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Supercritical fluid extraction of carotenoids and chlorophyll *a* from *Synechococcus* sp.

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Abstract

Supercritical carbon dioxide extraction of carotenoids and chlorophylls is an alternative to solvent extraction because it provides a high-speed extraction process with a simple purification stage. In the study described here, carotenoid and chlorophyll extraction with supercritical CO_2 was explored using dry biomass from a marine cyanobacterium *Synechococcus* sp. as the raw material. The operation conditions were as follows: pressures of 100, 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 an empirical correlation.

The results demonstrate that the highest extraction yield of total carotenoids is obtained operating at a pressure of 300 bar and a temperature of 50 °C. The most appropriate operating conditions to obtain the best yield in the extraction of chlorophyll *a* are 500 bar and 60 °C.

The highest carotenoids/chlorophylls (Carot/Chlor) 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.

Keywords: Supercritical fluid extraction; Carotenoids; Chlorophyll *a*; *Synechococcus* sp.; Microalgae

1. Introduction

The development of increasingly restrictive legislation concerning the origin of preservatives used in the production of alimentary products, along with the growing demand by consumers for substances from natural sources, has meant that the preparation of products by means of microalgae is a viable alternative to chemical synthesis [1,2]. On the other hand, methods of extraction of pigments involving conventional organic solvents, besides being limited by legislation, require several purification stages [3].

The production of carotenoids from microalgae constitutes a topic of great scientific and commercial importance within the alimentary and aquaculture fields [4]. The main interest in the use of carotenoids lies in the advantage that it is not affected, as other colourings are, by the presence of ascorbic acid, heating and freezing. Furthermore, this compound has a high colouring capacity that is sensitive even at levels of parts for million as a food colouring. Legislation allows the use of this colouring in processed foods such as margarine, cheese, ham and gelatine [5].

As far as chlorophylls in food technology are concerned, studies usually focus on avoiding the degradation of this material during processing and storage so that it is present in a natural form in food [6]. The use of this compound is authorized in food applications such as colouring in the manufacture of cold drinks and ice creams among others [7,8].

The application of the extraction technique with supercritical carbon dioxide to the production of alimentary preservatives has been widely studied in recent years due to the clear advantages of carbon dioxide as a solvent—these advantages include low toxicity, low cost and ease of separation of the extracted product [9–11]. The extraction of carotenoids by this technique represents an alternative to the conventional extraction method as the

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purification stage is simpler and the extraction time reduced [12]. Mendes et al. [13], Careri et al. [14] and Macías-Sánchez et al. [15] have applied the extraction of carotenoids with supercritical fluid to the microalgae *Chlorella vulgaris*, *Spirulina platensis* and *Nannochloropsis gaditana*, respectively, with satisfactory results.

Synechococcus sp. is a microalga that belongs to the cyanophyceae or blue-green algae group. This is a photosynthetic prokaryotic organism with chlorophyll *a* and is closer in character to other photosynthetic bacteria than eukaryotic algae and, as such, is classified as a cyanobacteria.

In the last decade, cyanobacteria *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC 7002 have been highlighted as suitable organisms for genetic modification [16] with the aim of enhancing β-carotene accumulation for biotechnological production. The pigment profile of *Synechococcus* strains is mainly composed of β-carotene and zeaxanthin (a dihydroxylated derivative of β-carotene)—as well as chlorophyll *a*, diverse glycocarotenoids and equinenone (a keto-carotenoid) as accompanying carotenoids to a varying extent depending on the strain [17]. Studies have shown that this prokaryotic organism is considered to be a favourable candidate for carotenoid production on a commercial scale [18].

The work presented here involved taking the experimental data and carrying out a multilevel factorial design in order to analyze the effect that the temperature and the operation pressure have on the extraction yield of carotenoids and chlorophyll from the microalga *Synechococcus* sp. on using supercritical carbon dioxide. The program Statgraphics Plus 5.1 (1994–2001, Statistical Graphics Corp.) was subsequently used to develop an empirical equation to predict the yields obtained in the carotenoid extraction processes. Finally, the relationship between the extraction yields obtained with supercritical carbon dioxide and methanol as a liquid solvent was investigated.

