OBTAINING TOCOTRIENOLS FROM ANNATTO SEEDS BY SFE OPERATING IN CONTINUOUS MODE AND INTEGRATION WITH LPSE TO OBTAIN BIXIN

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ABSTRACT

A process proposed in literature to obtain tocotrienols from annatto seeds was studied in a SFE system containing 2 vessels of 1 L each presenting ratios of height (H_B) to internal diameter (D_B) of 7.1 (E-1) and 2.7 (E-2). The SFE system was operated continuously at 40°C, 20 MPa and CO_2 flow rate of 20.7 g/min. The suitable extraction time, from the technical perspective, was set to 60 min, according to the kinetic parameters obtained from an overall extraction curve (OEC) with processing time of 360 min. The OECs were initially performed in batch mode (BM) and the kinetic parameters for the constant extraction rate (CER) period were: length of the CER period (t_{CER}) equal to 43±1 min for E-1 and 42±2 min for E-2; mass transfer rate (M_{CER}) of 0.22±0.01 g/min for E-1 and E-2; and total yield in the CER period (R_{CER}) of 50±1 g of extract/100 g of extractable for E-1 and 49±1 g of extract/100 g of extractable for E-2. No differences were observed between each group of kinetic parameters (t_{CER}; M_{CER}; R_{CER}) obtained in BM. Then, the continuous operating mode was carried out in four extraction stages of 60 min each with the extractors disposed in parallel, which enabled to improve the productivity of obtaining tocotrienols. Also based in literature we propose the integration of SFE with low-pressure solvent extraction (LPSE) using water and ethanol as solvents to remove bixin from the defatted annatto seeds. LPSE conditions were: 60°C and solvent mass to feed mass (S/F) ratio of 8 for ethanol; 50°C and S/F ratio of 8 for water. LPSE assays were performed in an agitated bed during 95 min. Using the partially defatted annatto seeds for LPSE enabled increasing the extraction efficiency of bixin. LPSE with ethanol was found to increase the bixin content in the extract. Thus, the process integration seems to be useful, mainly due to three cited aspects: (1) the initial removal of the oil layer via supercritical CO_2 favors the extraction of bixin, (2) LPSE is a low-cost methodology for extracting bixin and (3) the cost of raw material in the second step is null.

INTRODUCTION

Supercritical Fluid Extraction (SFE) of bioactive compounds from vegetal raw materials in batch mode is industrially undesirable. The pauses on the processes cause an increment of the operation time and, consequently, interfere on the productivity. SFE of target compounds using supercritical CO_2 in continuous mode is recommended, because using n ($n \geq 2$) extractor vessels at an industrial plant enables simulating a continuous operation mode by intercalating the charge/extraction/discharge steps of each vessel.

It is possible to find computational simulations of processes wherein the operations are performed in countercurrent mode with multi-stages to represent continuous extractions [1]. However, the lack of experimental information limits the performance of sequential studies

aiming to scale up, as experienced by Nunez *et al.* [1], Zabot *et al.* [2], Prado *et al.* [3], Prado *et al.* [4] and Pronyk *et al.* [5]. Firstly, information about continuous mode should be taken in laboratory scale to verify technical and economic feasibilities. After, the knowledge acquired might be used to encourage the transference of the studied technology to industrial scale. Regarding economic feasibility, there are simulations of the cost of manufacturing products from several vegetal raw materials [4, 6-9]; notwithstanding, information about continuous mode involving supercritical technology is still scarce.

From an implemented industrial plant operating in batch mode, we can change it for operating in continuous mode by adding valves, piping and extractor vessels. Indeed, the cooling, heating and pumping devices need to support handling with a large amount of solvent and extract. In such case, coupling other additional pumps is favorable to avoid pressure oscillation inside the beds during extraction step. Before performing a continuous mode extraction, we need to know the effective operation time on each bed (time of each stage) with the goal to obtain satisfactory yields which indicate the maximum productivity, also considering economics aspects (spent with labor, materials, etc.). This information is acquired by doing studies in batch mode to evaluate the behavior of OECs with respect to some variables, as temperature, pressure, solvent mass to feed mass (S/F) ratio, and so on.

