Supercritical CO₂ Extraction of *β*-Carotene from a Marine Strain of the Cyanobacterium *Synechococcus* Species

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Dynamic extraction of carotenoids from a marine strain of *Synechococcus* sp. (Cyanophyceae) with supercritical CO₂ (SC-CO₂) was investigated with regard to operation pressure and temperature effects on extraction efficiency. Extraction yield (milligrams of pigment per gram of dry weight) for SC-CO₂ was compared with the extraction yield for dimethylformamide (DMF). Carotenoids extracted with SC-CO₂ were *β*-carotene (Ct), zeaxanthin (Z), *β*-cryptoxanthin (Cr), and equinenone; chlorophyll *a* was poorly extracted, whereas myxoxanthophyll, another major carotenoid, was not extracted under any experimental condition. The highest relative yield, which is defined here as $y_r = [(\text{mg of pigment}_{SC-CO2}/\text{mg of pigment}_{DMF})] \times 100$, was $76.1 \pm 8.6\%$ for Ct, but it rose to $87.0 \pm 3.4\%$ when 15% ethanol was used as cosolvent. The pressure effect on y_r was found to be significant ($p < 0.05$) for both Cr and Z, along with total carotenoids, whereas the effect of square *T* (*TT*) was significant for only Ct. From empirical correlations, pairwise pressure (bar) and temperature (°C), respectively, for optimal extraction were determined to be (358, 50) for Ct, (454, 59) for Cr, and (500, 60) for Z. Cell disruption by sonication or detergent treatment of the biomass did not improve the extraction efficiency. Matrix structure together with material state could explain the low carotenoid extraction yield obtained with SC-CO₂ as compared to DMF in *Synechococcus* sp. However, the process can be applied to selective extraction of different carotenoids.

KEYWORDS: Supercritical carbon dioxide; carotenoids; *Synechococcus*; HPLC

INTRODUCTION

Most carotenoids commercially available at the present are chemically synthesized, but there is increasing demand for carotenoids from natural sources (*1*). *β*-Carotene is a highly conjugated polyprenoid hydrocarbon that is currently used in the food industry as a colorant as well as an additive because of its provitamin A activity. Furthermore, antioxidant and anticancer activities have been reported for *β*-carotene (*2*). Microalgae are considered to be a suitable natural source of carotenoids because of their rapid growth and easy manipulation; however, at present, carotenoid production from microalgae refers only to astaxanthin and *β*-carotene from *Haematococcus pluvialis* and *Dunaliella salina*, respectively, owing to the fact they are the only algae which accumulate enough pigment to make production cost-effective. During the past decade, the cyanobacteria *Synechocystis* sp. PCC6803 and *Synechococcus*

sp. PCC 7002 have been pointed out as very suitable organisms for genetic modification (*3*) with the aim of enhancing *β*-carotene accumulation for biotechnological production. The pigment profile of *Synechococcus* strains is mainly composed by *β*-carotene (Ct) and zeaxanthin (Z, a dihydroxylated derivative of *β*-carotene) besides chlorophyll *a* (Chl), with diverse glycocarotenoids and equinenone (a ketocarotenoid) as accompanying carotenoids to varying extents between different strains (*4*).

Pigment extraction and purification are two milestones in carotenoid production from microalgae (*5*). Supercritical carbon dioxide extraction (SC-CO₂) of carotenoids is an alternative to solvent extraction because it provides a high-speed extraction process and a simple purification (*6*). In addition, SC-CO₂ complies with legal requirements regarding low or null toxicity. The supercritical fluid extraction of carotenoids from the microalgae *Dunaliella salina*, *Chlorella vulgaris*, *Spirulina pacifica*, and *Nannochloropsis gaditana* has been reported in a number of papers with promising results (*7*−*11*). At an industrial scale, SC-CO₂ is applied to astaxanthin extraction from *H. pluvialis* for human consumption (*12*). SC-CO₂ extraction of carotenoids has a limitation regarding hydroxylated carotenoids

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(xanthophylls) due to their increased polarity compared to β-carotene (*10*). In addition, the extraction efficiency of a given solute depends on the type of matrix and particle size among a number of factors (*10*). Indeed, Subra et al. (*13*) showed that the inner structure of the matrix could interfere with β-carotene release due to interactions with the protein environment (*14*).

This study deals with SC-CO$_2$ extraction of carotenoids from dry biomass of a marine cyanobacterium *Synechococcus* sp. as raw material.

