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Supercritical fluid extraction of carotenoids and chlorophyll *a* from *Nannochloropsis gaditana*

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Abstract

Traditional methods for the extraction of carotenoids and chlorophylls from microalgae frequently require more than one extraction step with organic solvents, which are forbidden in the processing of food additives. In addition, further process steps are necessary for the separation of carotenoids from chlorophylls. Consequently, faster processing methods that are compatible with food production are extremely important.

The aim of this study was to ascertain the influence of pressure and temperature on the supercritical fluid extraction of carotene and chlorophyll from a freeze-dried powder of the marine microalgae *Nannochloropsis gaditana*. The operating conditions were as follows: pressures of 100, 200, 300, 400 and 500 bar and temperatures of 40, 50 and 60 °C. The extracts were analysed by measuring the absorbance at 665 and 480 nm. Empirical correlations were also developed.

The results demonstrate that it is necessary to work at a pressure of 400 bar and a temperature of 60 °C to obtain a significant yield in the extraction of the pigments. The best Carot/Chlor ratio was obtained at 200 bar and 60 °C. It was also found that excellent selectivity can be obtained under these operating conditions and this could enable the separation and purification of these kinds of extracted pigments.

Keywords: Supercritical fluid extraction; Carotenoids; Chlorophyll *a*; *Nannochloropsis gaditana*; Microalgae

1. Introduction

Microalgae are the subject of numerous research projects at an international level and this interest is due to their potential as a source of pigments (Ip, Wong, & Chen, 2003; Lorenz & Cysewski, 2000). A range of different pigments are present in microalgae but two in particular should be highlighted; the chlorophylls and the carotenoids.

The main interest in the use of carotenoids is based on the advantage that they are not affected—unlike many other dyes—by the presence of ascorbic acid or heating and freezing cycles. Furthermore, carotenoids are extremely strong dyes and already give the desired properties in food even at levels of parts per million. Carotenoids are increasingly used in food technology, mainly due to consumer pressure and more demanding regulations regarding the use of artificial dyes (Gordon & Bouernfeind, 1982).

With regard to the interest in chlorophylls in food technology, studies are mainly aimed at avoiding the degradation of the material during processing and storage so that it is present in a natural way in the food (Schwartz & Lorenzo, 1990). In addition, the use of chlorophylls is authorized in the dyeing of foodstuffs such as cold drinks and ice creams amongst others (Directive 94/36/CE, 1994; Madrid & Madrid, 1990).

Nannochloropsis gaditana is a microalga that belongs to the group of brown algae in the class Eustigmatophycea. This alga is used in aquaculture for the cultivation of fish, either directly or via rotifers, as it is a microalga with very stable behaviour during the

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cultivation process. The alga has adapted to the climatological conditions of the Bay of Cádiz and possesses a good nutritional profile (Lubián & Cañabate, 1987, 2001).

Studies on the morphology, ultrastructure and growth physiology of this system have been described in the literature (Lubián, 1982).

Nannochloropsis gaditana stands out as an important source of pigments of great commercial value. The major pigments present are chlorophyll *a*, beta-carotene, violaxanthin and vaucheriaxanthin.

Conventional methods based on the solvent extraction of these substances from natural matrices are time-consuming since they require multiple extraction steps and need large amounts of organic solvents, which are often expensive and potentially harmful.

Extraction with carbon dioxide under supercritical conditions constitutes an emerging technology in terms of environmental impact. The advantages in using carbon dioxide include its lack of toxicity, chemical inertness, low cost and ready availability (Hawthorne, 1990). Furthermore, the use of carbon dioxide is also beneficial in adding quality to the products obtained since this technique does not give rise to excessive heating, which usually has a negative effect on the thermolabile compounds.

Investigations carried out previously have demonstrated the feasibility of extracting pigments with supercritical carbon dioxide and examples include carotenoids, starting from carrots (Bath, Zhou, Kute, & Rosenthal, 1995), cabbages (Albino, Penteado, Lanças, & Vilegas, 1999) and microalgae (Mendes et al., 1995). These processes allow good extraction yields to be obtained. On the other hand, studies concerning the extraction of chlorophylls are scarce.

In the work described here, starting from experimental data, a factorial multilevel experimental design was carried out in order to analyse the effect of temperature and the operating pressure on the extraction yield of carotenoids and chlorophyll *a* when supercritical carbon dioxide is used as the solvent. Subsequently, the program STATGRAPHICS Plus 4.0 (1994–1999, Statistical Graphics Corp.) was used to develop empirical equations that are able to predict the yields obtained in the extraction processes for carotenoids and chlorophyll *a*. Finally, the yield of the supercritical carbon dioxide extraction process was compared with the conventional method using methanol as a liquid solvent.

