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EXTRACTION OF BIXIN FROM ANNATTO SEEDS USING SUPERCRITICAL CARBON DIOXIDE

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Abstract - The solubility of 93% pure bixin in supercritical carbon dioxide (SC-CO₂) and of the bixin present in annatto seeds (*Bixa orellana* L.) was measured. For the seeds, the measurements were made in a temperature range from 30 to 50°C and pressure between 10 and 35 MPa and for the pure bixin, at 40°C from 10 to 35 MPa. The main pigments of annatto seeds are bixin and norbixin, but the extracts only showed the presence of cis and trans-bixin, indicating that norbixin is not soluble in SC-CO₂. The annatto seeds used in the experiments contained about 2.7% bixin and 3.1% oil. In the seeds, the crossover point of solubility was at about 28 MPa and values for solubility were about ten times higher than those of the pure bixin, giving evidence that the oil acted as a co-solvent with the CO₂.

Keywords: Supercritical carbon dioxide; Annatto; *Bixa orellana* L.; Bixin; Solubility.

INTRODUCTION

The main pigments of annatto seeds are bixin and norbixin, whose structures are shown in Figure 1. These pigments are carotenoids that vary in tone between yellow and red, colours of importance in the food, pharmacological and cosmetic industries. In food industries these natural pigments are used in cheeses, sausages, meats and candies (Furtado, 2003). Normally, three main methods could be used to extract the pigment from the annatto seeds: vegetable oil extraction, alkaline solution and organic solvent extraction. In the first case, the pigment is obtained by abrasion of the exocarp submerged in warm vegetable oil (70 °C). When extracted by organic solvent, such as acetone and methanol, a product with higher pigment concentrations, from 3.5 to 5.2% bixin, can be obtained. In this case, after extraction, the solvent is removed and then the powder pigment is dissolved in vegetable oil. The water-soluble form of this pigment

is produced by abrasion of the annatto seed exocarp in alkaline solution, and the resultant product is the salt of norbixin (cis and trans) (Pimentel and Stringheta, 1999). Therefore, bixin is easily converted into norbixin by dissolving the bixin in an alkaline medium (Carvalho et al., 1993). The chemical, toxicological, and antioxidant properties, and degradations of bixin and norbixin have been extensively studied (Satyanarayana et al., 2003; Kiokias and Gordon, 2003; Scotter, 1995), but for SC-CO₂ only a few studies can be found in the literatures (Degnan et al., 1991; Jay et al., 1991; Chao et al., 1991; Nobre et al., 2006). Currently, micronization of natural bixin using SC-CO₂ as antisolvent is also being studied (Suo et al., 2005).

Considering the restrictions placed on the use of synthetic dyes by the World Health Organisation, interest in natural dyes is increasing. The annatto (*Bixa orellana* L.) seed pigments, bixin and norbixin, are amongst those most used in the food, pharmacological and cosmetic industries due to the

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intensity of their colours, their greater stability and the wide variety of tones from yellow to red. This range of colours is an additional advantage of the annatto carotenoids over other carotenoids, such as those of the carrot and beetroot, which only show their respective colours (Souza, 1998).

Extraction with supercritical fluids is a technique which, in principle, could substitute traditional extraction processes such as distillation and liquid-liquid and solid-liquid extractions. When compared to conventional processes, using supercritical CO₂ for extraction in foods offers the advantages of CO₂ being an inert gas, non-toxic, non-inflammable and cheap. Since it is a gas under normal conditions, it is easy to separate from the solute, obtaining a pure

final product without organic solvent residues. It also makes processing at relatively low temperatures possible, avoiding heat degradation of thermolabile components. The aim of this study was to measure the solubility of pure bixin in SC-CO₂ and the solubility of the bixin present in annatto seeds with oil present and to correlate the data by temperature and density of SC-CO₂ using the Chrastil equation. The power solubilisation of annatto pigments by supercritical CO₂ at different pressures and temperatures and the correlation of these data with empirical models is the first step in the study of this extraction of interest for industrial application, followed by a comparison with conventional methods.