MATERIALS AND METHODS

Cultures. The *Synechococcus* sp. strain 05/0201 from the Marine Microalgae Culture Collection of the Institute for Marine Sciences of Andalucía (CSIC, Spain) was used. Cells were grown in a 2-L benchtop photobioreactor (model Biostat-B, B Braun Biotech International GmbH) during 4 days, in natural seawater filtered through a 1.0 μm filter, sterilized in an autoclave (120 °C, 1 kg pressure, 15 min), and enriched with F/2 medium with double nitrate and phosphate concentrations. Culture conditions were as follows: irradiance of 300 ± 25 μmol m^{-2} s^{-1}, temperature of 35 °C, and agitation at 50 rpm. On the fourth day of culture growth cell biomass was harvested by centrifugation, washed once with 0.9% ammonium formate, frozen at -20 °C, and lyophilized. The resulting freeze-dried biomass was used as raw material for SC-CO$_2$ extraction. During the growth period, 1-mL samples were daily taken for pigment analysis.

Traditional Pigment Extraction. For solvent extraction, *N*,*N'*-dimethylformamide (DMF) was used as extractant in both samples taken out during growth and samples from the freeze-dried biomass. Cells were harvested by centrifugation, and the supernatant was discarded; then 1 mL of DMF was added to the pellet and kept overnight at 4 °C. For the freeze-dried biomass, an amount of 2–5 mg was weighed and 1 mL of DMF then added; after sonication, samples were kept overnight at 4 °C. For both fresh cells and dried biomass, the DMF extract was separated from the pellet and recovered by centrifugation, immediately filtered through 0.22 μm, and stored at 4 °C until analysis by high-performance liquid chromatography (HPLC).

Supercritical Fluid Extraction. Supercritical CO$_2$ extraction (SFE) was performed using an ISCO SFX 220 extractor with a 0.5 mL chamber; CO$_2$ was supplied by a syringe pump model 260DX, and the extraction flow rate was controlled by a micrometric valve at the outlet of the extractor. A scheme and further details can be found in ref *11*. The extraction was over 0.1 g of algal dry powder, and operation conditions were as follows: 200, 300, or 400 bar, and 40, 50, or 60 °C; solvent flow rate was 4.5 mmol min^{-1} measured in working conditions; and extraction time was up to 120 (two experiments) or 240 min. Extracts were collected in ethanol and stored at -4 °C until measurement.

Additional Biomass Treatments. To assess the effect on the extraction efficiency of the matrix in which the pigments are embedded, two treatments were assayed with the aim of enhancing the carotenoid extraction yield by facilitating CO$_2$ entry inside the cell and, hence, the access to pigment complexes. Both treatments were applied to lyophilized biomass and were sonication in 0.9% ammonium formate (3 mL, 10 cycles of 0.5 s each at an amplitude of 30 kHz for 5 times) and suspension in 1% Triton X-100 in distilled water (3 mL) for 1 h at 4 °C. The treated biomass was thereafter collected by centrifugation, washed once with 0.9% ammonium formate, and lyophilized again after it was harvested by a new centrifugation. Sonication is expected to break the cell wall, whereas the detergent effect is rather related to protein denaturation with consequent easy release of the pigment moiety. Pigments were extracted from the treated biomasses with DMF and SC-CO$_2$ (300 bar and 50 °C).

High-Performance Liquid Chromatography (HPLC). Pigments were analyzed by HPLC using a Waters 600E multisolvent delivery system coupled to a Waters 996 PDA detector. Elution was according to the gradient system described in ref *15*, using a reversed-phase C-18 column packed with Spherisorb ODS-2 (15 × 4 mm i.d., 5 μm particle size). Data were acquired three-dimensionally (absorbance–time–wavelength) over the wavelength range of 350–750 nm. A standard

of zeaxanthin was obtained by pigment purification using thin-layer chromatography (TLC) from a methanolic extract of *Nannochloropsis gaditana* (*15*), β-carotene was purchased from Sigma, and myxoxanthophyll, β-cryptoxanthin, and equinenone were identified by comparison of their spectral and chromatographic characteristics with those in the literature (*16, 17*). Standard curves (pigment concentration versus peak area) were developed for zeaxanthin and β-carotene, whereas myxoxanthophyll, β-cryptoxanthin, and equinenone were quantified according to the following expression: (mg of pigment)/(10^6 cells) = $(SFV_{ex} \times 10^3)/[E_{1cm}^{1\%}(V_iV_cN) \times 60]$, where S is the peak area, F is the elution flow (1 mL min^{-1}), V_i (μL) is the extract volume injected, V_{ex} is the extract volume, V_c is the culture volume over which the extraction was performed, $E_{1cm}^{1\%}$ (L g^{-1} cm^{-1}) is the specific extinction coefficient, and N is the cell density expressed in millions of cells per mL of culture. This equation is that shown in ref *16* but with slight modifications. Values of $E_{1cm}^{1\%}$ used for quantification were 2620 for β-carotene, 2540 for zeaxanthin, 2160 for myxoxanthophyll, 2386 for β-cryptoxanthin, and 2158 for equinenone (*17*).

All reagents used in this study were of HPLC grade and purchased from Merck Chemicals (VWR International S.L., Barcelona, Spain) or Scharlau Chemie (Scharlab S.L., Barcelona, Spain).

