Extraction of Astaxantine and Phycocyanine from Microalgae with Supercritical Carbon Dioxide

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Astaxantine from the microalga *Haematococcus pluvialis* and phycocyanine from the microalga *Spirulina maxima* have been obtained by supercritical extraction using carbon dioxide. Prior to extraction, the samples of microalgae were crushed by cutting mills (coffee mill) and then manually ground with dry ice (solid carbon dioxide). The *Haematococcus* extracts were analyzed by liquid chromatography, using astaxantin (purity of 98%) as a standard. Phycocyanine, being insoluble in carbon dioxide, was indirectly separated. Thus, lipid-soluble substances from spirulina were extracted and analyzed by liquid chromatography, using as a standard a solution prepared on the base of reagent (purity of 40%). For the astaxantine extraction, the results show that cellular wall breaking has an important effect on extraction efficiency. The addition of a cosolvent (9.4 mass % of ethanol) has little effect, reaching an extraction yield of about 1.7 mass %. The extraction yield is much below the expected lipid content in dry *Haematococcus* alga samples, which is known to be close to 30 mass %. The maximum total recovery of astaxantin, calculated from its initial and residual content in the alga (0.0147 and 0.0004, respectively), exceeds 97%. For phycocyanine extraction, the addition of cosolvent (10 mass % of ethanol) has a strong effect on the extraction yield of lipidic substances. This value goes from about 1.1% for extraction without cosolvent to about 1.7 mass % when the cosolvent is used. The total extraction yield of about 3 mass % corresponds well to the average lipid content of 3.27% in alga *Spirulina maxima* reported in the literature.

Introduction

Microalgae form an extremely diverse group of organisms yielding an almost unlimited range of chemicals. The main products currently being commercialized are carotenoids, phycobilins, fatty acids, polysaccharides, vitamins, sterols, and other biologically active compounds. The most advanced fine chemical derived from algae is β-carotene. Some species of algae (*Euglena, Haematococcus, Chlorella*) also produce, in significant quantities, other carotenoids such as astaxantin and canthaxantin. Phycocyanine is a water-soluble phycobili-protein present in some algae such as *Spirulina, Porphyndium*, and *Rhodella*.[1−3]

Carotenoid pigments such as astaxantine are used as dietary supplements in salmonoid fish diets as a method for inducing the desired coloration of the fish. Although other pigments could be used for these purposes, astaxantine is naturally retained by the fish flesh and is more stable.[4,5] Phycocyanine is among the most commercially promising biochemicals present in *Spirulina* and is used as a natural food colorant and as a biochemical tracer in immunoassays, among other uses.[1] Phycocyanine has no special odor, is nontoxic, and is slightly sweet. When dissolved in water, the pigment gives a brilliant blue color with a high red fluorescence.[6]

As a rule, extraction and purification of alga products are similar to those used for plant cells, yeasts, and bacteria, that is, processing with the aid of organic solvents.[7] Increased demand for "natural" products without any contact with chemicals motivates the development of new extraction methods.[8] Supercritical carbon dioxide, which presents some unique characteristics and is a nontoxic and fully "green" solvent, can be considered as a good candidate for alga treatment.[9] During the past decades, the application of supercritical fluids to several separation processes has been a matter of intensive research, specially those processes that use carbon dioxide as the extracting solvent.[10−12] Novel applications of supercritical fluid extraction include particle design for drug formulation and some biological processes such as cell lysis and sterilization.[13]

Experimental Section

Material and Methods. Dried samples of the microalgae *Haematococcus pluvialis* and *Spirulina maxima* were supplied by Chañar Blanco, La Serena, Chile. The algae were locally produced in artificial ponds and were dried in a stove at low temperature.[14,15] The raw material as industrially produced was prepared for extraction using supercritical carbon dioxide, as explained below.

In the extraction experiments, for *Haematococcus pluvialis* three runs were performed: in run 1, the samples were crushed by cutting mills prior to extraction; in run 2, the samples were crushed by cutting mills and then manually ground with dry ice (solid CO_2) prior to extraction. In both cases, extraction was done using pure supercritical carbon dioxide. A third run was performed using samples treated as in run 2 but extracted using supercritical carbon dioxide and ethanol (9.4 mass %) as a cosolvent.