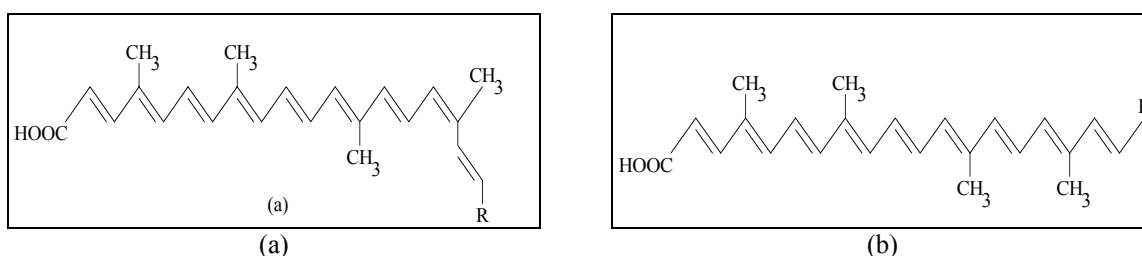


Figure 1: Molecular structures of bixin ($R=COOCH_3$) and norbixin ($R=COOH$): (a) α -(cis) and (b) β -(trans).

MATERIALS AND METHODS

Materials

Annatto seeds cultivated in the state of Bahia were acquired from the local market in Campinas, SP, Brazil. A batch of these seeds, available and commercialised on the national market, was characterised for density, porosity and bixin, moisture and oil contents, and was used in the solubility measurement experiments. Another two batches (Piava vermelha and Bico de Pato), from the state of Pará, were used to compare the solubility of the bixin from seeds of different origins. All the batches were packaged and stored at temperatures of about -10°C . The 93% pure bixin (diluted in dichloromethane) was produced and kindly provided by the Department of Chemical Engineering of the Federal University of Minas Gerais. An 18kg cylinder of liquid CO₂ with a purity of 99% was kindly provided by White Martins; chloroform with 99% purity was acquired from Merck, Chenco. All of the glassware was amber to protect samples from diffuse light.

Extraction and Solubility Measurement

The experimental apparatus used in this study is shown in Figure 2. It consisted of a 50 mL

equilibrium cell (stainless steel AISI 316, Suprilab, Campinas, Brazil) (7) immersed in a constant-temperature water bath controlled by a heater (Suprilab, Campinas, Brazil) to within $\pm 0.1^\circ\text{C}$. The CO₂ from the supply tank (White Martins, Campinas, Brazil) (1) was cooled to the liquid state (Refrigeration Bath Model TE 184 Tecnal, Campinas, Brazil) (2) and compressed into the equilibrium cell by a high pressure pump (Model AA 100S, Eldex Laboratories Inc., Napa, CA USA) (3). After reaching the equilibrium conditions of temperature, pressure and time (24 h), a sample of the extract mixture was transferred to a collector of known volume (8) and then collected in the glass tube (9) which contained chloroform. To recover the material remaining in the collector (8), a peristaltic pump (Model P-201 Tecnal, Campinas Brazil) circulated solvent through the collector by entrance valve (10). The experimental conditions were 30 to 50°C and 10 to 35 MPa. All experiments were done in triplicate with an equilibrium time of 24 hours, as defined in the preliminary tests. Preliminary tests had been necessary to define the period in which phase equilibrium would be reached. Amongst times tested it was observed that in 24h phase equilibrium was indubitably accomplished (Silva, 1999).