Statistics. An empirical model was developed from the correlation of the extraction yield (response variable) to both pressure and temperature (experimental variables) by using the Statgraphics Plus 5.1 (1994–2001, Statistical Graphics Corp.) utilities. The model provides information on the effect (positive or negative) of each experimental variable at a factorial multilevel, along with its significance for a 12 experimental data set (only mean values were considered). Significance is accepted for $p < 0.05$. Optimal conditions of extraction were then calculated from the model for each pigment.

RESULTS

A typical chromatogram for a DMF extract of *Synechococcus* sp. is shown in **Figure 1**. HPLC-detected pigments in this study are in good agreement with the current pigment profile reported for cyanobacteria (*4*). Mean values of total carotenoids (TC), β-carotene (Ct), β-cryptoxanthin (Cr), zeaxanthin (Z), and Chl content determined from DMF extracts of different biomasses ($n = 5$) are shown in **Table 1**.

Extraction Yield. Carotenoid extraction with SC-CO$_2$ from *Synechococcus* sp. dried biomass showed dependence on both pressure (P) and temperature (T). Over an extraction time of 240 min, a maximal content of 2.76 ± 0.89 mg/g of dry weight (DW) was rendered for TC at 500 bar and 60 °C (**Figure 2**). The optimal values for P and T regarding the highest extraction throughput differed among the three main pigments that were extracted with SC-CO$_2$, namely, Ct, Cr, and Z. The yield of SC-CO$_2$ extraction in relation to solvent (DMF) extraction (relative yield, y_r), which is defined here as the ratio between milligrams of pigment extracted with SC-CO$_2$ and milligrams of pigment extracted with DMF, with both amounts being referred to per gram of dry weight, and expressed in percent, that is $y_r = [(\text{mg of pigment}_{SC-CO2}/\text{mg of pigment}_{DMF})] \times 100$, is depicted in **Figure 3** for Ct, Cr, and Z with respect to P and T dependence. The highest values of y_r obtained in the different experiments were 71.6 ± 8.1, 90.3 ± 11.4, and $36.4 \pm 0.4\%$ for Ct, Cr, and Z, respectively. A significant ($p < 0.05$) positive effect of P on y_r was found for TC, Cr, and Z (**Table 2**), whereas no significant ($p > 0.05$) effect of T on y_r resulted for any of the pigments, the square T term (TT) being significant for Ct ($p = 0.04$) and slightly significant for Z ($p = 0.07$). The interaction between T and P was not significant for any of the pigments ($p > 0.05$).

Empirical correlations were determined for the relative extraction yield of SC-CO$_2$ to DMF (y_r) for TC, Ct, Cr, and Z (**Table 3**). From these correlations, the optimal values of P and

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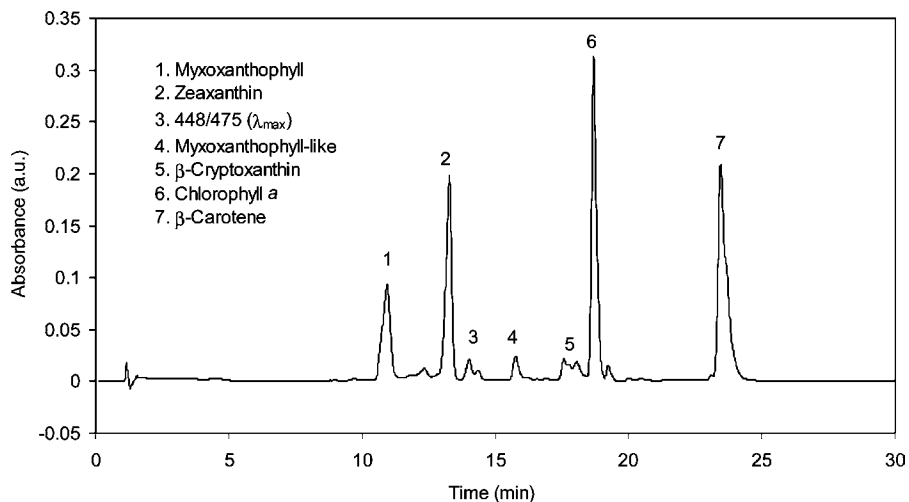*J. Agric. Food Chem.,* Vol. 53, No. 25, 2005   **9703**

Figure 1. Typical chromatogram from *Synechococcus* sp. identifying the main pigments.