2. Experimentation

2.1. Raw material

The raw material employed in the experiments was the cyanobacteria *Synechococcus* sp. type 05/0201 and this was obtained from the Marine Microalgae Culture Collection at the Instituto de Ciencias Marinas de Andalucia (CSIC, Spain). The biomass was freeze-dried. Cells were cultured in a Braun-Biotech bench-top photobioreactor with a 2 L vessel, using filtered and autoclaved natural seawater enriched with F/2 medium [19]. Experimental conditions were 35 °C, irradiance of 200–400 μmol m^{-2} s^{-1}, 50 rpm agitation speed and aeration with atmospheric air. CO_2 was automatically added to the culture to keep the pH at 8.5. The cultures were grown under these conditions for 3 days. Nitrate and phosphate were added daily. Samples (2 mL) were taken out on a daily basis for pigment extraction. Following the culture period, the cell biomass was harvested by centrifugation, the pellet washed with 0.9% ammonium formate in order to partially desalt the sample and, after further centrifugation, the pellet was freeze-dried.

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).

2.3. Supercritical extraction

Supercritical carbon dioxide extraction was performed using a micro-scale supercritical 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. A schematic representation of the equipment and further details can be found in a previous publication [15].

The operating methodology involved loading the extraction cartridge with approximately 0.1 g of the cyanobacteria 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 the pump was opened and the extractor was opened. The extractor was then pressurized with CO_2. A period of 15 min of static extraction was allowed to elapse.

When a balanced state had been attained, 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 was then carried out for 3 h.

The extracts were collected in glass tubes containing methanol. After the extraction process was complete, the solvent was removed with a nitrogen stream at a temperature of 40 °C. The extracted product dissolved in methanol (5 mL) was stored at 4 °C with the exclusion of light until subsequent analysis.

2.4. Experimental design

The experimental data were used in conjunction with the program Statgraphics Plus 5.1 (1994–2001, Statistical Graphics Corp.) to develop an empirical correlation that predicts the yields obtained in the extraction of carotenoids and chlorophyll *a*. A multilevel factorial design 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

The experiments carried out in a previous study with another microalga [15] indicate that the yields obtained in the extraction with acetone of pigments starting from freeze-dried microalgae

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

Temperature (°C)	Pressure (bar)	Yields		Relation Carot/Chlor
		Microgram carotenoids/milligram dry weight of microalga	Microgram chlorophyll *a*/milligram dry weight of microalga	
Supercritical CO_2				
40	100	0.071	0.001	71
50	100	–	–	–
60	100	0.004	0.000	–
40	200	0.386	0.002	193
50	200	1.225	0.053	23.113
60	200	0.405	0.004	101.25
40	300	0.748	0.023	32.522
50	300	1.511	0.078	19.372
60	300	0.880	0.019	46.316
40	400	0.896	0.037	24.216
50	400	1.179	0.157	7.510
60	400	1.188	0.170	6.988
40	500	1.185	0.256	4.629
50	500	1.022	0.149	6.859
60	500	1.328	0.715	1.857
Methanol extraction		1.353	4.096	0.330

Carotenoids/chlorophyll: Carot/Chlor.

are quite low in relation to those obtained with methanol. For this reason methanol was also selected as a solvent for the cyanobacteria *Synechococcus* sp.

A sample of the freeze-dried cyanobacteria *Synechococcus* sp. (0.1 g) was suspended in methanol (5 mL). The sample was sonicated for 10 min in a Select (Spain) ultrasound bath and then maintained at 4 °C for 24 h.

The supernatant liquid was recovered by centrifugation and stored until analysis was carried out. After six extraction cycles with methanol, the solvent did not show any colouration. Once the aforementioned process had been carried out the cyanobacteria sample hardly presented colour.

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).

The equations proposed by Wellburn [20] were used in the analysis:

$$C_a\,(\mu g/mL) = 16.72 \cdot A_{665.2} - 9.16 \cdot A_{652.4} \quad (1)$$

where C_a is the concentration of chlorophyll *a*, $A_{665.2}$ the absorbance at 665.2 nm and $A_{652.4}$ is the absorbance at 652.4 nm.

$$C_b\,(\mu g/mL) = 34.09 \cdot A_{652.4} - 15.28 \cdot A_{665.2} \quad (2)$$

where C_b is the concentration of chlorophyll *b*.

$$C_{\text{total carotenoids}\,(x+c)} = \frac{1000 \cdot A_{470} - 1.63 \cdot C_a - 104.96 \cdot C_b}{221} \quad (3)$$

where $C_{\text{total carotenoids}\,(x+c)}$ is the concentration of total carotenoids and A_{470} is the absorbance at 470 nm.

Although *Synechococcus* sp. has only chlorophyll *a* and not chlorophyll *b*, it is necessary to determine the parameter C_b as it appears in the other equations and it is not recommended to discount this parameter.