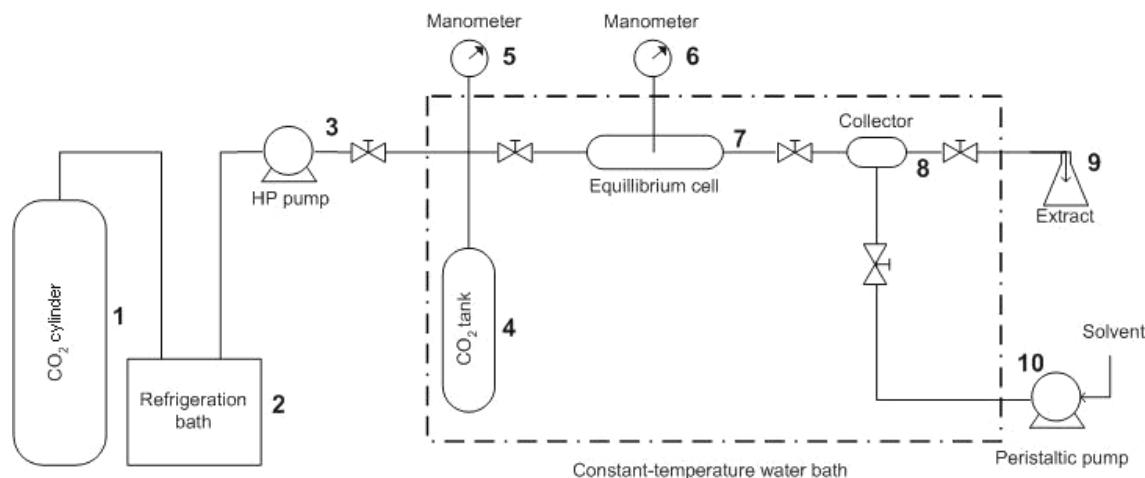


Figure 2: Experimental equipment (Static System).

Experimental Procedure for Measuring Solubility

Twenty seven grams of whole annatto seeds or approximately 0.005 g of 93% pure bixin adhering to inert glass beads with a mean diameter of 1.35 mm were placed in the 50 mL equilibrium cell. Liquid CO₂ was pumped using a refrigerated system to maintain it at -9°C at the entrance of the pump. The equilibrium cell was maintained at the desired temperature and pressure for 24 hours. Part of the extract was transferred to a collector (8) (volume of approximately 5.3 mL), maintaining the extractor pressure constant.

With the objective to check whether the insoluble solids influenced bixin solubility, another experiment was made. The seed pigments and oil were extracted with chloroform and then impregnated onto inert glass beads. This experiment was conducted under only one set of conditions, 40°C and 20 MPa.

Analytical Methods

The purity of the bixin was determined by diluting 0.0030 g bixin in 1000 mL chloroform and obtaining a reading for absorbance at 470 nm, obtaining an absorbance unit of 0.791 (AU=0.791), corresponding to 0.0028 g or 93.3% purity.

The bixin concentration in the extracts, obtained at different pressures and temperatures (Tables 1, 3 and 4), was also determined by spectrophotometer (Model 432 Femto, São Paulo, Brazil), at the same wave length (470 nm) as that of the pure bixin. The absorbance values (AU) had been used in the

solubility calculation, S (g bixin m⁻³), using the following equation, based on the Beer law:

$$S = \frac{AU}{E d} \frac{V}{V_C} \quad (1)$$

where

S = Solubility in g bixin m⁻³ CO₂

AU = Absorbance unit at 470 nm

E = Emittance 282.6 m³ of CHCl₃ kg⁻¹ bixin

V = Total extract volume (mL)

V_C = Collector volume (mL)

d = Cuvette size (cm)