2. Experimental

2.1. Raw material

The raw material employed in the experiments was the microalga *Nannochloropsis gaditana* and this was obtained from the Marine Microalgae Culture Collection of Instituto de Ciencias Marinas de Andalucía (CSIC, Spain). The biomass was freeze-dried after being cultivated in sea water enriched with $f/2$ medium (Guillard & Ryther, 1962) at 20 °C and aerated with atmospheric air. The illumination conditions were 75 $\mu mol\, m^{-2}\, s^{-1}$ from daylight fluorescence lamps. Once the sample had been obtained it was stored under vacuum in darkness until the extraction procedure was carried out.

2.2. Chemicals

Extractions were carried out with high purity carbon dioxide (99.995%) purchased from Carburos Metálicos (Spain).

Methanol (HPLC grade) from Panreac was used as the extraction solvent. The solvent was flushed with a stream of nitrogen from Air Liquid (France). Ethanol (instrumental analysis grade) was used as the collection solvent for the extracts.

2.3. Supercritical fluid extraction

The experimental development was carried out in a micro-scale supercrtical extraction apparatus obtained from Isco (Nebraska) (model SFX 220). The equipment consisted of an extractor, an SFX 200 controller, a restrictor and a syringe pump (Fig. 1).

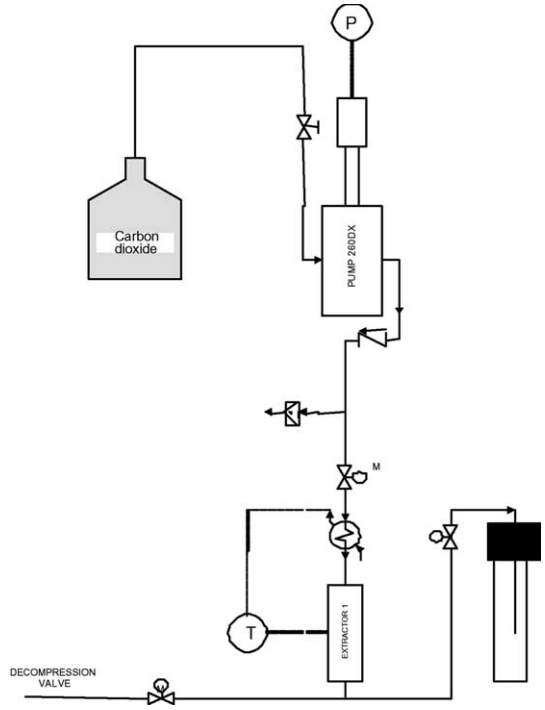

Fig. 1. Diagram showing the flow of the extraction process with supercritical carbon dioxide.

The extractor had an extraction area, a thermostatic system and a system of valves and connections. The extraction system consisted of two extractors with a maximum capacity of 10 ml each and a cartridge containing the sample was inserted into each extractor. Each extraction cartridge consisted of a stainless steel cylinder with a volume of 0.5 ml, a threaded closing mechanism and a 2 μm filter at both the inlet and outlet to avoid loss of sample.

The extracted sample emerged through a restrictor connected to extractor 1. This device consisted of a connector that enabled the extract to be bubbled through a solvent. Decompression of the carbon dioxide at the exit of the system often caused obstruction of the restrictor due to solidification of the CO_2. This problem was overcome by fitting a coaxial heater around the restrictor using a resistance connected to a temperature control system that enabled the exit of the restrictor to reach 150 °C. The flow was regulated with a manual micrometric valve that was also fitting with a thermostatically controlled heater.

The operating methodology involved loading the extraction cartridge with approximately 0.2 g of the microalga sample, which had previously been homogenized 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 pump was loaded with carbon dioxide until the operating pressure was reached in the pump. The automatic decompression valves of the extractor were closed, the valve connecting of the pump opened up and the extractor opened. The extractor was then pressurized with CO_2. A period of 15 min of static extraction was allowed to elapse.

When the system had attained a balanced state, the micrometric valve was opened up from the thermostatically controlled restrictor (at 60 °C) until a constant flow of 4.5 mmol/min was achieved. An extraction for a time of 3 h was then carried out. It was used an extraction time of 3 h because, practically, most of the carotenoids and chlorophyll *a*, extracted with supercritical carbon dioxide, were obtained during this period.

