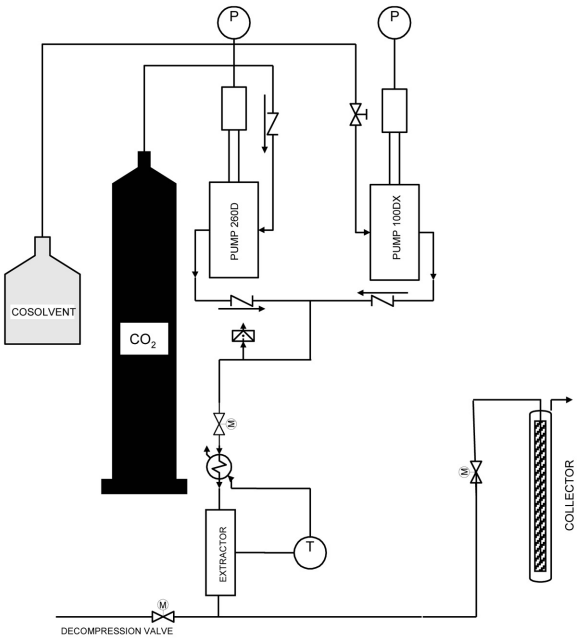

Figure 1. Diagram showing the flow of the extraction process with supercritical carbon dioxide and cosolvent.

2.4 Conventional extraction

With the aim of obtaining a reference value in order to compare the experimental results from supercritical extraction processes, methanol and DMF were used as solvents at atmospheric pressure. Several organic solvents, such as methanol and acetone, were used in the extraction of pigments from freeze-dried microalga. These experiments indicated that the use of acetone gives quite low yields. For this reason, methanol was selected as the solvent due to the higher yields obtained.

In relation to the methanol extraction, an amount of 0.200 g was used for *N. gaditana*, 0.100 g for *Synechococcus* sp. and 0.105 g for *D. salina*. Each sample was suspended in methanol (5 mL). The sample was sonicated for 10 min in a ultrasound bath (Spain, Selecta) and then kept at 4°C for 24 h. *D. salina* was sonicated for 3 min because this microalga has a structure that is more sensitive to sonication than the others. This time was, however, sufficient for the pigment extraction.

The supernatant liquid was recovered by centrifugation and stored until analysis was carried out. After a number of extraction cycles with methanol, the solvent did not show any colouration (seven cycles for *N. gaditana*, six cycles for *Synechococcus* sp. and *D. salina*). In the cases of *N. gaditana* and *D. salina*, the pellet remained greenish in colour but the *Synechococcus* sp. pellet became colourless.

With regard to the DMF extraction, the same amounts of raw materials and the same procedure were used as

Table 2. Carotenoid and chlorophyll yields obtained for an extraction time of 180 min

Pressure (bar)	Temperature (°C)	*N. gaditana*			*Synechococcus* sp.			*D. salina*		
		µg carotenoids/mg dry weight microalga	µg chlorophyll/mg dry weight microalga	Car/Chlor ratio	µg carotenoids/mg dry weight microalga	µg chlorophyll/mg dry weight microalga	Car/Chlor ratio	µg carotenoids/mg dry weight microalga	µg chlorophyll/mg dry weight microalga	Car/Chlor ratio
200	40	0.845	0.004	211.250	0.366	0.004	91.500	2.447	0.112	21.848
	50	0.997	0.000	–	0.295	0.002	147.500	0.539	0.017	31.706
	60	0.703	0.005	140.600	0.397	0.019	20.895	1.353	0.032	42.281
300	40	1.000	0.002	500.000	0.459	0.311	1.476	1.583	0.078	20.295
	50	2.100	0.228	9.211	1.860	0.286	6.503	4.536	0.200	22.680
	60	2.341	0.084	27.869	0.553	2.218	0.249	5.790	0.291	19.897
400	40	0.530	0.005	106.000	0.511	0.833	0.613	1.057	0.263	4.019
	50	2.250	0.059	38.136	0.557	0.572	0.974	1.709	0.210	8.138
	60	2.768	0.147	18.830	0.852	0.776	1.098	9.629	0.700	13.756
500	40	1.385	0.069	20.072	0.414	0.207	2.000	2.281	0.027	84.481
	50	2.405	0.325	7.400	0.668	1.803	0.370	4.093	0.350	11.694
	60	2.893	0.369	7.840	0.485	0.004	121.250	3.880	0.293	13.242
Methanol extraction		2.200	26.400	0.083	1.400	4.100	0.341	14.100	2.500	5.640
DMF extraction		6.900	41.500	0.166	3.300	9.600	0.344	27.700	3.100	8.935

for the methanol extraction. After a number of extraction cycles with DMF, the solvent did not show any colouration (nine cycles for *N. gaditana*, four cycles for *D. salina* and six cycles for *Synechococcus* sp.). The pellets did not retain any colour but it was observed that the pellet in the case of *Synechococcus* sp. was black. A possible explanation could be that this solvent reacts in some way with certain compounds contained in the microalga, for instance phycocyanines. These compounds are a kind of photosynthetic pigments that give the blue-green colour to cyanophytes.