Obtaining supercritical extracts in continuous mode is useful, because the processes involving this technology are reliable by producing extracts presenting high quality and free of solvents. Furthermore, as Meireles [10] mentioned, natural extracts do not compete with synthetic products with respect to cost. Natural extracts compete with synthetic products because they were elaborated using a natural resource, in addition they keep their original sensory quality.

In this context, the choice of the raw material is really important. Annatto was selected because it contains tocotrienols-rich lipid fraction [7, 11-13] and geranil geraniol [14, 15], which can be applied as nutritional supplement in food and beverages. Recently, annatto was identified as a rich resource of δ-tocotrienols, overcoming those other two known resources, palm oil and rice bran [7, 13]. Moreover, annatto extract is effective for preventing lipid peroxidation [16] and as antioxidant agent by combining the action of tocotrienols and bixin [17] to protect unsaturated fats against oxidative damages [18].

Annatto enables a great opportunity for doing process integration, because two target compounds are extracted with different solvents. The residue of obtaining tocotrienols-rich oil (defatted seeds) can be used for extracting bixin. Thus, the process is improved and more products are attained from this botanic matrix. In this case, the loaded material for extracting bixin is the residue coming from the first step (obtaining tocotrienols), being a low-cost material in the second step.

Thus, the objective of this study was based on the complete use of annatto seeds to: (1) obtaining tocotrienols-rich oil in continuous mode by SFE in a homemade laboratorial unit; and (2) developing process integration aiming to obtain bixin-rich extract by low-pressure solvent extraction (LPSE) with ethanol and water using the defatted seeds from step (1).

MATERIAL AND METHODS

Raw material

Annatto (*Bixa orellana* L.) seeds were obtained from local market of Campinas, Brazil. The seeds were packed in air impermeable bags and stored against light incidence at -18°C. The mean particle diameter (d_p) was determined according to Silva *et al.* [19]. The length, width and thickness of 20 randomly selected seeds were measured, and the geometric

mean was calculated. The moisture (U) was determined in duplicate by drying the samples at 105°C until constant mass. The procedures are described in AOAC [20]. The true density (ρ_r) of the annatto seeds was determined by pycnometry with helium gas at the Central Analytical Laboratory of the Institute of Chemistry/UNICAMP (Campinas, Brazil). The bed apparent density (ρ_a) was calculated by dividing the feed mass by the extraction vessel volume. The total porosity of the bed (ε) was calculated as $\varepsilon = 1 - (\rho_a/\rho_r)$.

Kinetic curves of obtaining annatto oil

Supercritical fluid extraction using CO_2 (SFE-CO_2) to obtain tocotrienols-rich oil from annatto was performed at 40°C and 20 MPa, as the best condition presented by Albuquerque and Meireles [7]. CO_2 (99.0% purity, Air Liquid, Campinas, Brazil) was used as the solvent. Firstly, the global yield of annatto extract (X_0) was obtained using the commercial Spe-ed unit (Applied Separations, 7071, Allentown-USA). The extraction was done using solvent mass to feed mass (S/F) ratio of 250. A 10 mL extractor was filled completely with 7.1 g of annatto and the CO_2 flow rate was maintained constant at 4.9 g/min. In SFE-2×1L unit [2], the beds were filled completely with 700 g of annatto for the experimental assays. All the experimental runs were duplicated. The extraction relative yield (R) was calculated using Equation 1.

$$R\,(g\ extract\ /\ 100 g\ extractable) = \frac{mass_{extract}(S/F)}{mass_{extract}(S/F = 250)} \cdot 100 \quad (1)$$

The experimental OEC data were fitted to a spline with 3 straight lines [21] using SAS 9.2® to estimate: the length of the constant extraction rate period – CER (t_{CER}); the mass transfer rate for the CER period (M_{CER}); the yield for the CER period (R_{CER}); the mass ratio of solute in the fluid phase at the extractor outlet for the CER period (Y_{CER}); the length of the falling extraction rate period – FER (t_{FER}); the mass transfer rate for the FER period (M_{FER}); the yield for the FER period (R_{FER}); the mass ratio of solute in the fluid phase at the extractor outlet for the FER period (Y_{FER}).