3. Experimental results

The yields of the carotenoid and chlorophyll extractions and the carotenoid/chlorophyll ratios obtained from the freeze-dried *Synechococcus* sp. are shown in Table 1. 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 extractions are indicated at the end of Table 1.

4. Discussion of the results

4.1. Analysis of experimental design

The results from the analysis of the experimental design are shown in Table 2. The estimated effects and interactions between the range of variables studied and the analysis of variance of the extraction process are also 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.

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

Variable	Carotenoids		Chlorophyll *a*	
	Effects	*p*-Value	Effects	*p*-Value
Temperature (*T*)	0.104	0.541	0.118	0.151
Pressure (*P*)	0.979	0.002	0.342	0.007
TT	−0.722	0.050	0.075	0.611
PP	−0.668	0.087	0.296	0.095
TP	0.139	0.564	0.210	0.080

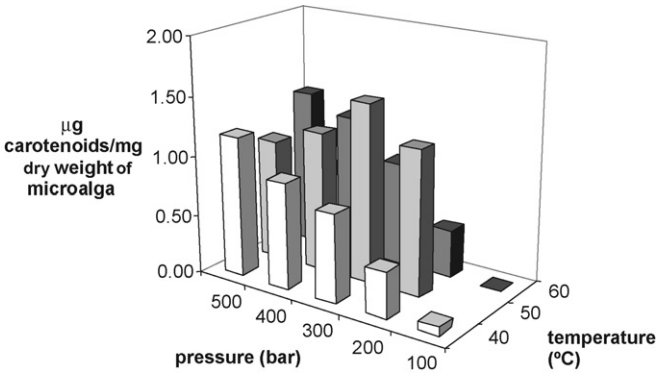

Fig. 1. Yield of carotenoid extraction for an extraction time of 180 min.

The results obtained show that pressure has a significant influence on the process (*p*-value < 0.05). This factor only has a positive influence on the yield of the carotenoid and chlorophyll *a* extraction.

4.2. Extraction yields of carotenoids

The experimental yields of carotenoid extraction, obtained at different pressures and temperatures for an extraction time of 180 min, are shown in Fig. 1.

4.2.1. Effect of pressure

It can be observed from Fig. 2 that at 40 and 60 °C the yield increases with pressure; the maximum value is obtained at 500 bar, while at 50 °C the maximum value is obtained at 300 bar. This behaviour can be attributed to a double effect as the pressure increases—an increase in the density of the supercritical carbon dioxide and a decrease in its diffusion coefficient [21]. On the one hand, an increase in the density of carbon dioxide is associated with an increase in its solvating power and this enhances the extraction process. On the other hand, the decrease in the diffusivity leads to a reduction in the interaction between the supercritical fluid and the solute contained within the matrix and this makes the yield of the extraction process decrease. At 40 and 60 °C the only effect produced is the increase in solvating power of the supercritical carbon dioxide.

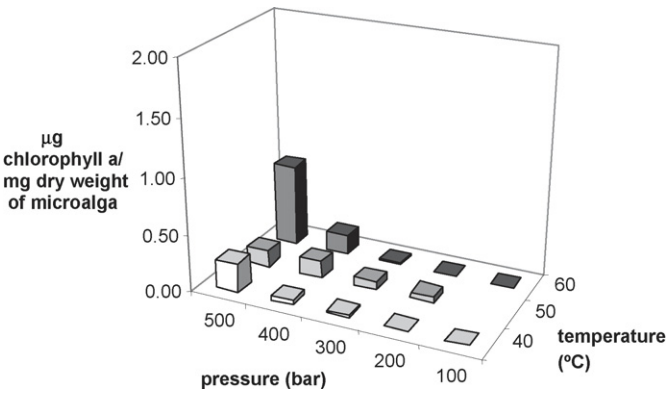

Fig. 2. Yield of chlorophyll *a* extraction for an extraction time of 180 min.

4.2.2. Effect of temperature

The highest yield is obtained at a temperature of 50 °C when the operating pressure is 200 and 300 bar, as can be seen in Fig. 1. On the other hand, at pressures of 400 and 500 bar the maximum extraction yield is obtained when the temperature is 60 °C and a slight decrease is observed at 50 °C at the higher pressure. The yield depends on a complex balance between the decrease in the supercritical carbon dioxide density and the increase in vapour pressure of these pigments as the temperature increases, which essentially represents the solubility of the pigments in the solvent. At 400 bar the solubility of the pigments in the solvent is higher due to the increase in the vapour pressure of carotenoids. At 200 and 300 bar this effect prevails up to a temperature of 50 °C. At 500 bar the decrease in the density of the supercritical carbon dioxide reduces the extraction yield down to a temperature of 50 °C [14,15,22].