To characterise the seeds, the following measurements were made: 1) the mean diameter was calculated by randomly separating 20 seeds from each sample and measuring the length, width and thickness, calculating the geometric mean of these three measurements; 2) the moisture content was calculated by oven drying at 105°C until constant weight was achieved (Rebocho, 1985); 3) the porosity of the extraction bed filled with seeds and apparent density were obtained by weighing a known volume of seeds and the real density, determined by pycnometry with gas helium (Central Analytical, IQ, UNICAMP) as follows: $\rho_s = \rho_a / (1 - \epsilon)$; 4) the bixin content of the seeds: extraction with chloroform; 5) the oil content was determined by Soxhlet extraction using petroleum ether adapted from AOAC 945.16 for 72 hours (AOAC, 1995), followed by drying at 105°C for 1 hour; 6) the fatty acid compositions of

the oils were identified by gas chromatography using a GC Agilent 6850 series GC system (Agilent Technologies, Wilmington, USA) with a DB-23 Agilent (50% cyanopropyl)-methylpolysiloxane, capillary column (60 m $\times$ 0.25 mm $\times$ 0.25 μ m; Serial US2201526H, Agilent Technologies, Wilmington, USA). The carrier gas was helium (1.0 mL/min, White Martins, Campinas, Brazil) with a split ratio of 1:50. The detector and injector temperatures were respectively 553 K and 523 K. The column was maintained at 383 K for 5 min., then raised from 383 to 488 K at 5 K/min and finally maintained at 488 K for 24 min. One microliter of the methyl esters was injected and the fatty acids were identified by comparison of the retention times with those of the methyl ester standards. The analyses were done in duplicate using a gas chromatograph equipped with a flame ionization detector (FID) and a column packed with Silar 10C.

Thermodynamic Modelling

The experimental values for solubility were correlated with temperature and CO₂ density, adjusting the parameters of the Chrastil equation (Chrastil, 1982).

$$S = \rho^k \exp\left(\frac{a}{T} + b\right) \quad (2)$$

where S (kg m⁻³) is the solubility; ρ (kg m⁻³) is the density of SC-CO₂; T (K) is the temperature; and k , a and b are parameters to be adjusted to the experimental data.

This equation was also fitted separately for each isotherm in the following way:

$$S = \rho^k \exp A \text{ or } \ln(S) = k \ln(\rho) + A \quad (3)$$

where $A = a/T + b$ is the parameter that depends on

temperature.

Since the light phase of the equilibrium mixture for the systems under study was only slightly concentrated in the solute, the density was considered to be that of pure CO₂. The values for density of CO₂ at the different temperatures and pressures were calculated using a computer programme that employs the empirical equation of Huang (Huang et al., 1985), which reproduces the experimental values of IUPAC (Angus et al., 1976).

RESULTS AND DISCUSSION

The solubility of 93% pure bixin at 40°C (Table 1) shows that these values (0.9 to 3.5 g m⁻³) are similar to those obtained by other authors. Solubilities of 1.0 to 3.0 g m⁻³ were obtained by Degnan et al. (1991) for 97% pure bixin with the temperature and pressure ranging from 40 to 55°C and from 20 to 47 MPa, respectively. Solubilities of 0.4 to 4.9 g m⁻³ were obtained by Jay et al. (1991) with the temperature and pressure ranging from 15 to 55°C and from 10 to 65 MPa, respectively. At 55°C, the data of this research (Degnan et al., 1991; Jay et al., 1991) agreed with respect to order of magnitude. However, at 40°C, the values found in this study were two times higher than those of Degnan et al. (1991) (e.g. at 30 MPa, values of 3.28 g m⁻³ as compared to 1.8 g m⁻³).

The annatto seed sample was characterised by its physical properties, bixin content and oil content. The values, reported in Table 2, were obtained from the mean of the four experimental analysis samples of seed. In general the data agreed with the values obtained by Faria (1998). The main fatty acids found in the seed oil, of which approximately 68% are unsaturated oleic and linoleic fatty acids are shown in Table 2. The composition of the seed oil is similar to that of soybean oil.

