Supercritical extraction of astaxanthin from *H. pluvialis* using ethanol-modified CO$_2$. Experiments and modeling.

A. Bustamante, P. Roberts, R. Aravena, J.M. del Valle

1 Departamento de Ciencia y Tecnología Química de los Alimentos, Facultad Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile. (proberts@uchile.cl)
2 Departamento de Ingeniería Química y Bioprocesos, Pontificia Universidad Católica de Chile, Santiago, Chile. (delvalle@ing.puc.cl)

**ABSTRACT**

*Haematococcus pluvialis* is the main natural source of astaxanthin. Extraction with SuperCritical (SC) carbon dioxide (CO$_2$) is an interesting choice for astaxanthin recovery because the medium-to-high yield, high selectivity for high-value compounds, and avoidance of thermal damage to labile bioactive compounds. In the case of heavy and/polar compounds such as astaxanthin, yield can be increased using GRAS polar co-solvents or modifiers such as ethanol. For this reason SC CO$_2$ extraction was carried out at 313-343 K and 30-55 MPa using pure or ethanol-modified (4 or 8% v/v) CO$_2$. Response surface analysis showed a positive effect of pressure and mild negative effect of temperature in the astaxanthin recovery. Besides, added ethanol improves astaxanthin recovery but up to a maximal using 4.5% (v/v). The model of Sovova (2006) fitted well experimental data for astaxanthin extraction using pure and ethanol-modified CO$_2$ under selected experimental conditions. Best-fitting model parameters suggest that improvement in astaxanthin recovery when adding ethanol is due to its large positive effect on mass transfer within unbroken *H. pluvialis* cysts rather than in the solubility in SC CO$_2$ or solute partition between SC CO$_2$ and pretreated cysts.

**Keywords:** Astaxanthin, Carbon dioxide, Co-solvent, Ethanol, *Haematococcus pluvialis*, Mass transfer, Supercritical extraction, Modeling.

**INTRODUCTION**

Currently there is growing interest in the development of functional foods or nutraceuticals based on natural ingredients to deliver health benefits to people or animals through nutrition. Astaxanthin (ASX) is a ketocarotenoid that belongs to the xanthophyll class and has a potent antioxidant activity, higher than lutein and β-carotene, and ≥100 times more than α-tocopherol [1]. Astaxanthin extracted from the microalga *Haematococcus pluvialis* is an interesting choice to achieve these benefits. However, astaxanthin-rich extracts, must be stabilized to prevent its oxidation during storage. Among other industrial alternatives, encapsulation is an interesting protective process [2]. However, the need of experimental studies to optimize the encapsulation process makes it necessary having large amounts of astaxanthin-rich extracts.

Astaxanthin can be extracted from *H. pluvialis* using organic solvents, but they exhibit limited selectivity, favour astaxanthin degradation, and extracts contain solvent residues potentially toxic. An alternative solvent without these limitations is SuperCritical (SC) carbon dioxide (CO$_2$). SC Extraction (SCE) is characterized by medium-to-high yield, high selectivity for high-value compounds, and avoidance of thermal damage to labile bioactive compounds [3]. Several researchers studied SC CO$_2$ extraction of astaxanthin from *H. pluvialis* reporting high yield, and high concentrations of astaxanthin in extracts depending on operating conditions [4-7]. Factors evaluated in these studies included sample pre-treatment; extraction temperature (313-353 K); extraction pressure (20-55 MPa); specific CO$_2$ rate (0.15-0.24 kg/min/kg (dry) microalgae); extraction time (1-4h); and co-solvent type and concentration (1.6-10% ethanol, 12% vegetable oil). Experimental results show that astaxanthin recovery generally increases with extraction pressure, extraction temperature, and added co-solvent; and decreases with specific CO$_2$ rate.