4.3. Extraction yields of chlorophyll a

The yields obtained in the extraction of chlorophyll *a* from cyanobacteria *Synechococcus* sp. are represented in Fig. 2 for different operating pressures and temperatures for an extraction period of 180 min.

4.3.1. Effect of pressure

It can be seen from Fig. 2 that the yields obtained in the extraction of chlorophyll *a* show the same trend as the yields obtained in the extraction of carotenoids when the operating temperatures are 40 and 60 °C. At an intermediate operating temperature (50 °C) the maximum extraction yield is obtained at a pressure of 400 bar. The explanation for this behaviour is similar to that proposed for the carotenoids; when the pressure is increased at a given 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 of the extraction process at higher pressures. However, at the same time a decrease in the diffusivity coefficient occurs.

4.3.2. Effect of temperature

It can be seen that the yields obtained in the extraction of chlorophyll *a* show a similar trend to the yields obtained in the extraction of carotenoids. The explanation for these findings is similar to that proposed for the carotenoid extraction; at 400 bar the solubility of the pigments in the solvent is higher

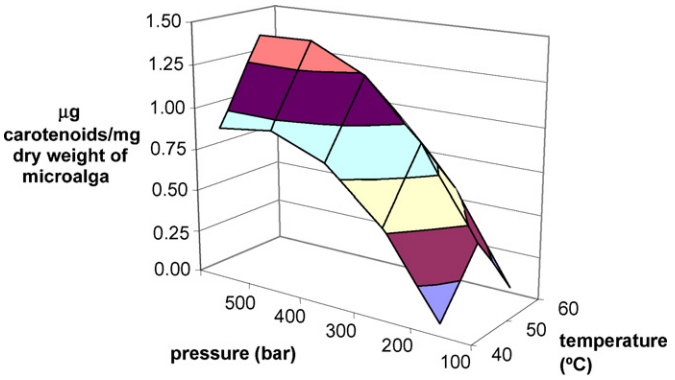

Fig. 3. Estimated yields of carotenoid extraction with supercritical carbon dioxide using the empirical correlation.

due to the increase in the vapour pressure of carotenoids. At 200 and 300 bar this effect prevails until a temperature of 50 °C is employed. At 500 bar the decrease in the density of carbon dioxide reduces the extraction yield down to 50 °C.

4.4. Empirical correlations

Empirical correlations were obtained using the experimental data and the program Statgraphics. These correlations relate the variables that influence the extraction process of carotenoids and chlorophylls with supercritical carbon dioxide.

Eq. (4) is the expression in the case of carotenoids:

$$R = -9.015 + 0.006 \cdot P + 0.356 \cdot T - 0.000 \cdot P^2 + 0.000 \cdot P \cdot T - 0.004 \cdot T^2 \quad (4)$$

where R is the yield of extracted carotenoids [µg/mg dry weight of microalga], T the temperature [°C] and P is the pressure [bar]. The resulting correlation coefficient is 0.90.

Eq. (4) is represented graphically in Fig. 3 for the different operating conditions. A detailed analysis of the graph indicates that the highest yield is obtained at a temperature of 51 °C. Experimentally a temperature of 50 °C was employed. In relation to the pressure, the proposed correlation provides a value of 449 bar. Experimentally the value employed was 300 bar. Other aspects, such as economic considerations, must also be taken into account when choosing an appropriate pressure for a process.

With regard to chlorophyll extraction, the correlation is as follows:

$$R = 1.524 - 0.004 \cdot P - 0.048 \cdot T + 0.000 \cdot P^2 + 0.000 \cdot P \cdot T + 0.000 \cdot T^2 \quad (5)$$

where R is the yield of extracted chlorophyll a [µg/mg dry weight of microalga], T the temperature [°C] and P is the pressure [bar]. The resulting correlation coefficient is 0.87.

Eq. (5) 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 500 bar and a temperature of 60 °C.