Table 1. Typical Values (Mean ± Standard Deviation, $n = 5$) of Chlorophyll, Myxoxanthophyl, β-Carotene, β-Cryptoxanthin, Zeaxanthin, and Total Carotenoid Contents of the Dried Biomass of *Synechococcus* Species That Was Used as Raw Material for Supercritical CO$_2$ Extraction[a]

	pigment (mg of pigment/g of dw)					
	Chl	Myx	Ct	Cr	Z	TC
lyophilized biomass	5.40 ± 0.72	0.48 ± 0.03	2.15 ± 0.39	0.12 ± 0.01	1.79 ± 0.44	4.93 ± 0.98

[a] Pigments were extracted with dimethylformamide (DMF).
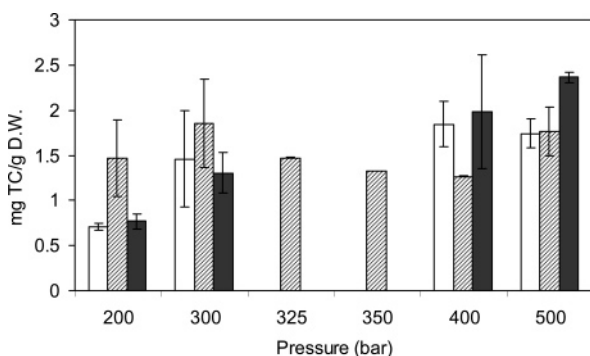

Figure 2. Total carotenoid content (mg pigment/g dry weight) extracted with SC-CO$_2$ under different operation conditions of pressure and temperature, over an extraction period of 240 min. Pigments were quantified by means of HPLC. Values correspond to the mean ± standard deviation from two to four replicates of different biomasses. Bars: white, 40 °C; slashed, 50 °C; gray, 60 °C.

Table 2. Estimated Effects of Pressure (P) and Temperature (T) on the Extraction Process for the Different Carotenoids from *Synechococcus* Species ($n = 12$)[a]

	total carotenoids		β-carotene		β-cryptoxanthin		zeaxanthin	
variable	effect	p	effect	p	effect	p	effect	p
T	3.38	0.59	1.94	0.84	10.36	0.47	6.86	0.23
P	18.53	0.03	2.27	0.83	54.35	0.01	19.98	0.02
TP	5.99	0.48	8.65	0.52	6.40	0.74	7.03	0.40
TT	−3.86	0.72	−41.88	0.04	−8.38	0.74	18.15	0.07
PP	−7.77	0.50	−22.43	0.24	−43.39	0.13	4.99	0.60

[a] Significant influence is accepted for $p < 0.05$.

T for the highest extraction yield were calculated, which are given by the following pairwise (P, bar; T, °C): (500, 60), (358, 50), (454, 59), and (500, 60) for TC, Ct, Cr, and Z, respectively.

Ct was always the predominant carotenoid in the SC-CO$_2$ extracts. The highest percentage of Ct in relation to TC extracted
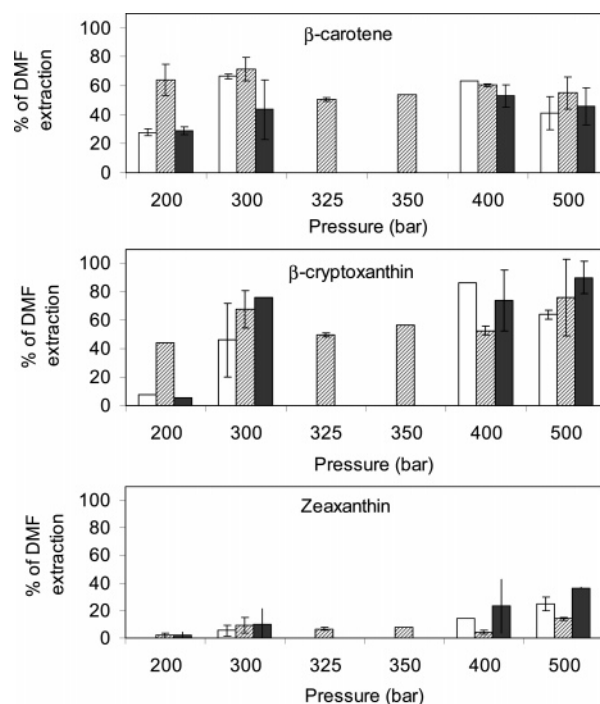

Figure 3. Relative extraction yield, $y_r = [(\text{mg of pigment}_{SC-CO_2})/(\text{mg of pigment}_{DMF})] \times 100$, for β-carotene, β-cryptoxanthin, and zeaxanthin. Values are mean ± standard deviation from two to four replicates of different biomasses. Bars: white, 40 °C; slashed, 50 °C; gray, 60 °C.

(94.54 ± 5.64%) was found to be at 200 bar and 40 °C (**Figure 4A**), even though more Ct was extracted at 300 bar and 50 °C. In contrast, increasing the operation *P* and/or *T* the contents of Cr and Z relative to TC rose. In **Figure 4B**, the UV−vis spectra of different SC-CO$_2$ extracts are shown overlaid to the DMF extract spectrum for a comparative purpose. These figures illustrate the poor extraction of chlorophyll *a* (Chl), this fact leading to a high purity of carotenoids in the SC-CO$_2$ extracts.