SFE-CO_2 of tocotrienols-rich oil in continuous mode

We selected the condition wherein the kinetic behaviors were similar between the beds by analyzing the results obtained in batch mode and based on the results obtained by Zabot *et al.* [2]. Thus, S/F of 2 was used to extract annatto oil during 60 min. This condition represents the best productivity of the process for each stage based on the evaluation of the kinetic parameters. The beds were filled completely with 700 g of annatto for each stage.

Selecting the ideal extraction time also depends on the analysis of the cost of manufacturing the oil to understand the best period to finish the extraction on each stage. Commonly, this period is near to t_{CER}. Once the economic analysis was not performed in this study, the time was selected based on the profile of the OECs in batch mode (directly linked to the kinetic parameters). In such case, the extraction time was fixed in 60 min and, consequently, the cycle time was 120 min. The cycle time was then divided as follows 5 min for loading, 5 min for pressurizing; 20 min for static time; 60 min for dynamic extraction; 25 min for depressurizing; and 5 min for unloading. To understand it better, Figure 1 illustrates the sequence of operation. It is possible to obtain extract continuously (red bars) and the stages 2, 3 and 4 always starts after the predecessor stage has run half of the processing time. Therefore, the total operating time with 4 stages was 310 min, including a cleaning period at the end of the operation. The process variables were maintained constant and equal to the batch mode. On each stage, eight samples of extract were collected in 0.1 L glass flasks (separation at ambient pressure) in gradual intervals.

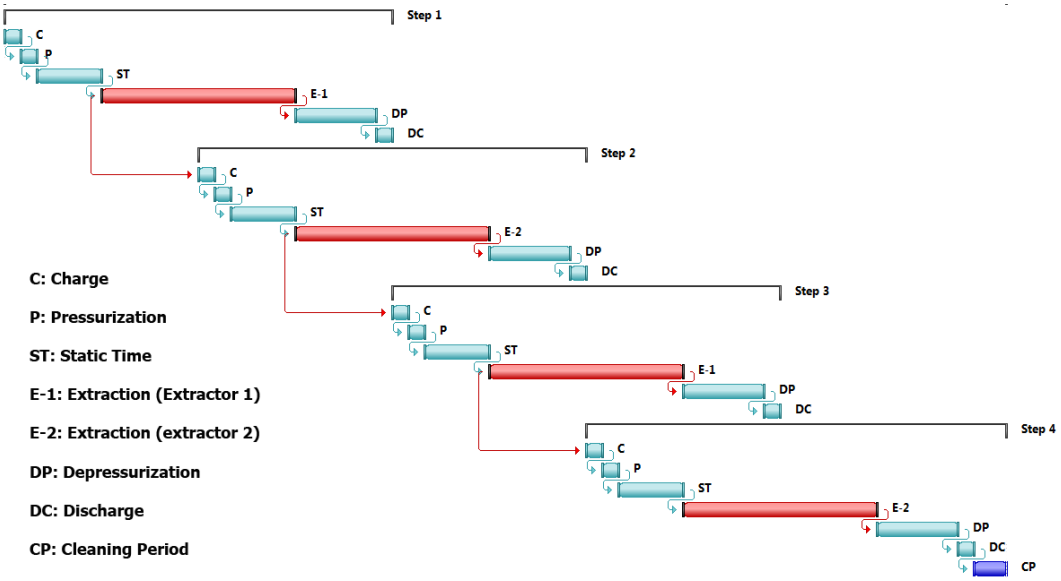

Figure 1: Gantt chart of operating in continuous mode in SFE-2×1L unit.