With the objective of obtaining an astaxanthin rich extract for encapsulation, we used response surface (RS) analysis to evaluate the effect of extraction temperature (313-343 K), extraction pressure (30-55 MPa), and ethanol concentration (0-8% v/v) on astaxanthin recovery in 5-h SC CO$_2$ extraction experiments. Results were not as expected in that temperature had a negative effect and pressure had not as large positive effect as reported by others. Because our extraction time (5 h) is higher than used in previous works (1-4 h), we
speculated that anomalies were due to kinetic effects during extraction and recognized the need to evaluate the effect of extraction time on astaxanthin recovery. Thus, the main objective of this work was measuring and modelling kinetic curves of astaxanthin extraction under selected conditions to evaluate the effect of extraction time on astaxanthin recovery. Complementarily, we show results of the RS analysis study mentioned above.

**MATERIALS & METHODS**

**Sample.** Whole dried cysts of *H. pluvialis* containing 2.7% water were supplied by Astax S.A. (Iquique, II Región, Chile). Cysts underwent grinding in a bench top ring mill pulveriser (Rocklabs, Auckland, New Zealand).

**Treatments.** Extractions were carried out in a one-pass laboratory SCE device (Spe-ed SFE 7071, Applied Separations, Allentown, PA). Three-gram samples of disrupted microalgae were mixed with 9 g of Celite (Merck, Darmstadt, Germany) and loaded in a 50-cm³ extraction vessel. Extractions were carried out at 313, 328, or 343 K, and 30, 42.5, or 55 MPa, and using 4.5 g/min of pure or ethanol-modified (4 or 8% v/v) CO₂. Whereas sampling in RS experiments occurred during a 5 h extraction period, in kinetic studies under selected experimental conditions, samples were taken every 10 minutes during the first hour, an hourly during the subsiding four hours. Extract samples were collected in pre-weighed glass vials and recovered extracts were assessed gravimetrically. Astaxanthin content in extract samples was determined by UV/VIS analysis in a spectrophotometer (ATI UNICAM, Cambridge, UK). Extracts were dissolved in acetone, and the amount of astaxanthin estimated quantified using the optical extinction coefficient of astaxanthin in acetone ($E_{\text{1% cm}^{-1}}=2100$) at $\lambda=470$ nm [8].

**Experimental design.** RS analysis was used to evaluate the effects of three independent variables (temperature, $T$; pressure, $P$; and ethanol concentration, $E$) on astaxanthin recovery. Experiments were carried out according to a face centered design with three levels. Table 1 shows results of experiments for the $2^3$ fractional factorial design considering a duplicate at the center. A quadratic equation was used to estimate the astaxanthin recovery as function of $T$, $P$, and $E$. The coefficients of the RS equation were estimated by using Design-Expert Software, version 8.0.1 (Stat-Ease, Inc., Minneapolis, MN).

**Modeling.** Extraction curves were modelled using the model of Sovova [9] that is based on differential mass balance equation in a packed bed operating under plug flow conditions. This model considers a tissue containing a fraction of broken cells ($r$) and a remainder of intact cells. The assumed extraction process has two steps. First, the solute is transferred from intact to broken cells with a flux that is proportional to concentration gradient between these cells and an internal mass transfer coefficient ($k_{a0}$). Second, the solute is transferred from broken cells to SC CO₂ with a flux that is proportional to the concentration gradient between the two and an external mass transfer coefficient ($k_{afa}$). The driving force for this second step uses as the concentration in the broken cells the one in a SC CO₂ phase that is in equilibrium with it. This equilibrium is determined by the solubility of astaxanthin in SC CO₂ under extraction conditions ($y_s$), a transition concentration in the solid ($x_t$) when equilibrium stops being determined by solubility, a partition coefficient for astaxanthin between SC CO₂ and *H. pluvialis* ($K$) as proposed by Perrut [10].

CO₂ properties were calculated using RFPROP (NIST, U.S) linked to Excel. A bed porosity of 36% was adopted as for a bed of regularly packed spherical particles [11]. The differential equations were solved with the Runge-Kutta method in Matlab 7.0 (Math Works, Natick, MA). Both equilibrium parameters ($y_s$, $x_t$, and $K$) and the mass transfer parameters ($k_{afa}$, $k_{a0}$; and $r$) were best-fitted to experimental data.