Table 3. Empirical Correlations Determined for the Different Pigments Using the Software Statgraphics Plus 5.1 (Statistical Graphics Corp.)[a]

pigment	empirical correlation	r^2
TC	$y_r = -29.51 + 8.28 \times 10^{-2}P + 1.40T - 1.73 \times 10^{-4}P^2 + 2.00 \times 10^{-3}PT - 1.93 \times 10^{-2}T^2$	0.62
Ct	$y_r = -472.57 + 0.21P + 20.03T - 4.98 \times 10^{-4}P^2 + 2.88 \times 10^{-3}PT - 0.21T^2$	0.60
Cr	$y_r = -198.31 + 0.75P + 3.79T - 9.64 \times 10^{-4}P^2 + 2.13 \times 10^{-3}PT - 4.02 \times 10^{-2}T^2$	0.74
Z	$y_r = 246.99 - 0.13P - 9.55T + 1.11 \times 10^{-4}P^2 + 2.34 \times 10^{-3}PT + 9.07 \times 10^{-2}T^2$	0.85

[a] y_r is the relative yield to DMF extraction (mg$_{SC-CO2}$/mg$_{DMF}$) × 100, P is pressure, and T is temperature. r^2 is the correlation coefficient.

Table 4. Mean Values (± SD, n = 2–4) of the Constant a (Milligrams per Gram of Dry Weight) Resulting from the Fit of Time Course Data for SC-CO$_2$ Extraction to a Logarithmic Function [mg of Pigment/g of Dry Weight = $a \times$ Ln(t) + b, Where t Is Time and b an Additional Constant To Be Determined from the Fit]

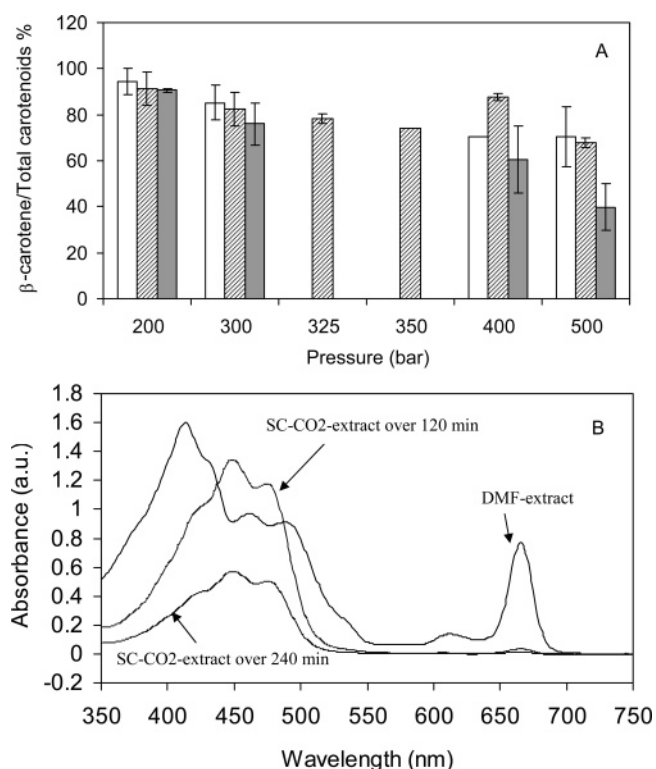|	total carotenoids			β-carotene		
pressure (bar)	40 °C	50 °C	60 °C	40 °C	50 °C	60 °C
200	0.17 ± 0.02	0.46 ± 0.02	0.19 ± 0.01	0.14 ± 0.01	0.40 ± 0.00	0.17 ± 0.01
300	0.29 ± 0.08	0.57 ± 0.11	0.27 ± 0.13	0.26 ± 0.08	0.49 ± 0.10	0.21 ± 0.13
400	0.38 ± nd[a]	0.51 ± 0.01	0.37 ± 0.12	0.25 ± n.d.	0.45 ± 0.01	0.16 ± 0.01
500	0.39 ± 0.04	0.39 ± 0.10	0.53 ± 0.06	0.21 ± 0.08	0.26 ± 0.03	0.17 ± 0.10

[a] nd indicates that the SD is <0.01.

Figure 4. (**A**) Relative content of β-carotene to total carotenoids, in percentage, extracted with SC-CO$_2$, under different extraction conditions of pressure and temperature, over an extraction period of 240 min. Bars: white, 40 °C; slashed, 50 °C; gray, 60 °C. (**B**) UV–vis spectra of different extracts for comparative purposes.