2.5 Analysis methods

The determination of the total concentration of carotenoids and chlorophyll was carried out by measuring the absorbance of the different samples using a spectrophotometer (Japan, Hitachi U-2010).

The equations proposed by Wellburn [25] were used in the analysis of carotenoids and chlorophylls. These equations are given in a previous publication [19].

3 Results and discussion

The yields of the carotenoid and chlorophyll extractions and the carotenoid/chlorophyll (Car/Chlor) ratios obtained from the three freeze-dried microalgae (*N. gaditana*, *Synechococcus* sp. and *D. salina*) are shown in Table 2. The yields are expressed as micrograms of pigment *per* milligram dry weight of microalga. These values were obtained for an extraction time of 180 min for the different extraction conditions studied. The yields of the methanol and DMF extractions at atmospheric pressure are given at the end of Table 2.

The experimental results for carotenoid and chlorophyll extraction yields, using carbon dioxide + 5% molar ethanol as the solvent system, are analysed in this section. These yields were obtained for an extraction time of 180 min.

3.1 Effect of pressure and temperature on the extraction yield of carotenoids

The experimental results in terms of carotenoid extraction yields obtained for the three microalgae under the pressure and temperature conditions indicated previously are presented in Figs. 2 – 4.

The results obtained using *N. gaditana* as the raw material are represented in Fig. 2 and it can be seen that for each temperature the extraction yields increase with pressure. This behaviour can be attributed to an increase in the density of the solvent system with pressure at the operating temperatures considered, a change that makes the diffusivity of the system higher. At 40°C, the trend in the experimental results is similar to the developments observed at 50 and 60°C. Nevertheless, there is a slight decrease in the yield at 400 bar of pressure.

The same behaviour can be observed when the effect of temperature is analysed. For each pressure, an increase in temperature produces an increase in the carotenoid extraction yields. However, at 200 bar a decrease in the yield is observed when the temperature is 60°C. This behaviour is similar even when the extraction process is carried out on the same microalga without cosolvent [18]. The slight reduction in the carotenoid extraction yield at 60°C, when the operating pressure is 200 bar, is due to the decrease in the density of the supercritical fluid and, as a consequence, in its solvating power. On

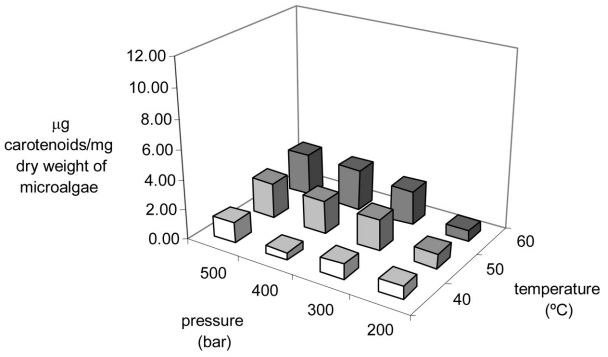

Figure 2. Yield of carotenoid extraction with supercritical carbon dioxide + 5% molar of ethanol from *N. gaditana*, for an extraction time of 180 min.

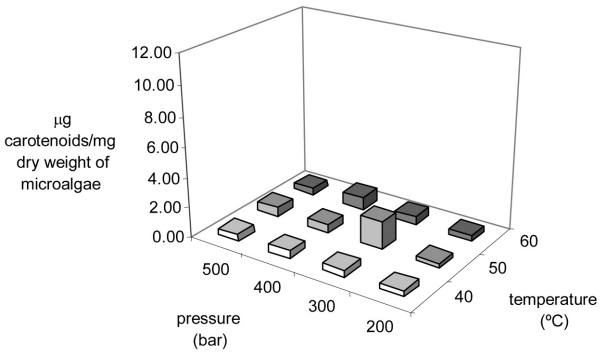

Figure 3. Yield of carotenoid extraction with supercritical carbon dioxide + 5% molar of ethanol from *Synechococcus* sp., for an extraction time of 180 min.

the other hand, for pressures up to 200 bar it can be seen that the increase in vapour pressure of carotenoids, due to the increase in the temperature and diffusivity, compensates for the reduced density of supercritical carbon dioxide and the extraction of these pigments therefore occurs with high efficiency [21, 26]. At 300, 400 and 500 bar, the density of the supercritical carbon dioxide and its solvent power are higher, giving rise to slight variations compared to the results obtained at 200 bar.