Obtaining bixin-rich extract by LPSE

Bixin-rich extract was obtained by low-pressure solvent extraction (LPSE). Ethanol and water were the solvents. As Rodrigues *et al.* [22] concluded, the bixin yields were larger for S/F = 8 than for S/F = 4; S/F = 8 was used. The assays were carried out in an agitated bed during 95 min. The conditions of temperature were 60°C for ethanol and 50°C for water [22].

The operational procedure consisted in loading 8 g of annatto seeds in erlenmeyers and adding 64 g of solvent. The erlenmeyers were placed in a shaker (Marconi, MA 420, Piracicaba, Brazil) under 297 rpm. After 95 min, the solution was filtered and ethanol was removed from the extracted mixture using a rotary vacuum evaporator (Logen Scientific, LSCS-1/52C, Diadema, Brazil) at 40°C. The aqueous extracts were freeze-dried (Liotop, L101, São Carlos, Brazil). The extract mass was determined with an analytical balance (Radwag, AS200/C/2, Radom, Poland). The assays were duplicated.

The extract yield ($R_{extract}$) was calculated as the ration of the extracted mass to the mass of raw material used (dry basis). The bixin total yield (B_{TY}) was obtained by exhaustive extraction using acetone (Êxodo Científica, Hortolândia, Brasil) until the defatted seeds became colorless. B_{TY} was used to calculate the bixin relative yield (B_{RY}), considering the bixin extracted ($B_{extracted}$) in the experimental assays (Equation 2):

$$B_{RY}\,(g\ bixin\ /\ 100 g\ annatto) = \frac{R_{extract} \cdot B_{extracted}}{B_{TY}} \tag{2}$$

Chemical analyses

The content of bixin in the extracts was measured according to FAO/WHO methodology [23] as adapted by Albuquerque and Meireles [7]. The extracts (≈5 mg of each sample) were diluted in acetone to yield suitable concentrations of bixin for analysis. Sample absorbance was measured at 487 nm with an UV–vis spectrophotometer (Hach DR/4000 U Loveland, Colorado, USA), and the bixin content was calculated according to the Lambert–Beer law (Equation 3), using $E_{1cm}^{1\%}$ = 3090 [23].

$$B_{extracted}\,(g\ /\ g\ extract) = \frac{A \cdot 10^4}{E_{1cm}^{1\%}} \cdot \frac{V_1 \cdot V_2}{m \cdot v_a} \tag{3}$$

Where: A is the absorbance; $E_{1cm}^{1\%}$ is the specific absorbance [23], V_1 and V_2 are the volumes of the dilutions (mL); v_a is the volume of the aliquot (mL); and m is the mass of extract (μg).

RESULTS

Annatto

Annatto seeds presented U = 16.0±0.4 (g/100 g of annatto), ρ_r = 1.37±0.01 (g/cm^3) and d_p = 3.69±0.04 (mm). X_0 value was 3.05±0.03 g/100 g of annatto. Table 1 shows the operational information defined for the continuous operation.

Table 1: Parameters defined to each extraction bed of SFE-2×1L for continuous operation.

Extractor	E-1	E-2
Raw material (kg)	0.7	0.7
ρ_a (kg/m^3)	700±1	700±1
ε (-)	0.49	0.49
CO_2 flow rate (g/min)	20.8±0.2	20.6±0.1
S/F (-)	2.1±0.1	2.0±0.1

Yield of tocotrienol-rich oil

Figure 2 presents the extraction kinetics of annatto oil in batch mode for the two beds, of SFE-2×1L, disposed in parallel configuration: E-1 (H_B/D_B = 2.7) and E-2 (H_B/D_B = 7.1). Although the H_B/D_B ratios are different, the accumulated yields in E-1 and E-2 during the constant extraction rate period are equal. Table 2 displays the kinetic parameters obtained for each experimental run. No differences were observed between each group of parameters, indicating a valid criterion for geometry shift and scale up for this raw material.