Significant Chl amounts were extracted at only the highest P and T, as well as over the longest extraction times, that were assayed in this study. Myxoxanthophyll (Myx), another major carotenoid from *Synechococcus* sp. (**Figure 1**), was not extracted under any experimental condition. The ketocarotenoid equinenone, with a polarity intermediate between those of Ct and Cr, was also currently extracted with SC-CO$_2$, but the extraction yield could not be assessed for this pigment because it coelutes with Chl in the DMF extract. Nonetheless, equinenone amounted to a mean value of 3.8 ± 0.9% of the total carotenoids extracted with SC-CO$_2$. Inverse effects on the extraction throughput were

observed for T and P regarding this pigment; thus, the higher P and lower T, the higher throughput was found (data not shown).

Extraction Kinetics. There was a correspondence between the extraction yield and the extraction kinetics. Typical curves of the extraction time course for total carotenoids (TC) showing the pressure (left-side panels, T = 50 °C) and temperature (right-side panels, P = 300 bar) effects are depicted in **Figure 5** for TC, Ct, and Z. A steady state was not attained over the extraction period of 240 min at any of the operation conditions used in this study. Lines in **Figure 5** correspond to the fit of the experimental data for TC and Ct to a logarithmic equation of the form (mg of TC/g of DW) = a Ln(t) + b, where t is the time (min) and a and b are constants to be determined from the fit (R^2 > 0.9); kinetic data for Z fitted better to a second-order polynomial equation (R^2 > 0.9). Ct was the main carotenoid extracted over the first period of extraction, whereas there was a delay in Z extraction that was reduced with increased operation P and T. Accordingly, short extraction times enhanced the Ct proportion in the SC-CO$_2$ extract, with a relative content >95% under defined extraction conditions of P and T. Values of the constant a (mg of pigment/g of DW) are provided in **Table 4** for TC and Ct.

Additional Treatments. Because the use of a modifier has been shown to improve extraction efficiency (*10*, *18*, *19*), additional experiments were carried out in which 15% ethanol was used as cosolvent to SC-CO$_2$. The relative extraction yield of SC-CO$_2$ to DMF was slightly higher in those samples extracted with 15% ethanol as cosolvent for operation conditions of 300 bar and 50 °C (**Table 5**). Nonetheless, in contrast to Careri et al. (*10*), yields of 100% were not achieved.

Sonication led to solubilization of phycobilins in the aqueous medium, whereas chlorophyll-like pigments along with phycobilins were released to the aqueous medium in the detergent treatment (**Figure 6**). The y_r values for these treatments are shown in **Table 5**. Lower efficiencies were rendered in the treated biomasses than in the corresponding nontreated biomass (100% CO$_2$).

DISCUSSION

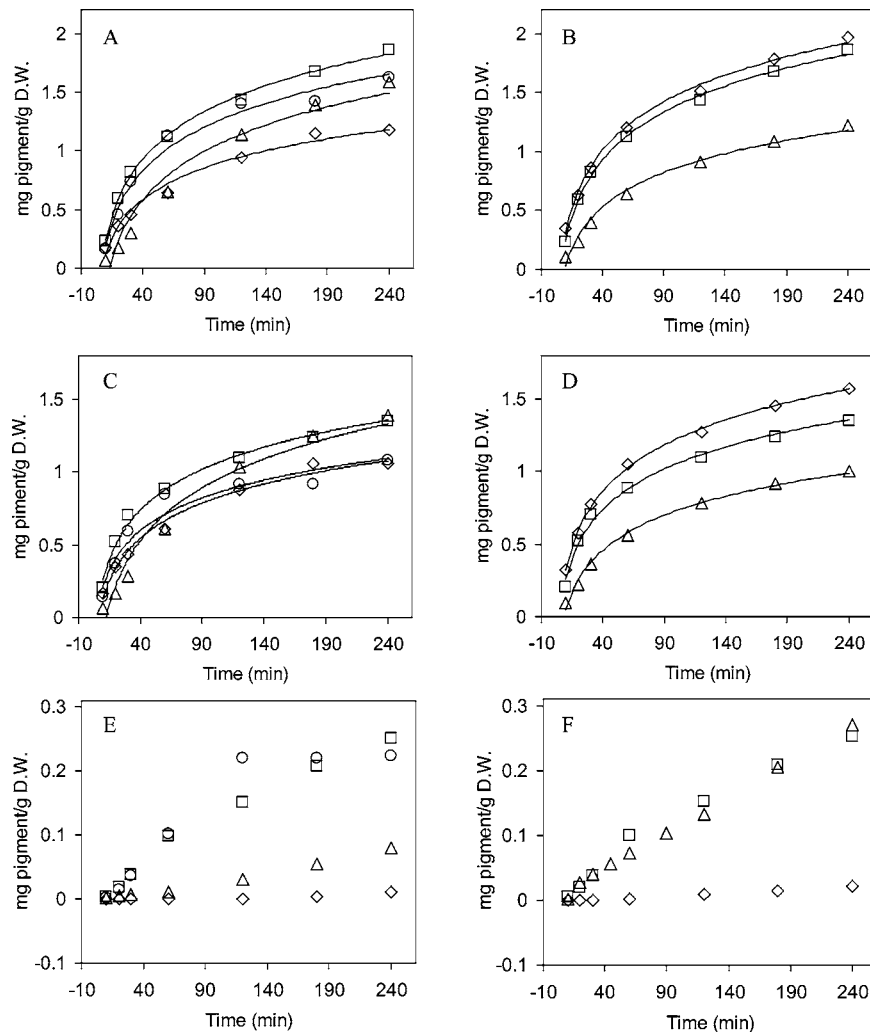Maximal y_r obtained in this study was lower than y_r reported by other authors for pigment extraction with SC-CO$_2$ from a