In the extraction experiments, for *S. maxima* two runs were performed: in run 1, the samples were crushed by cutting mills prior to extraction and then extracted using pure supercritical carbon dioxide; in run 2, the samples were treated as in run 1 but extracted using supercritical

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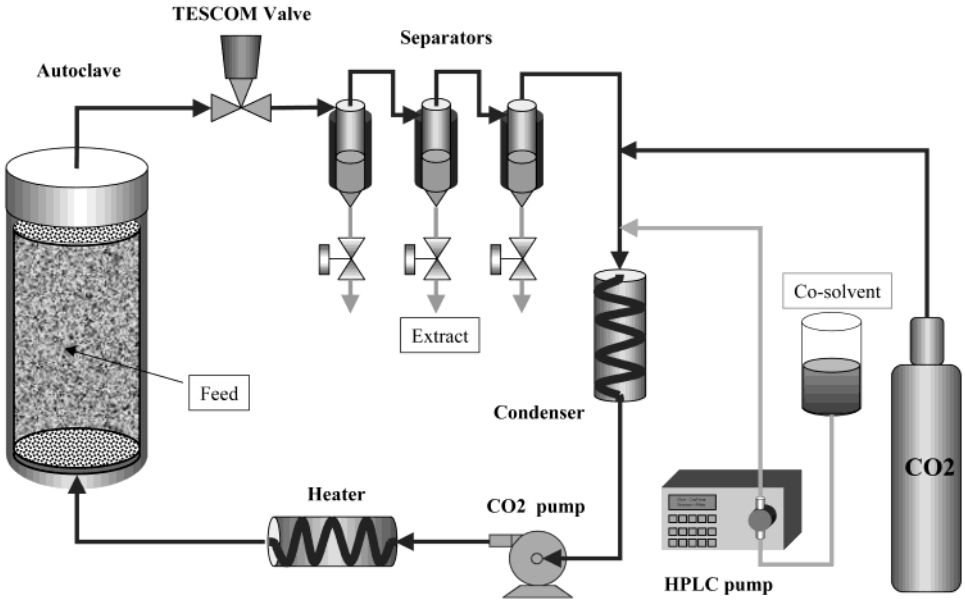

Figure 1. Flow-sheet of the SFE-500 SEPAREX pilot plant.

Table 1. Details on the Equipment Used and Some Variables for the Analysis of the Extract Containing Astaxantine and Phycocyanine

	astaxantine extraction	phycocyanine extraction
column	Spherisorb ODS I, 5μ, 80A (250 mm × 4.6 mm i.d.)	Spherisorb ODS I, 5μ, 80A (250 mm × 4.6 mm i.d.)
mobile phase	methanol/acetonitril (9:1 by volume)	HPLC quality water (0.1% of TFA)
sample dissolution	HPLC grade dichloromethane	
flow rate	1 mL·min^{-1}	1 mL·min^{-1}
column temperature	60 °C	60 °C
column pressure	300 bar	300 bar
detector	UV (490 nm)	UV (220 nm)
sample injection	20 μL	100 μL
standard	astaxantin (purity of 98%), Sigma	prepared on the basis of chemical (purity of 40%), Sigma

carbon dioxide and ethanol (10%) as a cosolvent. Since the blue pigment phycocyanine is not soluble in carbon dioxide, soluble materials that are primarily composed of lipid-soluble compounds were extracted. The pigment phycocyanine remained with the residue inside the extractor vessel.

Extraction Unit. A schematic diagram of the pilot plant, a Bench Scale SFE-500 SEPAREX unit, is given in Figure 1. Solvent (CO_2), from the cylinder, was delivered through a pipe to the condenser. Liquid CO_2 reaches the inlet of the high-pressure pump rated up to 300 bar. Compressed fluid was fed to the heater prior to entering the extraction vessel. The unit contained an extraction container of 450 mL, closed with stainless steel porous disks. After percolating through the feed bed, the fluid was expanded into three high-performance separators where extract is precipitated. Fluid leaving the last separator was recycled to the cooling exchanger. An HPLC pump was used to feed the fluid that contained the desired concentration of cosolvent.

Analytical Methods. An HP 1100 liquid chromatograph (Hewlett-Packard) consisting of an autosampler, a binary solvent pump, a column oven, and two detectors (variable UV and light scattering detector LSD) was used. The system was controlled by a PC with the appropriate software. Table 1 gives details on the equipment used and some variables for the analysis of the extract.

Results and Discussion

The extraction of astaxantin and phycocyanine was evaluated in terms of the yield extract for the different runs described above: two runs for the extraction of phycocyanine and three runs for the extraction of astaxantin.