The increase in both the vapour pressure of carotenoids and the diffusion coefficient of the solvent, on increasing the temperature, compensate for the decrease in the density. The overall result of these changes is a higher extraction yield.

It can be seen from Fig. 3 that, on using *Synechococcus* sp., at 40 and 60°C the higher value in carotenoids is obtained when the pressure is 400 bar. At 50°C, however, the maximun carotenoid extraction yield is obtained at a pressure of 300 bar. The same trend is observed when the solvent does not include cosolvent, as described in a previous publication [19]. This behaviour can be attributed to a double effect; as the pressure increases the solvent power of the system (CO_2 + cosolvent) increases and its diffusivity

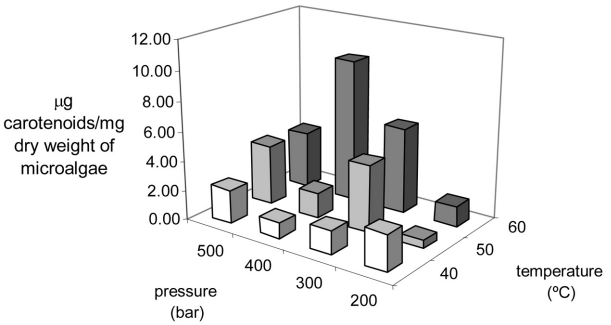

Figure 4. Yield of carotenoid extraction with supercritical carbon dioxide + 5% molar of ethanol from *D. salina*, for an extraction time of 180 min.

decreases. The prevalence of one or other effect explains the trend observed in the experimental values.

With regard to effect of temperature, it can be seen from Fig. 3 that at pressures of 200 and 400 bar the yields increase up to 60°C. At 300 and 500 bar the maximum yield is obtained at 50°C and this decreases on increasing the temperature further. This trend is due to the fact that the increase in the diffusivity of the solvent system with temperature is balanced by the decrease in its solvent power.

Finally, the experimental yields for the extraction of carotenoids from *D. salina* are shown in Fig. 4. It can be seen that at 40°C an increase in pressure from 200 to 400 bar leads to a progressive decrease in the carotenoid extraction yields, which increase again when the pressure is 500 bar. At 60°C the opposite trend is seen; from 200 to 400 bar the extraction yield increases gradually until the maximum value is obtained. On the other hand, at 50°C a considerable reduction in the yield is observed, as shown in Fig. 4.

The effect of temperature on the yields indicates that at 300 and 400 bar an increase in temperature up to 60°C causes an increase in the carotenoid extraction yields. However, when the operating pressure is 500 bar an increase in temperature to 50°C produces a slight decrease in the extraction yield.

At 200 bar the yield decreases on changing the temperature from 40 to 50°C and then increases at 60°C.

3.2 Effect of pressure and temperature on the chlorophyll extraction yield

It is worth pointing out that *D. salina* is a microalga that has chlorophyll *a* and *b* and, therefore, the extraction yields presented in this work represent the sum of both of these. *N. gaditana* and *Synechococcus* sp. contain only chlorophyll *a*.

The yields obtained in the extraction of chlorophyll from the three microalgae studied are represented in Figs. 5 – 7 for different pressures and operation tempera-

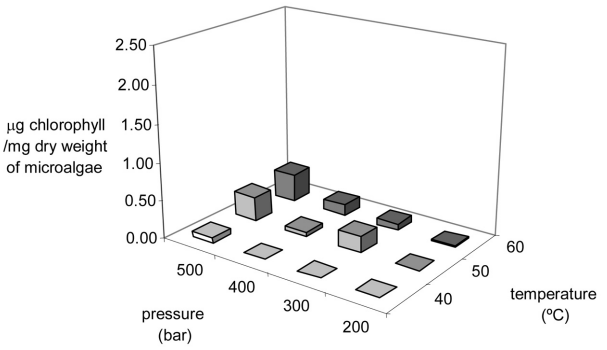

Figure 5. Yield of chlorophyll extraction with supercritical carbon dioxide + 5% molar of ethanol from *N. gaditana*, for an extraction time of 180 min.

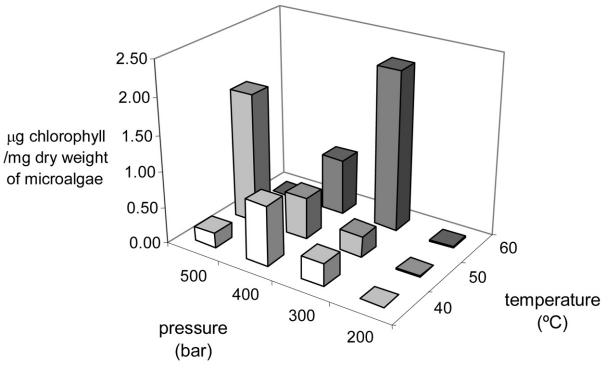

Figure 6. Yield of chlorophyll extraction with supercritical carbon dioxide + 5% molar of ethanol from *Synechococcus* sp., for an extraction time of 180 min.