**RESULTS & DISCUSSION**

**Response Surface Analysis.** Table 1 presents results of astaxanthin recovery in the RS experiments. Depending on extraction conditions and ethanol added, astaxanthin recoveries ranged 43-82.8% (based on an astaxanthin content of 18.14 mg ASX per gram of extract-free algae). These results agreed with others in literature on SC CO₂ extraction of astaxanthin from *H. pluvialis* using SC CO₂ (recoveries ranging from 36 to 84% depending on the sample pretreatment and extraction conditions [4-7]).
The use of ethanol as co-solvent improves astaxanthin recovery, possibly due to an improvement in the negative effect of temperature on astaxanthin recovery observed in this work.

This study than others in literature), especially in experiments using ethanol as a co-solvent, may explain the difference in astaxanthin recovery. Although temperature may improve astaxanthin recovery, its degradation during extractions (longer in experiments at 343 K using pure CO$_2$) over pressure solubility increases because of the more pronounced increase in volatility and solubility of the compressible) solubility decreases with temperature because of the decrease in density, but above the cross-over pressure (at near-critical pressure, where CO$_2$ is highly compressible) solubility decreases with temperature because of the decrease in density, but above the cross-over pressure solubility increases because of the more pronounced increase in volatility and solubility of the solute. The “cross-over” pressure for natural compounds is generally slightly above the critical pressure (7.3 MPa) of the CO$_2$, particularly in the case carotenoids where it is below 20 MPa [12, 13]. Because of this, the 4.8% decrease in astaxanthin recovery as a result of an increase in temperature of 15 K at 30 MPa (cf. third term in Eq. 1) was unexpected. Also, this is just the opposite observation of others [6,7], who report astaxanthin extraction form H. pluvialis, where temperature had a positive effect at high pressures (although not at low pressure [7]). The negative effect of temperature observed in the present work may be due to the oxidation of astaxanthin with the oxygen present in the CO$_2$ or in the vials where extract samples were collected and kept during the 5-h experiments [14]. The oxidation of astaxanthin increases with storage time and the temperature [15]. Furthermore, ethanol-mediated isomerization of astaxanthin favors oxidation [16]. So, although temperature may improve astaxanthin recovery, its degradation during extractions (longer in this study than others in literature), especially in experiments using ethanol as a co-solvent, may explain the negative effect of temperature on astaxanthin recovery observed in this work.

The use of ethanol as co-solvent improves astaxanthin recovery, possibly due to an improvement in the solvent power of SC CO$_2$ for polar compounds. Indeed, the solubility of β-carotene in CO$_2$ at 50 MPa and 328 K increases ca. 2.5-fold when adding 1.6% ethanol [13]. However, our results indicate that astaxanthin recovery is maximized when ca. 4.5 % (v/v) ethanol is added (cf. fourth and fifth terms in Eq. 1). This phenomenon was observed previously in SC CO$_2$ extraction of astaxanthin [6] using SC CO$_2$ by Machmudah et al. [6], who observed a lower astaxanthin recovery using 7.5% than 5% (v/v) ethanol.

| $P$ | 30 | 30 | 30 | 30 | 30 | 42.5 | 42.5 | 42.5 | 42.5 | 42.5 | 42.5 | 42.5 | 42.5 | 55 | 55 | 55 | 55 | 55 |
| $T$ | 343 | 343 | 313 | 313 | 328 | 328 | 328 | 328 | 328 | 328 | 343 | 313 | 343 | 343 | 313 | 313 | 328 |
| $E$ | 0 | 8 | 0 | 8 | 4 | 0 | 8 | 4 | 4 | 4 | 4 | 4 | 4 | 0 | 8 | 0 | 8 | 4 |
| $R$ | 43.0 | 56.8 | 60.7 | 63.5 | 67.3 | 60.7 | 63.5 | 71.7 | 68.9 | 68.4 | 73.9 | 64.6 | 65.7 | 72.3 | 76.2 | 82.8 |

Table 1. Results of extraction experiments. $P$: Pressure (MPa); $T$: Temperature (K); $E$: Ethanol Concentration (%); and $R$: Astaxanthin Recovery (%).