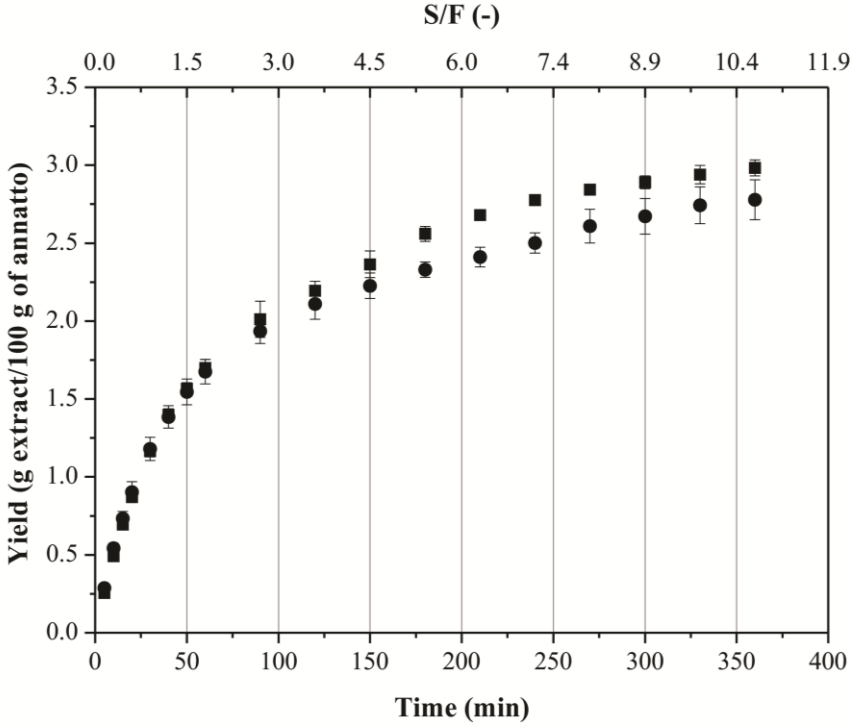

Figure 2: OEC for tocotrienols-rich oil obtained in SFE-2×1L unit: (■) E-1; **(●)** E-2.

SFE-CO_2 occurs in two different steps of mass transport. The first step consists in external mass transfer rate, when the solute is available on the particles surface to be solubilized by the solvent in the CER period. During the extraction process, the kinetic for obtaining bioactive compounds is modified along the time due to the solute depletion inside the bed. Thus, the second step consists in a falling extraction rate, which represents this solute depletion. It is desirable to maintain the extension of the extraction time on each stage, in a continuous mode operation, close to the first step (CER period), aiming to reach the maximum productivity. As can be seen in Figure 2, the yields during CER period were approximately 50%, which means the extraction of 50% of the extractable solute (X_0 = 3.05±0.03 g/100 g of annatto) from the biological matrix. In this way, an extraction stage consisted of 60 min and an operation cycle of 120 min. The results of two cycles are shown in Figure 3. In this figure we can see an extraction of 2.5 more extract in continuous mode than in batch mode for a defined time (240 min).

Nunez *et al.* [1] simulated an extraction unit containing multi-extractors (≥3). The extractors were connected together using a simulated moving bed system operating in countercurrent mode. In this plant, supercritical CO_2 is contacted first with the more exhausted substrate and then successively with progressively fresher substrate. The proposed configuration presents some restrictions, because the solute is removed from the bed up to reaching its maximum solubility in the solvent. Commonly, the solubility of bioactive compounds is low in supercritical CO_2. For instance, in this study the average Y_{CER} was 10.4±0.1 g extract/kg of CO_2, indicating a low value (≈1%).