The extracts were collected at different time intervals in glass tubes containing ethanol. These tubes were changed at different extraction times. After the extraction process was complete, the solvent was removed with a nitrogen stream 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.

2.4. Experimental design

Starting from the experimental data and with the help of the program STATGRAPHICS Plus 4.0 (1994–1999, Statistical Graphics Corp.), an empirical correlation was developed that predicts the yields obtained in the extraction of carotenoids and chlorophyll *a*. 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 chlorophyll *a* (dependent variables) when supercritical carbon dioxide is used as the solvent. 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 minimize errors.

2.5. Methanol extraction

Several organics solvents, as methanol and acetone, were used in the extraction of pigments from freeze-dried microalgae for this research. These experiments indicated that the use of acetone gives quite low yields. For this reason, methanol was selected as the solvent as the yields obtained are higher.

A sample of the freeze-dried microalga *Nannochloropsis gaditana* (0.2 g) was suspended in methanol (5 ml). The sample was sonicated for 10 min in a Select (Spain) ultrasound bath and then kept at 4 °C for 24 h.

The supernatant liquid was recovered by centrifugation and stored until analysis was carried out. After 14 extraction cycles with methanol, the solvent did not show any coloration—even though the pellet remained greenish in color.

2.6. Analysis methods

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

In the case of carotenoids, the equation proposed by Strickland and Parsons (1968) was used:

$$C_{\text{carot}}(\mu\text{g/ml}) = 4A_{480} \quad (1)$$

where A_{480} is the absorbance at 480 nm.

For analysis of chlorophyll the expression proposed by Talling and Driver (1963) was used:

$$C_{\text{chlor}}(\mu\text{g/ml}) = 13.9A_{665} \quad (2)$$

where A_{665} is the absorbance at 665 nm.

3. Experimental results

The yields of the carotenoid and chlorophyll extractions are shown in Table 1 and are expressed as μg of pigment per mg dry weight of microalga. The carotenoid/chlorophyll ratios are also shown. These values were obtained for an extraction time of 180 min for the

Table 1
Carotenoid and chlorophyll yields obtained for an extraction time of 180 min

Temperature (°C)	Pressure (bar)	Yield of carotenoids (μg carotenoids/mg dry weight microalga)	Yield of chorophyll (μg chlorophyll *a*/mg dry weight microalga)	Relation Carot/Chlor
40	100	0.00	0.00	–
50	100	0.00	0.00	–
60	100	0.00	0.00	–
40	200	0.152	0.290	0.524
50	200	0.152	0.371	0.410
60	200	0.125	0.090	1.389
40	300	0.208	0.807	0.258
50	300	0.248	1.076	0.230
60	300	0.250	1.399	0.179
40	400	0.125	1.088	0.115
50	400	–	1.000	–
60	400	0.343	2.238	0.153
40	500	0.104	0.809	0.129
50	500	0.180	1.390	0.129
60	500	0.252	2.097	0.120
Methanol extraction		*0.8*	*18.5*	*0.043*

(Carotenoids/Chlorophylls): Carot/Chlor.

different extraction conditions studied. The yields of the methanol extractions are indicated at the end of Table 1.

4. Discussion of the results

4.1. Carotenoid extraction

The experimental yields for the extraction of carotenoids are shown in Fig. 2. These yields were obtained at different pressures and temperatures for an extraction time of 180 min.

The results from the analysis of the experimental design are given in Table 2. 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 of 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.

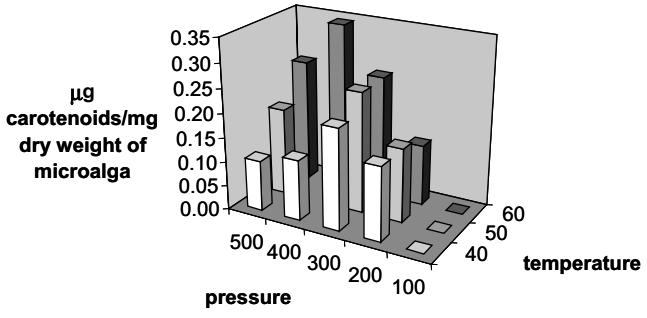

Fig. 2. Yield of carotenoid extraction for an extraction time of 180 min.