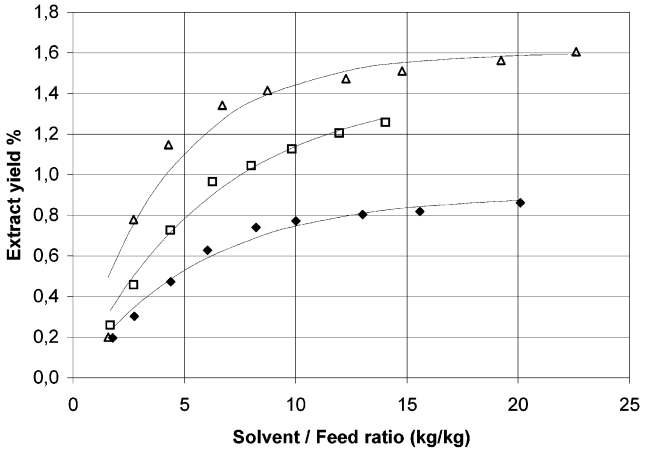

Figure 2. Extract yield in the extraction of astaxantine from *H. pluvialis* by supercritical carbon dioxide (60 °C and 300 bar): run 1, once-ground alga and pure CO_2 (◆); run 2, twice-ground alga and pure CO_2 (□); run 3, twice-ground alga and CO_2 + ethanol (△).

Astaxantin Extraction. Figure 2 shows the extraction profiles at 60 °C and 300 bar with pure CO_2 (run 1 and 2) and CO_2 + ethanol (run 3). The total extract yield for run 1 (slightly crushed alga) was lower than 1 mass %. For run 2, twice ground, the yield increases significantly, reaching values close to 1.3 mass %. This fact reveals the importance of cellular wall breaking on extraction efficiency. The addition of cosolvent (9.4% of ethanol) into supercritical

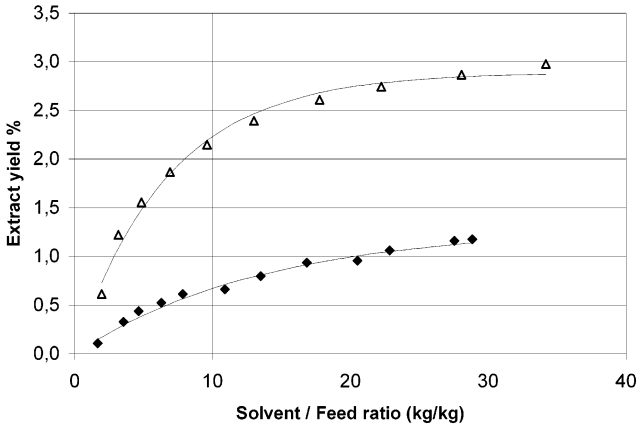

Figure 3. Extract yield in the extraction of phycocyanine from *S. maxima* by supercritical carbon dioxide (60 °C and 300 bar): run 1, ground alga and extraction with pure CO_2 (◆); run 2, ground alga and extraction with CO_2 + ethanol (△).

Table 2. Astaxantin Content in Samples of *H. pluvialis*

		S/F ratio	astaxantin content
run no.	sample	kg/kg	mass %
1	feed (ground)		0.0088
	HE 1/1	1.79	0.0259
	HE 1/4	6.04	0.0254
	HE 1/9	20.09	0.2362
	residue 1		0.0052
2	feed (2 times ground)		0.0147
	HE2/1	1.66	0.0455
	HE2/4	6.26	0.0980
	HE2/9	14.03	0.3693
	residue 2		0.0018
3	HE3/1	1.58	0.3094
	HE3/4	6.72	1.7504
	HE3/7	14.79	1.7472
	residue 3		0.0004

Table 3. Phycocyanine Content in Samples of *S. maxima*

			phycocyanine content
run	solvent	sample	mass %
1	pure CO_2	feed	6.55
		residue	8.30
2	CO_2 + 10% ethanol	feed	6.99
		residue	8.49

CO_2 increases only a little the extraction yield to about 1.6 mass %. In any case, the extraction yield is much below the expected lipid content in dry *Haematococcus* alga samples, thought to be nearly 30 mass %.