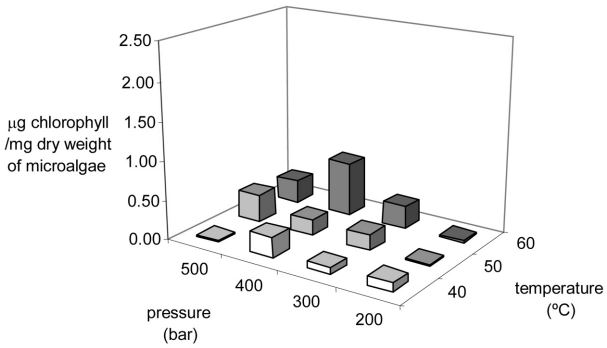

Figure 7. Yield of chlorophyll extraction with supercritical carbon dioxide + 5% molar of ethanol from *D. salina*, for an extraction time of 180 min.

tures. All of the results correspond to an extraction time of 180 min.

The yields obtained in the extraction of chlorophyll from *N. gaditana* are shown in Fig. 5. It can be seen that at a pressure of 200 bar the yield was negligible. However, at pressures below this value there were considerable chlorophyll extraction yields, particularly at temperatures of 50–60°C. At 40°C the yields are considerable when the operating pressure is 500 bar.

The maximum extraction yield for all of the operating temperatures studied was achieved at a pressure of 500 bar.

The same trend is observed when the extraction process was carried out in the absence of cosolvent, as found in a previous study [18], although the yields obtained are considerably lower in this case.

With regard to the effect of temperature, it can be seen that at 400 and 500 bar the extraction yield increases as the temperature is increased. In this case the highest yield is obtained at 60°C, which is due to the fact that the increase in temperature favours the solvent diffusivity. However, at 300 bar the maximum yield was obtained at 50°C.

The yields obtained in the extraction of chlorophyll from the microalga *Synechococcus* sp. are represented in Fig. 6. It can be seen that at 40 and 50°C the maximun extraction yields are obtained at 400 and 500 bar, respectively. However, at 60°C the highest value is obtained when the operating pressure is 300 bar; above this value the yields diminish considerably.

As far as the effect of temperature is concerned, negligible yields were obtained at 200 bar for all operating temperatures studied. At 300 bar, the chlorophyll extraction yield reached its maximum value when the temperature was 60°C. However, at a pressure of 500 bar the extraction yield was higher at 50°C.

This trend can be attributed to the variations in diffusivity and density of the solvent system with pressure and temperature. The overall result depends on which effect prevails in each case.

Finally, the yields obtained in the extraction of chlorophyll from *D. salina* are represented in Fig. 7.

When the effect of pressure is analysed, it is observed that an increase in pressure up to 500 bar favours the extraction yield of chlorophyll when the operating temperature is 50°C. On the other hand, at 60°C the maximum yield is obtained at 400 bar whereas at 40°C the yield only increases when the pressure is between 300 and 400 bar.

In terms of the effect of temperature, at 200 bar an increase in the temperature from 40 to 50°C leads to a reduction in the chlorophyll extraction yield. At 300 bar, the yields increase as the temperature increases up to 60°C. At 400 bar the maximum yield is also obtained at 60°C. However, at 500 bar the highest extraction yield is obtained at 50°C and a slight decrease is observed as the temperature is increased to 60°C.

3.3 Empirical correlations

Starting from the experimental data and with the help of the program STATGRAPHICS Plus 5.1 (1994–2001, Statistical Graphics), empirical correlations were developed

Table 3. Empirical correlations for the extraction process of carotenoids and chlorophyll from microalgae with supercritical carbon dioxide + 5% ethanol