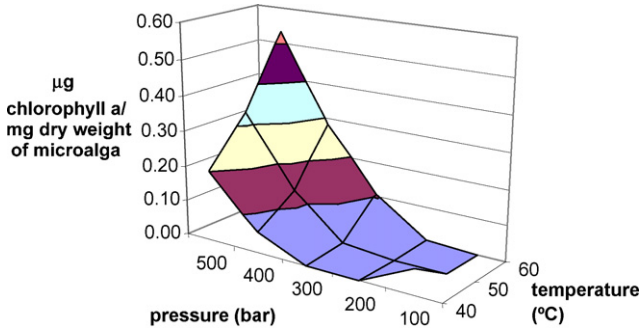

Fig. 4. Estimated yields of chlorophyll a extraction with supercritical carbon dioxide using the empirical correlation.

4.5. Relationship between yields of carotenoids and chlorophyll a

Analysis of the results in Table 1 shows that for each operating pressure the ratio of the yields carotenoids/chlorophylls (Carot/Chlor) varies in a different way with temperature. At a pressure of 200 bar, the ratio between carotenoids and chlorophyll increases as the operating temperature is increased up to 60 °C. Therefore, the increase in temperature leads to a higher selectivity between the two pigments.

At 400 bar the ratio Carot/Chlor decreases as the temperature is increased, meaning that selectivity between the pigments under investigation is more difficult to achieve.

At pressures of 300 and 500 bar the ratio Carot/Chlor is opposite, while at 300 bar the ratio decreases as the operating temperature increases from 40 to 50 °C, at 500 bar the ratio initially increases and then decreases as the temperature is raised up to 60 °C. At 300 bar, however, an increase in temperature above 50 °C leads to an increase in the Carot/Chlor ratio.

The highest ratio is obtained at 200 bar and 60 °C. Under these operating conditions a higher selectivity is achieved and this should facilitate the separation and purification of the two extracted pigments.

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.

4.6. Comparison with other algae

In an effort to compare this process with those carried out by other authors on other microalgae, literature yields of carotenoid extraction are presented in Table 3. The extraction yields from Mendes et al. [13] were obtained from the SFE curves of carotenoids that these authors present in Fig. 3 of their paper (mg carotenoids/5 g of dry weight of microalgae versus volume of carbon dioxide).

The results found by Careri et al. [14] cannot be compared with *Synechococcus* sp. because they only reported the extraction yields of the major carotenoids from Spirulina Pacifica (zeaxanthin, β-cryptoxanthin and β-carotene) and did not give the total carotenoid yields.

Table 3
Comparison of extraction yields with others microalgae studied by other authors

	Operating conditions	Carotenoids (μg pigments/mg dry weight of microalgae)	β-Carotene (μg pigments/mg dry weight of microalgae)	β-Cryptoxanthin (μg pigments/mg dry weight of microalgae)	Zeaxanthin (μg pigments/mg dry weight of microalgae)	Chlorophyll *a* (μg pigments/mg dry weight of microalgae)
Nannochloropsis gaditana[a]	200 bar–40 °C	0.152				0.290
	200 bar–50 °C	0.152				0.371
	300 bar–40 °C	0.208				0.807
	300 bar–50 °C	0.248				1.076
Chlorella vulgaris[b]	200 bar–40 °C, 136 min	0.011				
	200 bar–55 °C, 198 min	0.008				
	350 bar–55 °C, 252 min	0.080				
Spirulina Pacifica[c]	350 bar–80 °C, 70 min, 15% ethanol				0.480	
	350 bar–76 °C, 100 min, 15% ethanol			0.075.5		
	350 bar–60 °C, 100 min, 15% ethanol		1.1808			

[a] Macías-Sánchez et al. [15]. *Nannochloropsis gaditana*. Extraction time 180 min.
[b] Mendes et al. [13]. *Chlorella vulgaris*. Flow rate at 0.4 L/min.
[c] Careri et al. [14]. *Spirulina pacifica*.

A comparison between *C. vulgaris* and *Synechococcus* sp. shows that it is possible to obtain total carotenoid extraction yields from *Synechococcus* sp. that are higher than those using the microalgae studied by Mendes et al. [13].

A comparison between *N. gaditana* and *Synechococcus* sp. shows that the latter microalga is a better raw material for the extraction of total carotenoids than *N. gaditana* because the extraction yields are higher. Nevertheless, the best extraction yields of chlorophyll *a* are obtained from *N. gaditana*.

5. Conclusions

In the pressure range selected in these experiments the extraction of carotenoids and chlorophyll *a* begins at 200 bar.

The highest extraction yield of total carotenoids is obtained operating at a pressure of 300 bar and a temperature of 50 °C. The most appropriate operating conditions to obtain the best yield in the extraction of chlorophyll *a* are 500 bar and 60 °C.

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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