Astaxantin content in the alga samples is presented in Table 2 and Figure 3. As seen in Table 2, astaxantin content depends strongly on sample preparation and extraction conditions. Similar to extraction yield, when a more efficient milling procedure yielded more extract, the concentration of astaxantin is also higher in samples from feed ground twice (run 2). This shows that solvent properties markedly favor the extraction of astaxantin. For pure CO_2, astaxantin is removed only partially, and its content in the extract does not exceed 0.4%. For CO_2 with 9.4% of ethanol as a cosolvent, astaxantin extraction is improved, reaching concentrations higher than 1.7%. The total recovery of astaxantin (run 3), calculated from its initial and residual content in the alga (0.0147 and 0.0004, respectively), exceeds 97%.

Phycocyanine Extraction. Table 3 shows the phycocyanine content in the residue of the extraction with CO_2. It should be noticed from Table 3 that the amount of phycocyanine increased in the residue as a result of removing the CO_2-soluble substances. Figure 2 shows the extraction profiles at 60 °C and 300 bar with pure CO_2 (run 1) and CO_2 + ethanol (run 2). The total extract yield for run 1 (extraction without cosolvent) was of the order of 1.1%. For run 2, in which 10% ethanol is added as a cosolvent, the extraction yield increased to about 3%. The total extraction yield of about 3% corresponds well to the average lipid content of 3.27% in alga *S. maxima* reported in the literature.[1]

Table 4. Models for the Extract Yield (Y) as a Function of the Solvent/Feed Ratio (X) for the Extraction of Astaxantine and Phycocyanine with Supercritical CO_2 at 60 °C and 300 bar

			deviation	
extraction	method	model: $Y = \alpha(1 - e^{-\beta X})$	$\%D$	$\lvert\%D\rvert$
astaxantine from *H. pluvialis*	once-ground alga and extraction with pure CO_2	$\alpha = 0.8995$, $\beta = 0.1772$	−3.4	6.9
	twice-ground alga and extraction with pure CO_2	$\alpha = 1.4387$, $\beta = 0.1572$	−3.9	6.3
	twice-ground alga and extraction with CO_2 + ethanol	$\alpha = 1.6008$, $\beta = 0.2344$	−4.6	9.6
phycocyanine from *S. maxima*	once-ground alga and extraction with pure CO_2	$\alpha = 1.3029$, $\beta = 0.0720$	−0.2	8.8
	once-ground alga and extraction with CO_2 + ethanol	$\alpha = 2.8905$, $\beta = 0.1480$	−0.6	4.9

Models for the Extract Yield. The extract yield (Y) of astaxantine and of phycocyanine as a function of the solvent/feed (S/F) ratio (X) correlates reasonable well using an exponential asymptotic function of the form

$$Y = \alpha(1 - e^{-\beta X}) \quad (1)$$

In this model, α and β are empirical constants obtained from the experimental yield obtained for the different runs as a function of the solvent/feed ratio. The models fulfill the limit that $Y = 0$ for $X = 0$ (zero yield for zero solvent flow) and asymptotically tend to the empirical maximum, represented by the parameter α. Table 4 shows the values of the parameters for the different runs. The last two columns show the average and absolute deviations of the model prediction with respect to the raw data for each run. These deviations are defined, for a set of N data, as

$$D\,\% = (100/N)\sum[(Y_{calc} - Y_{exp})/Y_{exp}]_i \quad (2)$$

$$|D\,\%| = (100/N)\sum|(Y_{calc} - Y_{exp})/Y_{exp}|_i \quad (3)$$

Figures 2 and 3 show the experimental results (dots) and the model calculations (solid lines). The proposed model is shown to correlate the data in an acceptable form for interpolation and extrapolation purposes.

Conclusions

From the results obtained and the analysis of the data, the following conclusions can be drawn for both products:

For astaxantine: (1) The astaxantin content in the extract strongly depends on sample preparation and extraction conditions. (2) Solvent properties markedly favor the extraction of astaxantin. The yield slightly increases (from around 1.3 mass % to close to 1.6 mass %), but the concentration increases by 4 times (from around 0.35% to

close to 1.75%). (3) The total recovery of astaxantin, using well-ground alga and CO_2 with 9.4% ethanol (run 3), exceeds 97%.

For phycocyanine: (1) The addition of cosolvent drastically increases the yield, from 1.1% to 3%. (2) The solvent/feed ratio has a stronger effect when ethanol is used as a cosolvent, especially at a high solvent/feed ratio. (3) The total extraction yield of about 3 mass % corresponds well to the average lipid content of 3.27% in alga *S. maxima* reported in the literature.

For both: The model representing the extraction yield (Y) as a function of the solvent/feed ratio (X) for astaxantine and phycocyanine extraction was found to be accurate enough for interpolation or extrapolation purposes.

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