Table 2
Estimated effects and the analysis of variance of the process for the carotenoid and chlorophyll extraction with supercritical carbon dioxide

Variable	Carotenoids		Chlorophyll *a*	
	Effects	*p*-value	Effects	*p*-value
Temperature (*T*)	0.076	0.004	0.566	0.012
Pressure (*P*)	0.181	0.000	1.622	0.000
TP	0.108	0.004	0.785	0.013
TT	−0.018	0.619	0.229	0.481
PP	−0.282	0.000	−0.581	0.132

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

Effect of pressure. It can be observed from Fig. 2 that, for a given temperature, there is a maximum in the extraction yield at intermediate pressures. Any further increase in pressure causes an increase in the fluid density and this could have a double effect: an increase in the solvating power of the supercritical fluid and a reduced interaction between the fluid and the matrix as a consequence of a decrease in the diffusion coefficient at higher density (Mantell, 2000).

It should also be borne in mind that the microalga used has a cellular wall that is very thick and leads to great resistance to mass transfer. This phenomenon explains the variation observed in the yields obtained in the carotenoid extraction within the pressure range selected for each of the temperatures studied.

Effect of temperature. It can be seen that, for each pressure, an increase in the temperature generally

produces an increase in the yield of the carotenoid extraction. This yield depends on a complex balance between the decrease caused in the density of the supercritical carbon dioxide, which would be expected to reduce the yield, and the increase in the vapour pressure of these pigments as the temperature increases, which should make the solubility of the pigments in the solvent more favourable. At a pressure of 200 bar, the effect that prevails on increasing the temperature is the decrease in the density of supercritical carbon dioxide and, therefore, a reduction in its solvating power results.

On the other hand, at pressures greater than 200 bar the increase in the vapour pressure of carotenoids that occurs on increasing the temperature is compensated by the decrease in the density of supercritical carbon dioxide. This situation favours the extraction of the carotenoids (Baysal, Ersus, & Starmans, 2000; Careri et al., 2001).

4.2. Chlorophyll a extraction

The yields obtained in the extraction of chlorophyll from the microalga *Nannochloropsis gaditana* are represented in Fig. 3 for different pressures and operation temperatures. All the results correspond to an extraction time of 180 min.

The results of the experimental design analysis are gathered in Table 2. An estimate of the effects and interactions between the range of studied variables and the analysis of variance of the process are presented. The results obtained show that temperature, pressure and crossed interactions significantly influence the process (p-value <0.05) and that the influence is positive.

Effect of pressure. It can be seen (Fig. 3) that the yields obtained in the extraction of chlorophyll *a* follow the same trend as the yields obtained in the carotenoid extraction. The maximum yields obtained at temperatures 40 and 60 °C correspond to 400 bar and that at a temperature of 50 °C is 500 bar. The explanation for this behaviour is similar to that proposed for the carotenoids, i.e. when the pressure increases for each studied temperature, the solvating power of the carbon dioxide is increased. This increase favours the extraction process but is offset to some degree by a decrease in the diffusion coefficient, which reduces the penetration capacity of the solvent and diminishes the yield at higher pressures.

Effect of temperature. At 200 bar the yield of the chlorophyll *a* extraction process decreases as the temperature is increased. However, at 300, 400 and 500 bar the increase in temperature is translated into an increase in the yield of the process.

The explanation for this trend is again similar to that proposed for the carotenoid extraction. At 200 bar the extraction yield of chlorophyll *a* decreases due to a decrease in the density of the supercritical carbon dioxide and, therefore, of the solvating power effect prevails over the increase in the vapour pressure of the chlorophyll at this pressure. At higher pressures the effect of the increase in the vapour pressure prevails for chlorophyll *a*, thus favouring the solubility of this pigment and increasing its extraction yield.

4.3. Empirical correlations

Empirical correlations were obtained using the experimental data and the program STATGRAPHICS Plus 4.0 (1994–1999, Statistical Graphics Corp.). Eq. (3) is the expression in the case of carotenoids

$$R = -0.233163 + 0.00492577 \cdot T + 0.00121779 \cdot P - 0.0000923077 \cdot T^2 + 0.00002705 \cdot P \cdot T - 0.00000353013 \cdot P^2 \quad (3)$$

where R is the yield of extracted carotenoids expressed in μg carotenoids per mg dry weight of microalga, T is the temperature (°C) and P the pressure (bar). The resulting correlation coefficient is 0.94.

Eq. (3) is represented graphically in Fig. 4 for the different operating conditions. A detailed analysis of the graph leads to the same conclusions as deduced previously; the highest yield is obtained at a pressure of 400 bar and a temperature of 60 °C.