Microalgae		Empirical correlations
N. gaditana	Carotenoids	$R = -6.454 + 9.969\,e^{-3} \cdot P + 0.199\,T - 2.442\,e^{-5}\,P^2 + 2.339\,e^{-4} \cdot PT - 2.336\,e^{-3} \cdot T^2$ Correlation coefficient: 0.97
	Chlorophyll	$R = 4.547 + 2.322\,e^{-2} \cdot P - 0.307\,T - 2.326\,e^{-5}\,P^2 - 1.041\,e^{-4} \cdot PT + 3.124\,e^{-3} \cdot T^2$ Correlation coefficient: 0.53
Synechococcus sp.	Carotenoids	$R = -9.732 + 1.192\,e^{-2} \cdot P + 0.341\,T - 1.806\,e^{-5} \cdot P^2 + 1.835\,e^{-5} \cdot PT - 3.4045\,e^{-3} \cdot T$ Correlation coefficient: 0.62
	Chlorophyll	$R = -8.979 + 2.556\,e^{-2} \cdot P + 0.186\,T - 2.463\,e^{-5} \cdot P^2 - 1.308\,e^{-4} \cdot PT - 1.195\,e^{-3} \cdot T^2$ Correlation coefficient: 0.55
D. salina	Carotenoids	$R = 13.858 + 3.161\,e^{-2} \cdot P - 0.835\,T - 8.093\,e^{-5} \cdot P^2 + 6.222\,e^{-4} \cdot PT + 7.833\,e^{-3} \cdot T^2$ Correlation coefficient: 0.75
	Chlorophyll	$R = 0.446 + 2.866\,e^{-3} \cdot P - 4.176\,e^{-2}T - 7.583\,e^{-6} \cdot P^2 + 6.305\,e^{-5} \cdot PT + 3.013\,e^{-4} \cdot T^2$ Correlation coefficient: 0.82

that predict the yields obtained in the extraction of carotenoids and chlorophyll from the microalgae used in this study. A multilevel factorial design study was carried out to determine the effect of temperature and pressure (experimental variables) on the extraction yield of carotenoids and chlorophylls (dependent variables) when supercritical carbon dioxide + 5% ethanol is used as a solvent system. On the basis of this design, a total of 15 experiments were carried out in a single block and in a random way in order to minimise errors. The pressure and temperature conditions are specified in Section 2.3.

3.3.1 Empirical correlations for the carotenoid extraction process

Empirical correlations, as shown in Table 3, were obtained using the experimental data and the program STATGRAPHICS. These correlations relate the variables that influence the extraction process of carotenoids with supercritical carbon dioxide + ethanol.

R is the yield of extracted carotenoids (μg/mg dry weight of microalga), T the temperature (°C) and P is the pressure (bar).

The results from the analysis of the experimental design are given in Table 4. The estimated effects and interactions between the range of variables studied and the analysis of variance of the extraction process are given. The sign associated with each of the effects indicates a positive or negative influence on the yield caused by the dependent variable. The degree of significance of each factor is represented in the table by its p-value; when a factor has a p-value smaller than 0.05 it influences the process in a significant way for a confidence level of 0.95.

The results obtained from the microalga *N. gaditana* show that temperature, pressure and the interaction of both variables significantly influence the process ($p < 0.05$). All of these factors have a positive influence on the yield of the carotenoid extraction.

Table 4. Estimated effects and the analysis of variance of the process for the carotenoid and chlorophyll extraction with supercritical carbon dioxide + cosolvent

	Microalgae	Variable	Effects	p-value
N. gaditana	Carotenoids	Temperature (T)	0.944	0.002
		Pressure (P)	1.369	0.000
		TP	0.702	0.022
	Chlorophylls	Temperature (T)	−0.628	0.400
		Pressure (P)	0.520	0.519
		TP	−0.312	0.749
Synechococcus sp.	Carotenoids	Temperature (T)	0.134	0.683
		Pressure (P)	0.058	0.872
		TP	0.055	0.900
	Chlorophylls	Temperature (T)	0.415	0.506
		Pressure (P)	0.534	0.438
		TP	−0.392	0.636
D. salina	Carotenoids	Temperature (T)	3.321	0.086
		Pressure (P)	1.823	0.344
		TP	1.867	0.423
	Chlorophylls	Temperature (T)	0.209	0.092
		Pressure (P)	0.213	0.111
		TP	0.189	0.225

With regard to *Synechococcus* sp. and *D. salina*, it is observed that the variables studied do not significantly influence the process because the p-value is greater than 0.05.

Empirical correlations from Table 3 are represented graphically in Fig. 8 for the different operating conditions. A detailed analysis of the graph for *N. gaditana* (Fig. 8a) shows that the maximum carotenoid extraction yield is obtained between 400 and 500 bar and at 60°C. This behaviour leads to the same conclusions as deduced previously.

Analysis of the graph for *D. salina* (Fig. 8b) leads to the same conclusions as deduced previously; the highest yield is obtained between 400 and 500 bar and at a temperature of 60°C.