Experimental results were fitted to full second order response surface model but after deleting the least significant term one at a time up to the point were significant the 5% level. Equation 1 predicts astaxanthin recovery ($R$, %) as a function of temperature ($T$, K), pressure ($P$, MPa), and ethanol concentration ($E$, % v/v).

$$R = 72.20 + 7.01 \left( \frac{P - 24.5}{12.5} \right) - 4.80 \left( \frac{T - 55}{15} \right) + 2.43 \left( \frac{E - 4}{4} \right) - 9.51 \left( \frac{E - 4}{4} \right)^2$$  \hspace{1cm} [1]
On the other hand, added ethanol may not affect only astaxanthin solubility, but also its partition between ethanol-modified SC CO$_2$ and the substrate [17]. Co-solvents can also swell the substrate thus improving inner mass transfer within the solid matrix [18].

**Kinetic curves of extraction and modelling.** Figure 1 shows kinetic curves of astaxanthin extraction as a function of specific CO$_2$ consumption under selected conditions within the experimental region. Extraction rate is very slow after 2 h (180 g CO$_2$/g microalgae), which indicates that longer extraction times do not contribute to astaxanthin recovery to a great extent. Besides, solubility (related to the initial slope of the curves) improves when increasing temperature, so that shorter extractions are enough to reach a given recovery at high temperature. Consequently, a 5-h extraction at high temperature could favor astaxanthin degradation as previously speculated with regards to RS experiments.

Kinetic curves were modeled using the model of Sovova [9] for the extraction of vegetable matrixes containing broken and intact cells. Although microalgae are unicellular organisms without connection between cells, caking phenomena has been observed for us in *H. pluvialis* extraction (unreported results) as well as by others using various substrates when extracting small particles of hygroscopic materials [3]. Consequently, caking results in a “soft connection” between cells, which may exhibit a tissue-like behaviour. The model of Sovova [9] applies to four different types of extraction curve depending on the solute content in the solid and equilibrium partition for the solute between the solid matrix and solvent. Simplified solutions of the model for the four curve types were fitted to the experimental data, resulting type B curve the one that fitted the data best under all conditions tested (unreported results). Type B curves are characterized for solute-matrix interactions represented by the sorption isotherm model proposed by Perrut et al [10], and can divided into three zones. The first two zones exhibit constant (but different) extraction rate (see Figure 1) determined by the recovery of astaxanthin from broken cells. In the first zone, astaxanthin concentration in broken cells is enough to saturate the SC CO$_2$ stream (concentration $y$) before it leaves the extractor. In the second zone, astaxanthin concentration in the broken cells is below the level $x$ where there is not enough to saturate the CO$_2$ stream, and extraction rate is determined by solute-matrix interactions characterized by a constant partition coefficient $K$. Finally, in the third zone mass transfer from intact to broken cells controls extraction rate. Figure 1 shows the experimental data and best-fitting curves for the SC CO$_2$ extraction of *H. pluvialis* astaxanthin using pure and ethanol-modified CO$_2$, and Table 2 reports the best-fitting model parameters as a function of the extraction conditions. The equilibrium parameters were estimated using the simplified model of Sovova [9], which assumes that the external resistance to mass transfer is much lower than internal resistance to mass transfer.

![Figure 1. Kinetic curves of astaxanthin extraction from *H. Pluvialis*](image-url)
Table 2. Fitted parameters of kinetic curves.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Extraction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure (P, MPa)</td>
<td>55 42.5 55 42.5</td>
</tr>
<tr>
<td>Temperature (T, K)</td>
<td>313 328 313 328</td>
</tr>
<tr>
<td>Ethanol (E, % p/p)</td>
<td>0 0 4 4</td>
</tr>
<tr>
<td>Solubility ($y_s$, 10$^4$ kg/kg)</td>
<td>3.08 1.83 2.01 1.25</td>
</tr>
<tr>
<td>Partition coefficient ($K$, 10$^3$)</td>
<td>4.71 4.11 7.23 2.57</td>
</tr>
<tr>
<td>Transition concentration ($x_t$, 10$^3$ kg/kg)</td>
<td>5.91 7.50 12.3 8.93</td>
</tr>
<tr>
<td>Grinding efficient ($r$, %)</td>
<td>58 58 58 58</td>
</tr>
<tr>
<td>Internal mass transfer resistance ($k_{fas}$, 10$^6$/s)</td>
<td>0.91 0.29 12.7 3.41</td>
</tr>
<tr>
<td>External mass transfer resistance ($k_{fas}$, 1/s)</td>
<td>1.82 2.15 1.92 2.35</td>
</tr>
</tbody>
</table>