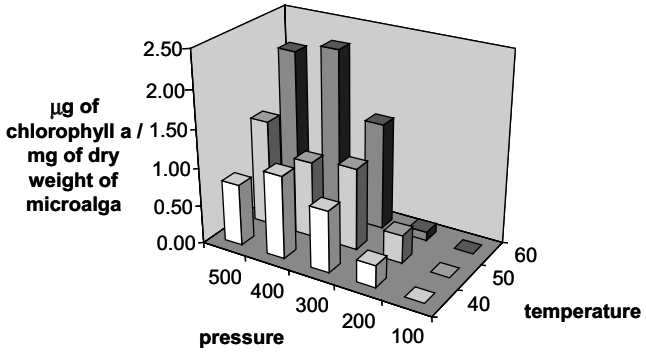

Fig. 3. Yield of chlorophyll *a* extraction for an extraction time of 180 min.

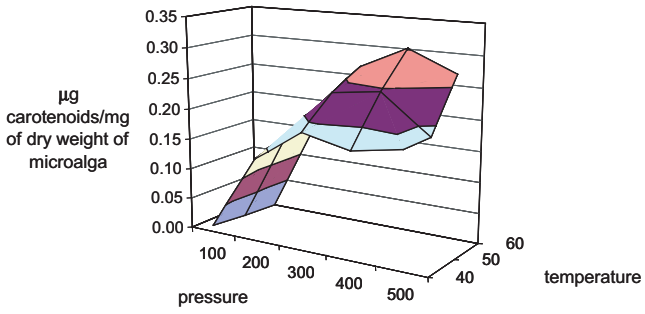

Fig. 4. Estimated yields of carotenoid extraction with supercritical carbon dioxide using the empirical correlation.

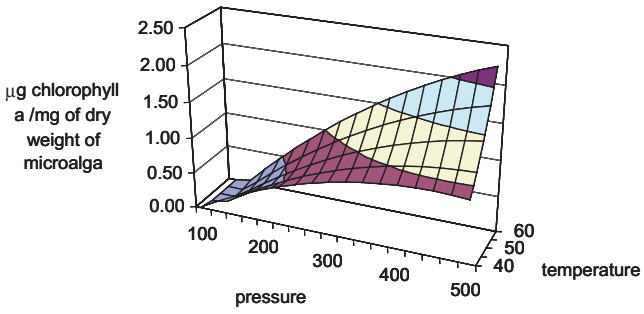

Fig. 5. Estimated yields of chlorophyll *a* extraction with supercritical carbon dioxide using the empirical correlation.

With regard to chlorophyll extraction, the correlation is as follows

$$R = 3.43203 - 0.00140362 \cdot P - 0.14499 \cdot T - 0.00000725952 \cdot P^2 + 0.0001963 \cdot P \cdot T + 0.001144 \cdot T^2 \quad (4)$$

where R is the yield of chlorophyll *a* extraction expressed in μg per mg of dry weight of microalga, T is the temperature (°C) and P the pressure (bar). The resulting correlation coefficient is 0.90.

Eq. (4) is represented graphically in Fig. 5 for the different operating conditions.

A detailed analysis of the graph indicates that the maximum yield predicted from Eq. (4) does not coincide with that obtained experimentally, which was 400 bar and 60 °C.

4.4. Comparative analysis between supercritical fluid extraction and methanol extraction

Analysis of the results in Table 1 shows that the ratio of the yields Carotenoids/Chlorophylls (Carot/Chlor) decreases as the operation pressure increases. The highest ratio is obtained at 200 bar and 60 °C. Under these operating conditions a higher selectivity is obtained and this should facilitate the separation and purification of the two extracted pigments. However, at 500 bar the smallest ratio is obtained for the yields of the two pigments Carot/Chlor and this is not influenced significantly by an increase in the operating temperature.

The Carot/Chlor ratio in the extraction with supercritical carbon dioxide is always higher than that obtained through methanol extraction. This suggests that the supercritical extraction process is more selective than the conventional one.

5. Conclusions

In the pressure range selected in these experiments the extraction of carotenoids and chlorophyll *a* begins at 200 bar. The highest extraction yields are obtained when operating at 400 bar.

The most appropriate operating temperature to obtain the best yields in the extraction of carotenoids and chlorophyll is 60 °C. It is not advisable to increase the temperature beyond this point as thermal degradation of the resulting extracts may occur.

Supercritical carbon dioxide is a suitable solvent for the extraction of carotenoids because of the low polarity of these compounds. Given this property, the carbon dioxide extraction process is selective in the presence of more polar pigments such as chlorophyll *a*.

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