The graph for *Synechococcus* sp. (Fig. 8c) shows that the maximum yield is obtained between 300 and 400 bar and, once again, this leads to the same conclusions as

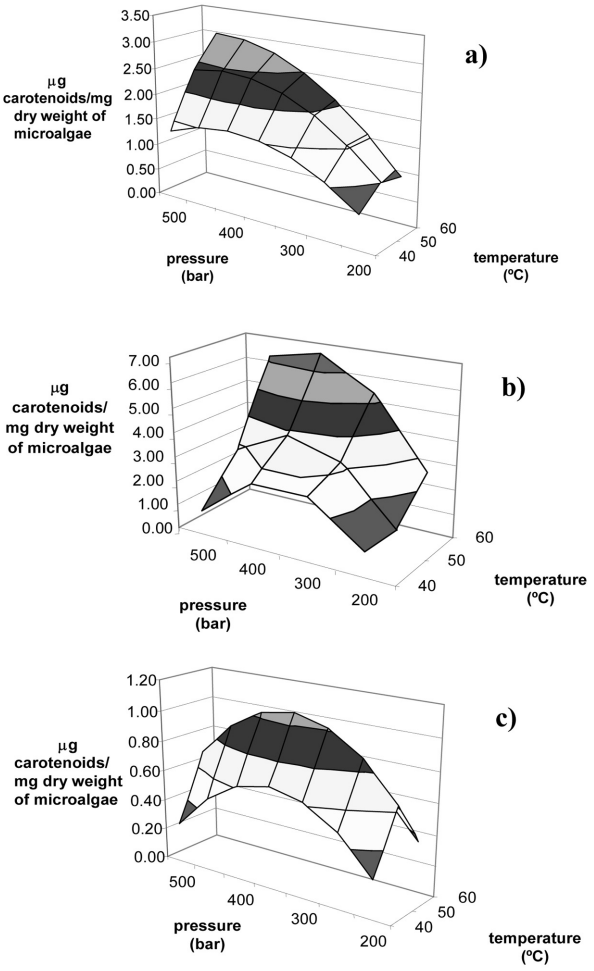

Figure 8. Estimated yields of carotenoid extraction with supercritical carbon dioxide + 5% ethanol using the empirical correlation. (a) *N. gaditana*, (b) *D. salina*, (c) *Synechococcus* sp.

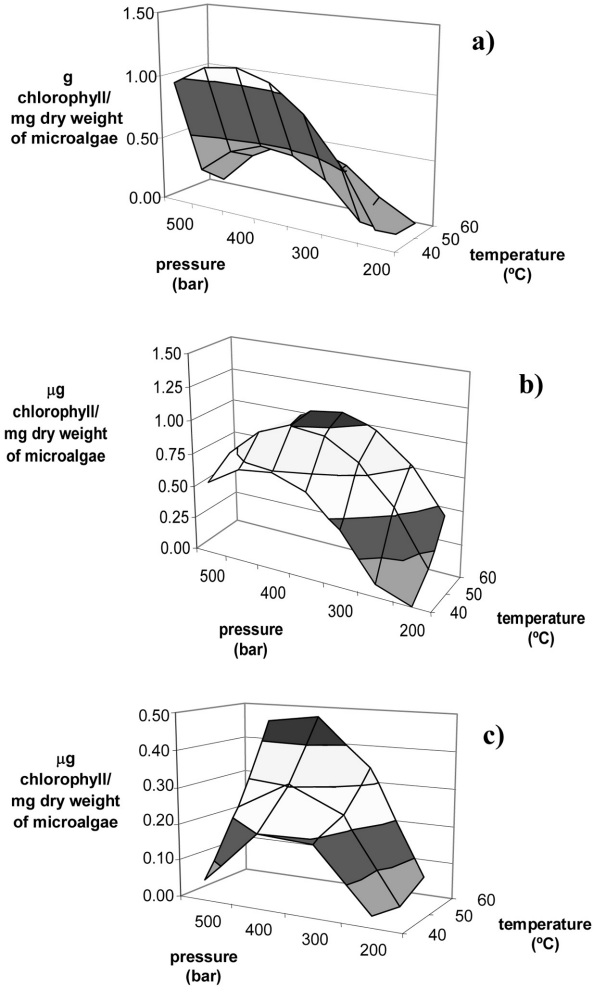

Figure 9. Estimated yields of chlorophyll extraction with supercritical carbon dioxide + 5% ethanol using the empirical correlation. (a) *N. gaditana*, (b) *Synechococcus* sp., (c) *D. salina*.

deduced experimentally. In relation to temperature, the graph indicates that the highest yield is obtained at a temperature of 51°C. Experimentally a temperature of 50°C was employed.

3.3.2 Empirical correlations for the chlorophyll extraction process

Empirical correlations were obtained using the experimental data and the program Statgraphics. These correlations relate the variables that influence the extraction process of chlorophylls with supercritical carbon dioxide + ethanol. The results of the experimental design analysis are gathered in Table 3.

R is the yield of extracted chlorophyll (μg/mg dry weight of microalga), *T* the temperature (°C) and *P* is the pressure (bar).