Table 1: Solubility of 93.3% pure bixin in CO₂ at 40°C

P (MPa)	ρ_{CO_2} (g.L ⁻¹)	S (g.m ⁻³) $\pm \sigma$
10	629.3	0.93 $\pm$ 0.04
15	781.6	2.14 $\pm$ 0.11
20	840.8	2.80 $\pm$ 0.07
25	880.2	3.06 $\pm$ 0.02
30	910.3	3.28 $\pm$ 0.07
35	935.0	3.54 $\pm$ 0.04

σ : standard deviation

Table 2: Characterisation of the annatto seeds

Annatto seeds		Fatty acid composition of oil		
Bixin content (% weight)	2.78 ± 0.01	Code	Name	% weight
Oil content (% weight)	3.14 ± 0.01	C10:0	C – Capric	10.7
Moisture content (%)	11.3 ± 0.1	C16:0	P – Palmitic	16.4
Seed diameter (mm)	3.50 ± 0.02	C18:0	S – Stearic	4.63
Porosity of seed bed (%)	44.0 ± 0.3	C18:1	O – Oleic	33.9
Apparent density (kg.m^{-3})	686 ± 5	C18:2	Li – Linoleic	34.3
True density (kg.m^{-3})	1220 ± 20	-	-	-

Table 3 contains the data on solubility of the seed bixin and Figure 3, the values represented as a function of pressure for each isotherm and as a function of temperature for each isobar. The solubility values for the bixin seed were substantially higher than those for the pure bixin, up to more than ten times higher; for example, at 35 MPa and 40°C, the solubility of pure bixin was 3.54 g m^{-3} but for seed bixin under the same conditions, the solubility was 43.9 g m^{-3} , indicating that the oil acted as a co-solvent with the CO_2 in the extraction of bixin, considerably improving its solubility. Because the oil

is highly soluble in supercritical CO_2 (Stahl et al., 1980) and bixin is an oil-soluble pigment (Pimentel and Stringheta, 1999), the solubility of bixin is significantly enhanced by the action of the oil present in the seeds. Figure 3 also shows a crossover point at about 28 MPa, a condition whereby solubility is independent of temperature. Above 28 MPa, an increase in temperature at constant pressure contributed favourably to an increase in solubility, the opposite occurring below 28 MPa, where a decrease in temperature at constant pressure favoured an increase in solubility.

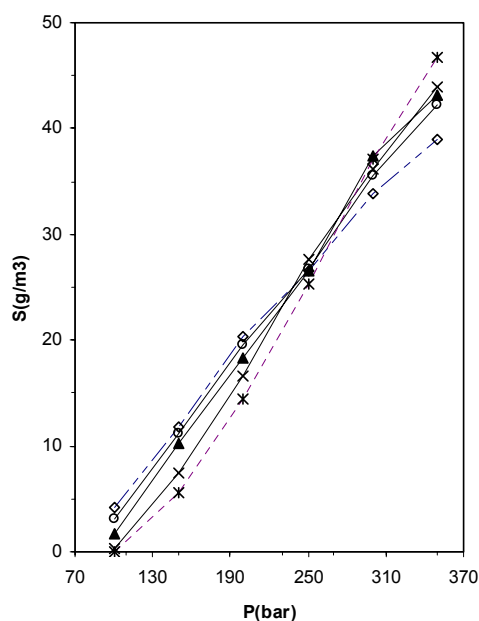


Figure 3a: Solubility of seed bixin as a function of pressure at different temperatures; $\diamond$ 40°C; $\square$ 35°C; $\blacktriangle$ 40°C; $\times$ 45°C and $*$ 50°C.