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Figure 5. Typical curves for time course evolution of pigment extraction from *Synechococcus* sp. with SC-CO$_2$ for total carotenoids (**A**, **B**), β-carotene (**C**, **D**), and zeaxanthin (**E**, **F**). Lines are drawn after the fit of experimental data to the following equation: mg of pigment/g of DW $= a \times \text{Ln}(t) + b$, where t is time and a and b are constants to be determined from the fit. Left side ($T = 50\ °C$): $\diamond$, 200 bar; $\square$, 300 bar; $\triangle$, 400 bar; $\bigcirc$, 500 bar. Right side ($P = 300$ bar): $\diamond$, 40 °C; $\square$, 50 °C; $\triangle$, 60 °C.

Table 5. Relative Extraction Yield (Percent) of SC-CO$_2$ to Dimethylformamide, $y_r = (\text{mg of Pigment}_{SC-CO2}/\text{mg of Pigment}_{DMF}) \times 100$, for Different Treatments[a]

pigment	100% CO$_2$	15% Et/ 85% CO$_2$	sonicated biomass[b]	Triton X-100 treated biomass[b]
TC	37.5 ± 9.9	58.7 ± 2.3	17.4	20.5
Ct	71.6 ± 8.1	87.0 ± 3.4	36.4	41.6
Cr	7.9 ± 13.1	63.6 ± nd	26.2	20.2
Z	9.3 ± 5.4	25.4 ± 1.3	1.9	2.5

[a] Operation conditions were 300 bar and 50 °C. [b] From a single experiment yet compared with DMF extraction from the same biomass.

Figure 6. Spectra of 0.9% ammonium formate solutions resulting from sonicated dried biomass and detergent (Triton X-100)-treated dried biomass.

microalga-based raw biomass (*8−11*), with the exception of Cr. Sanal et al. (*20*) also found the yield of SC-CO$_2$ extraction to be lower than the yield of solvent extraction in apricot pomace. Because *Synechococcus* sp. shares the cyanobacterial character with *Spirulina pacifica*, results similar to those reported by Careri et al. (*10*) were expected to be obtained in this study. Nonetheless, these authors reported extraction yields close to 100% over much shorter times of dynamic extraction (70−100 min), as well as no variation of the optimal extraction pressure (350 bar) among Ct, Cr, and Z. Because the operation process is rather a standardized one, it is likely that differences in the extraction yield between this study and those aforementioned
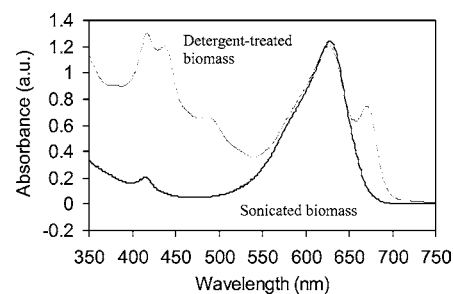
also using algal biomass have stemmed from the biomass state and characteristics.

Thus, even though compound solubility is primarily controlled by the density and temperature of the extraction fluid, the extraction efficiency also relies on the nature and state of the raw material due to the fact that matrix complexity, along with the lipid and protein environment, may impose constraints to pigment release and solubility in SC-CO$_2$ (*10*, *21*). Favati et al. (*14*) obtained a rapid and full removal of Ct from a leaf protein concentrate at 50 and 70 MPa (500 and 700 bar, respectively) over a 130−160 min extraction period (800 L of CO$_2$ at a flow

rate of 5−6 L/min). Furthermore, Ct, Cr, and Z recoveries were not enhanced in the detergent-treated biomass (see **Table 5**). Hence, protein influence is likely to be ruled out as responsible for the low extraction throughput. Stahl et al. (*22*) pointed out that carotenoid solubility was influenced by the entrainment effect of other lipids; likewise, Mendes et al. (*8*) resolved two zones in the extraction curve, a first one being due to solubility of cell outer lipids, and a second one being controlled by SC-CO_2 diffusion within the cells. The latter authors also found recovery enhancement in crushed cells. Because the lipid environment is expected to differ only slightly between *Synechococcus* sp. and *S. pacifica*, differences in extraction yield do not seem to have relied on any lipid-related factor. In addition, y_r was not enhanced in *Synechococcus* cells disrupted by sonication. Therefore, it seems likely that the matrix nature affected only the extraction efficiency in *Synechococcus* sp. in regard to SC-CO_2 diffusion and interaction with the solutes.