The results from the analysis of the experimental design are given in Table 4. The estimated effects and interactions between the range of variables studied and the analysis of variance of the extraction process are given.

The results obtained from the three microalgae show that the variables studied do not significantly influence the process because the *p*-value is greater than 0.05.

Empirical correlations for chlorophyll extraction are represented graphically in Fig. 9 for the different operating conditions. A detailed analysis of the graph for *N. gaditana* (Fig. 9a) shows that the maximum chlorophyll extraction yield is obtained between 400 and 500 bar of pressure. This behaviour leads to the same conclusions as deduced previously. With regard to temperature, the graph indicates that the highest yield is obtained at a temperature of 40°C. Experimentally a temperature of 60°C was employed.

The graph for *Synechococcus* sp. (Fig. 9b) shows that the maximum yield is obtained between 300 and 400 bar

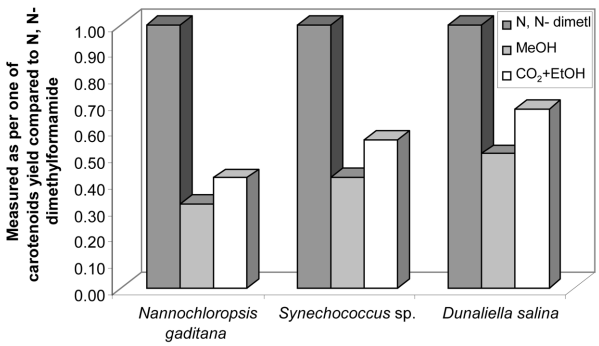

Figure 10. Measured as per one of carotenoid yield compared to extraction with DMF. *N,N*-dimetl: DMF; MeOH: methanol; CO_2 + EtOH: carbon dioxide + ethanol.

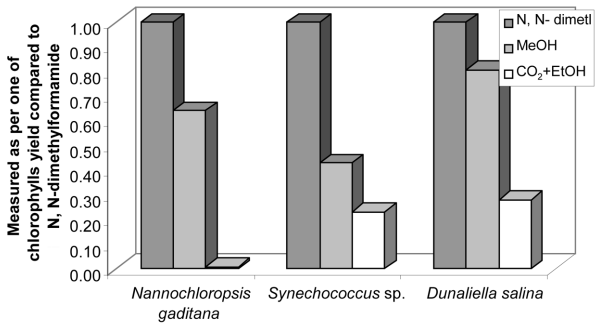

Figure 11. Measured as per one of chlorophyll yield compared to extraction with DMF. *N,N*-dimetl: DMF; MeOH: methanol; CO_2 + EtOH: carbon dioxide + ethanol.

and this leads to the same conclusions as deduced experimentally. In relation to temperature, the graph indicates that the highest yield is obtained at a temperature of 58°C. Experimentally a temperature of 60°C was employed.

Analysis of the graph for *D. salina* (Fig. 9c) leads to the same conclusions as deduced previously; the highest yield is obtained between 400 and 500 bar and at a temperature of 60°C.

3.4 Comparative analysis between supercritical fluid extraction and conventional extraction

The data for carotenoids and chlorophyll yields from the three microalgae on using CO_2 + 5% ethanol as the solvent system can be compared to extraction yields using DMF as the solvent (Figs. 10 and 11).

In relation to *N. gaditana*, the maximum extraction yields of carotenoids and chlorophyll represented in Figs. 10 and 11 were obtained at 500 bar and 60°C.

The methanol extraction from this microalga allows the extraction of larger amounts of chlorophyll than carotenoids. This is not unexpected as methanol is a good solvent for the extraction of polar compounds such as chlorophyll.

The carotenoid extraction yields with CO_2 + 5% ethanol are higher than the yields of chlorophyll, as can be seen in Figs. 10 and 11. Firstly, one can envisage that the use of a cosolvent such as ethanol could favour chlorophyll extraction over carotenoids due to its polarity. However, as the results demonstrate, this is not the case. This behaviour can be attributed to the particular cellular structure of *N. gaditana*, which has a very thick cell wall. The use of cosolvent can produce modifications in wall structure and favour carotenoid extraction.

The caroteonid extraction yields obtained on using cosolvent are higher than those with methanol as the solvent system. With regard to the selectivity of the process, the Car/Chlor ratio is 7.84 in the case of an extraction with cosolvent while this ratio is 0.08 for methanol extraction (see Table 2). This suggests that the supercritical extraction process with cosolvent is more selective than the methanol one. This finding should facilitate the separation of the two extracted pigments.

In relation to *Synechococcus* sp., the maximum extraction yield of carotenoids represented in Fig. 10 was obtained at 300 bar and 50°C. However, the chlorophyll extraction yield represented in Fig. 11 was obtained at 300 bar and 60°C.