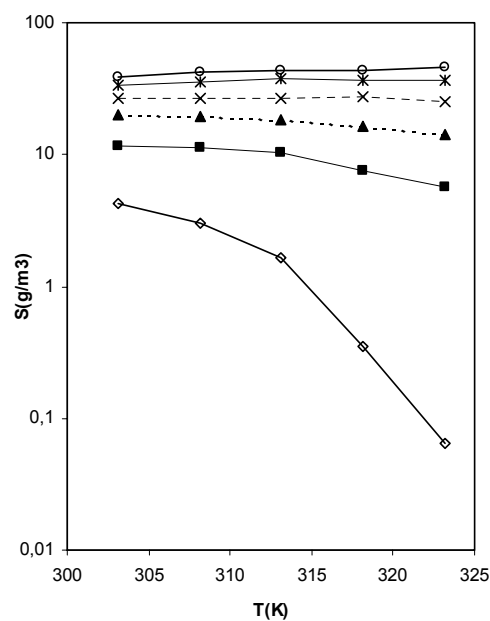


Figure 3b: Solubility of seed bixin as a function of temperature at different pressures; $\diamond$ 10MPa; $\blacksquare$ 15MPa; $\blacktriangle$ 20MPa; $\times$ 25MPa; $*$ 30MPa and $\circ$ 35MPa.

Table 3: Solubility S ($\text{g}\cdot\text{m}^{-3}$) of annatto seed bixin and CO_2 density ρ

T ($^{\circ}\text{C}$)	P (MPa)	ρ ($\text{g}\cdot\text{L}^{-1}$)	S ($\text{g}\cdot\text{m}^{-3}$) $\pm \sigma$
30	10	773.5	4.23 ± 0.57
	15	848.4	11.8 ± 0.8
	20	891.5	20.3 ± 0.7
	25	910.0	26.5 ± 0.6
	30	948.4	33.8 ± 1.4
	35	969.7	38.9 ± 1.0
35	10	714.5	3.05 ± 0.38
	15	816.5	11.2 ± 0.2
	20	866.7	19.6 ± 0.2
	25	902.0	26.7 ± 1.5
	30	929.5	35.6 ± 0.5
	35	952.5	42.2 ± 0.8
40	10	629.3	1.67 ± 0.20
	15	781.6	10.2 ± 0.9
	20	840.8	18.3 ± 0.2
	25	880.2	26.5 ± 0.6
	30	910.3	37.4 ± 1.2
	35	935.0	43.1 ± 0.6
45	10	500.8	0.35 ± 0.02
	15	743.3	7.47 ± 0.3
	20	813.6	16.6 ± 0.1
	25	857.8	27.7 ± 0.6
	30	891.3	36.2 ± 0.3
	35	917.4	43.9 ± 0.3
50	10	388.6	0.064 ± 0.02
	15	701.1	5.66 ± 0.18
	20	785.2	14.4 ± 0.1
	25	834.8	25.3 ± 1.1
	30	870.8	37.1 ± 0.2
	35	899.5	46.7 ± 0.1

 σ : standard deviation

Two factors are important in the dissolution of a solute, solvent density and temperature, expressed as the increase in solute vapour pressure. These contributions are clearly evident when analysing the values according to the Chrastil equation. It can be observed in Table 3 that at 10 MPa the density decreased from 770 g L^{-1} at 30°C to 390 g L^{-1} at 50°C , showing a reduction to half the original value, whilst at 35 MPa, the density remained practically the same, going from 970 g L^{-1} (30°C) to 900 g L^{-1} (50°C), a reduction of only 8%.

In analysing the effect of insoluble solids on bixin solubility, when oil and pigment extracted from the seeds were impregnated onto glass beads, it was

observed at 40°C and 20 MPa that solubility was 20.8 g m^{-3} compared to 18.3 g m^{-3} for solubility in the seeds, as shown in Table 4, indicating that the solid matrix had little effect on solubility.

The experimental values for bixin solubility are presented in terms of $\ln(S)$ versus $\ln(\rho)$ as shown in Eq. (3) and represented in Figure 4. The curves shown are linear but not parallel to one another, indicating the impossibility of fitting parameter k as a constant in the Chrastil equation (Chrastil, 1982). Since a good fit could not be obtained for the equation for the whole temperature range, the following form was chosen: $S = \rho^k \exp A$ with the values for parameters k and A shown in Table 5.