Regarding the solubility of astaxanthin in pure CO$_2$, results show an increase with density, as expected. However, the extrapolation of the data of de la Fuente et. al. [12] for solubility of free astaxanthin in SC CO$_2$ suggest that the solubility of free astaxanthin is 3.55×10$^{-6}$ kg ASX/kg CO$_2$ at 55 MPa and 313 K, and 1.18×10$^{-5}$ kg ASX/kg CO$_2$ at 42.5 MPa and 328 K. Differences in estimated solubility between kinetic experiments with natural products, and phase equilibrium experiments with isolated solutes can be explained by differences in the molecule, which in the case of H. pluvialis’ astaxanthin is mostly esterified with one or two fatty acids [19], and co- or anti-solvent effects of other compounds in the microalgae extract, mainly triglycerides [20]. Considering differences in solubility between free and esterified lutein (ca. 2×10$^{-5}$ [13] versus 3.6×10$^{-6}$ kg lutein/kg CO$_2$ [21], respectively, at 313 K and 33 MPa), a 6-fold decrease in the solubility of astaxanthin as compared to its free counterpart was expected, unlike experimentally observed. Hence, the high solubility values experimentally observed are possibly explained by the co-solvent effect of other microalgae component.

From Table 2, it is apparent that the solubility of astaxanthin esters in blend of CO$_2$ and ethanol is slightly lower that in pure CO$_2$, which does not agree with previous reports for others carotenoids, whose solubility increased in the presence of the co-solvent [13]. The change in the other parameters that characterize the sorption isotherm indicate that the ethanol negatively affect solute-matrix interactions ($K$ decreases and $x_t$ increases) at 328 K and 42.5 MPa. The same as in the experiment at other condition (313 K and 55 MPa), we expected as favorable effect of ethanol addition on astaxanthin partition, because ethanol should compete with a polar solute for sorption sites in the substrate [17].

The kinetic parameters were fitted using the complete model proposed for Sovová [9]. The fraction of broken cells ($r$) depends only of the raw material, so we forced this condition when best-fitting the data. The high value of $r$ suggests an efficient disruption process that breaks about 60% of the cell priors to extraction. On the other hand, in all cases $k_{fas}$ is much larger than $k_{fas}$, as reported by Sovová [9] for extraction of biological compounds using SC-CO$_2$. Moreover, the high value of $k_{fas}$ implicates that external mass transfer is of limited importance in the extraction process [9]. Added ethanol improved the mass transfer between broken and intact cells (Table 2), being the $k_{fas}$ with co-solvent 10 times higher than pure CO$_2$. Possibly this difference is due to a permeabilization / rupture of cells walls and/or swelling of unbroken cysts by ethanol [18, 22], thus improving internal mass transfer during extraction.

Considering all results of kinetic studies summarized in Fig. 2 and Table 2, it seems that the improvement in astaxanthin recovery when adding ethanol is due to its large positive effect over the mass transfer process rather than its effect in equilibrium.

CONCLUSIONS

The results show that in the experimental region an increase of pressure has a positive effect in the extraction, while an increment of temperature has a positive effect at a (estimated value) 4.5% v/v level. So, within the explored experimental region best extraction conditions with SC CO$_2$ are 55 MPa, 313 K, and 4.5% (v/v) ethanol, that allowed recovering 84% of all astaxanthin in 5 h.

The model of Sovová [9] fitted well astaxanthin extraction curves using both pure and ethanol-modified CO$_2$. Kinetics curves suggested that long extraction times are not necessary, that could even encourage astaxanthin degradation, particularly when using high temperatures. Model parameters suggest that the positive effect of added ethanol is due to enhancement of the mass transfer from unbroken cells.
REFERENCES