It can be seen from Fig. 10 that supercritical fluid extraction of carotenoids using ethanol as a cosolvent gives rise to better yields than extraction with methanol alone.

The supercritical extraction of chlorophyll does not follow the same trend as the carotenoid extraction. In this case, methanol extraction leads to higher yields of this pigment.

The solvent system CO_2 + 5% ethanol is able to penetrate more easily across the cellular wall of *Synechococccus* sp. than methanol alone. This cellular wall is thinner than that of *N. gaditana*. In addition these pigments are found near to the cellular membrane because this microalga does not have chloroplasts. In this way, the cellular structure contributes positively to the extraction process.

With regard to the selectivity of the process, the extraction with supercritical carbon dioxide + ethanol at 300 bar and 50°C is a more selective process than the extraction with methanol. The Car/Chlor ratios (see Table 2) are 6.50 and 0.34, respectively, meaning that the separation of these pigments is possible.

Finally, the maximum extraction yield of carotenoids and chlorophylls from *D. salina* (Figs. 10 and 11) was obtained at 400 bar and 60°C.

It can be seen in Fig. 10 that the presence of cosolvent leads to an increase in the carotenoid extraction yield in comparison to the methanol extraction. On the other hand, chlorophyll extraction with supercritical carbon dioxide + ethanol (see Fig. 11) is lower.

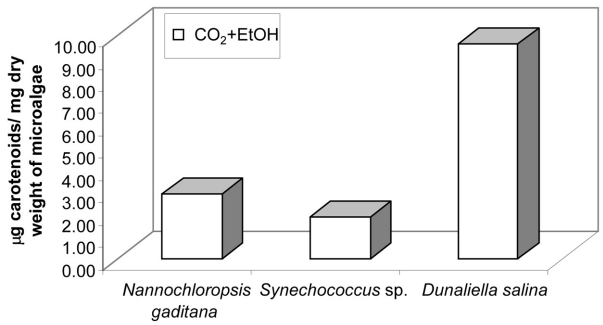

Figure 12. The highest yields of carotenoids using supercritical carbon dioxide + 5% ethanol.

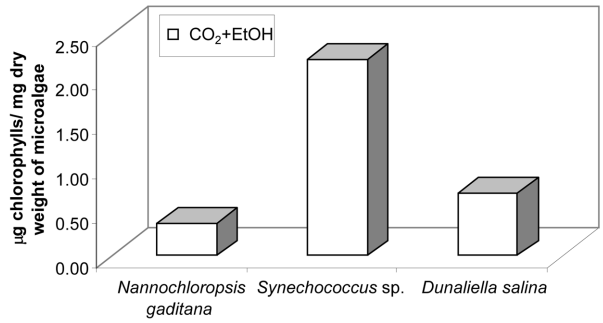

Figure 13. The highest yields of chlorophyll using supercritical carbon dioxide + 5% ethanol.

The Car/Chlor ratio is 5.64 for the methanol extraction whereas for the supercritical extraction it is 13.75. This result indicates that the supercritical extraction is more selective than the methanol extraction and the separation of these pigments is therefore possible.

3.5 Comparative analysis of the extraction processes from the microalgae studied using supercritical carbon dioxide + ethanol

The highest extraction yields of carotenoids and chlorophyll from the three microalgae are represented in Figs. 12 and 13.

It can be seen in Fig. 12 that the maximum levels of carotenoids are extracted from *D. salina* when the solvent system is CO_2 + 5% ethanol. The maximum total content of this pigment is present in *D. salina*, as compared to the other two algae, and this could be the reason why the extraction yields are higher.

It is necessary to point out that the extraction process using *Synechococcus* sp. as the raw material is more efficient than that using *N. gaditana* because it extracts more amount of carotenoids present in the microalga, as shown in Fig. 10.

Finally, as far as the chlorophyll extraction is concerned, it can be seen from Fig. 13 that the largest amount of this pigment was obtained from *Synechococcus* sp.

4 Conclusions

The highest extraction yields of carotenoids and chlorophyll with CO_2 + 5% ethanol from *N. gaditana* are obtained at 500 bar and 60°C. In the case of the microalga *D. salina*, the maximum yields for both pigments are obtained at 400 bar and 60°C, the same operating temperature as for *N. gaditana*.

With regard to *Synechococcus* sp., the best carotenoid extraction yields are obtained at 300 bar and 50°C. In relation to chlorophyll extraction yields, the highest values are obtained at 60°C and at the same pressure as for carotenoids.

Comparison of the results obtained for the three microalgae shows that the largest amount of carotenoids is extracted from *D. salina*. This is due to the higher total content of carotenoids present in this microalga in comparison to the other two.

In relation to the chlorophyll extraction, the highest extraction yield is obtained from *Synechococcus* sp.

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