Cygnarowicz and Seider (*18*) reported the highest solubility for Ct in SC-CO_2 to be at 343 K (69.9 °C) and 405 bar. Optimal conditions for Ct extraction from *Synechococcus* sp. were determined in this study to be 358 bar and 50 °C. Because an increase in *P* leads to an enhancement of fluid density and solvating power, but with concurrent reduction of the interaction between the fluid and the matrix (*10*, *23*, *24*), it can be concluded that the extraction efficiency of Ct in *Synechococcus* sp. is primarily governed by the residence time of the supercritical fluid within the matrix, with analyte desorption being the limiting step (*24*). This hypothesis is also underpinned by the fact that kinetic extraction curves did not attain an asymptotic value under any operation conditions. Therefore, and taking into account the results of Careri et al. (*10*), the possibility of improving the extraction efficiency for Ct in *Synechococcus* sp. is considered through operating static extraction for a short period before dynamic extraction is applied (*24*).

To compare SC-CO_2 and solvent extraction yields, many authors have used acetone, hexane, or THF/methanol (1:1) for solvent extraction (*8−10*). Some organic solvents are poor extractants for pigments from microalgae and cyanobacteria (*25*), a fact that might lead to overestimation of SC-CO_2 yield as compared to the solvent yield. Therefore, we used DMF for solvent extraction (24 h, 4 °C) (*4*). A higher ratio of the cis form to the all-trans form for Ct in *S. pacifica* might have also increased recovery owing to the higher solubility in SC-CO_2 of the cis form (*9*).

Polarity increases from Ct to Z through Cr due to the introduction of up to two hydroxyl groups in the Ct basic hydrocarbon structure. From our results it seems evident that increases in both *P* and *T* are necessary to extract a carotenoid-based compound as polarity rises. This finding could be explained by taking into account the opposite effects that result from the increase in both *P* and *T*. Thus, the rise in *T* leads to a higher value of the vapor pressure with concurrent higher solubility, although simultaneous reduction of density occurs; this latter feature is compensated for by the increase in *P* but at the expense of the concurrent fact that interaction with the matrix is negatively affected. Indeed, Favati et al. (*14*) reported a recovery for lutein (70%), a Z isomer, lower than for Ct under the same operation conditions. The optimal conditions for TC extraction with SC-CO_2 were calculated to be at 500 bar and 60 °C, whereas the optimal conditions for Ct, which was always the predominant pigment in the SC-CO_2 extracts (see **Figure 3**), were 358 bar and 50 °C. This initially unexpected result can be explained by taking into account that the relative proportion of Ct to TC is substantially reduced as *P* and *T* rise,

while the contribution of Z and Cr to TC concurrently increases. Consequently, the TC amount extracted with SC-CO_2 substantially augments with *P* and *T*, the maximal extracted TC amount then being rendered at 500 bar and 60 °C (see **Figure 2**).

The use of ethanol as cosolvent has been shown to improve extraction efficiency owing to increased solubility of the analytes, along with modifications of the matrix characteristics and concurrent enhanced interaction with the supercritical fluid (*10*, *19*), which in turn facilitates the desorption of the analytes from the sample matrix (*24*). Cygnarowicz and Seider (*18*) pointed out that ethanol function is dehydration. In this study, a substantial increment of y_r was measured when 15% ethanol was used as modifier in comparison to y_r measured for 100% CO_2 (at 300 bar and 50 °C). Because the increment was notably more pronounced for Cr and Z than for Ct, we conclude that solubility was mainly enhanced by the increased polarity of the extractant with 15% ethanol (*24*). However, alteration of the matrix environment, with concurrent easier access of SC-CO_2 to the pigments, is likely to have also occurred, this fact having facilitated pigment desorption. Consequently, matrix alteration, which could have also involved dehydration of the remaining interstitial water molecules, would explain the increase in y_r for Ct, a pigment with a rather unpolar character (*24*).

In summary, the interaction of SC-CO_2 with the pigment matrix seems to be the factor responsible for the relatively low extraction yields that were obtained in this study. A short period of static extraction, along with the use of 15% ethanol as modifier, is believed to improve the extraction process with SC-CO_2 and, thereby, further research is needed on this issue. Selective extraction of the different pigments from *Synechococcus* sp., in particular, β-carotene, with purities >95%, may be achieved by properly adjusting the operation conditions.

ABBREVIATIONS USED

SC-CO_2, supercritical carbon dioxide; TC, total carotenoids; Ct, β-carotene; Cr, β-cryptoxanthin; Z, zeaxanthin; chl, chlorophyll *a*; Myx, myxoxanthophyll; HPLC, high-performance liquid chromatography; *P*, pressure; *T*, temperature; DMF, *N,N′*-dimethylformamide.

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