Table 4: Effect of a solid matrix on bixin solubility

		$S(\text{g}\cdot\text{m}^{-3})$
20 MPa and 40°C	In seeds	18.3 ± 0.2
	In glass balls	20.8 ± 0.7

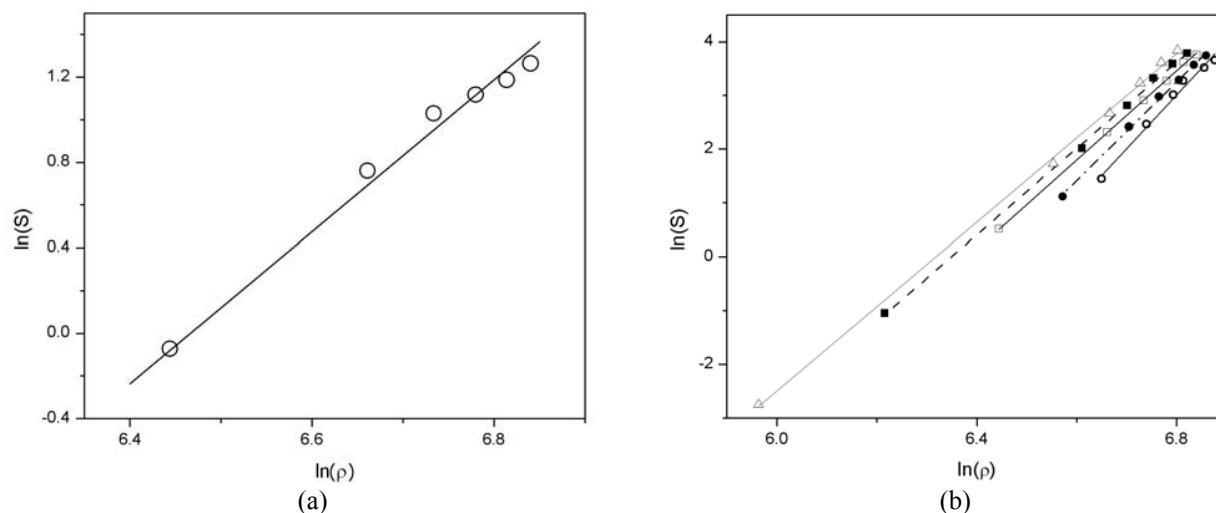


Figure 4: Solubility of bixin as a function of density fitted using the Chrastil equation: a) 93% pure bixin and b) seed bixin at different temperatures; ○ 30°C; ● 35°C; □ 40°C; ■ 45°C and △ 50°C.

Table 5: Parameters of the Chrastil equation as a function of temperature for the solubility of pure bixin and of that from seeds

	Temperature (°C)	k	A	R ²
Bixin from seeds	30	10.0085	-71.9562	0.98702
	35	9.18417	-66.1105	0.99823
	40	8.28635	-59.7947	0.99949
	45	8.03156	-57.9026	0.99908
	50	7.84666	-56.4822	0.99918
93% bixin	40	3.55983	-29.9272	0.98901

CONCLUSIONS

The values found for solubility were higher than those found by other researchers. As observed, the presence of oil in the seeds acted as a co-solvent with the CO₂, increasing solubility to more than ten times that of pure bixin. The insoluble solids of the seeds did not affect the solubility of the bixin, behaving as inert materials. The Chrastil equation could only be applied to one isotherm at a time, since the parameter k of the equation varied with temperature. The results presented in this work show that solubility of the main pigment present in annatto seeds, being oil-soluble, was greatly improved by the presence of oil in these seeds. These high solubility values show that the supercritical fluid technology can be used industrially to extract these pigments, offering the advantage of being clean, free of organic solvent and accordingly apt for use in the pharmaceutical, cosmetic and food industries.

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