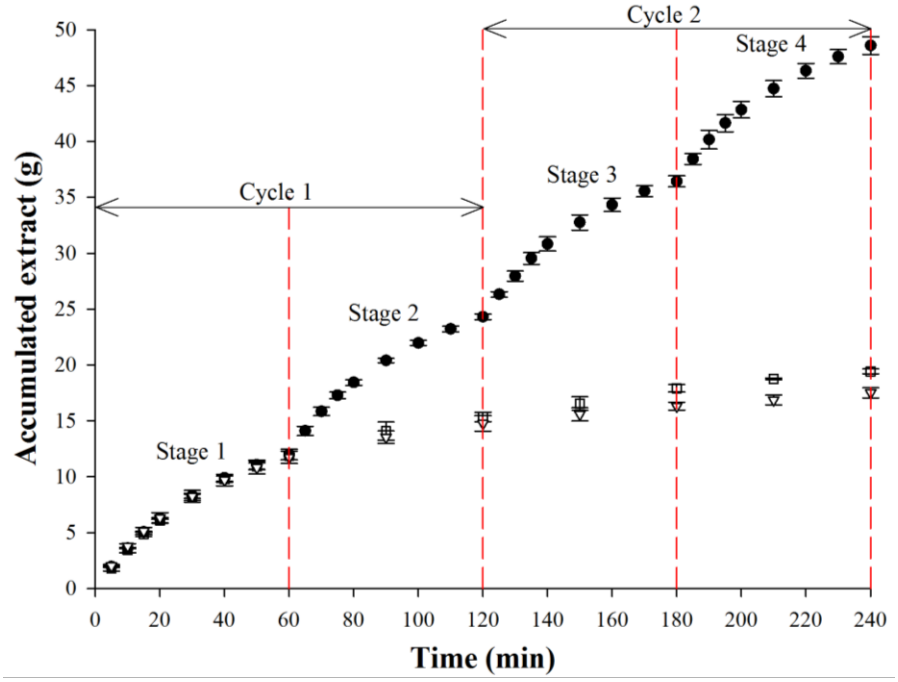

Figure 3: Extraction kinetics of tocotrienols-rich oil performed in continuous mode (●) and in batch mode for E-1 (□) and E-2 (▽).

Considering that the CO_2 might be recycled in both configurations (parallel, this study; countercurrent, Nunez *et al.* [1]), the less supply of CO_2 in the countercurrent mode is not a differential. Comparing both cited configurations, the driven forces of mass transfer are larger for the parallel mode. Therefore, the mass transfer rates are more pronounced in this mode, because the solutes are removed from the vegetal matrix using fresher solvent than that used in countercurrent mode. Thus, the solute concentration gradient between the phases is high for the parallel model.

Table 2: Data estimated for the kinetics of tocotrienol-rich oil in batch mode.

Extractor	t_{CER} (min)	M_{CER} (g/min)	R_{CER} (%)	Y_{CER} (g extract/kg CO_2)
E-2	42±2	0.22±0.01	49±1	10.5±0.7
E-1	43±1	0.218±0.006	50±0	10.4±0.2
Extractor	**t_{FER} (min)**	**M_{FER} (g/min)**	**R_{FER} (%)**	**Y_{FER} (g extract/kg CO_2)**
E-2	131±35	0.10±0.02	71±2	5.0±1.2
E-1	170±40	0.09±0.01	83±5	4.5±0.8

Process integration: obtaining bixin

The process integration for obtaining bixin and tocotrienols-rich oil was succeeded. Using ethanol (S/F = 8.0; T = 60°C; t = 95 min) we obtained 3.4±0.3 g of bixin/100 g of annatto. However, the recovery of bixin was lower when water was used, indicating 0.08±0.02 g of bixin/100 g of annatto. As cited on literature, annatto seeds contain from 1.0 to 6.3 g of bixin/100 g [7, 19, 24]. In such case, the results obtained in this study are in agreement with these articles. As Albuquerque and Meireles [25] presented, the bixin concentration in the extract is approximately 30%. According to our results, the bixin contents were 30% (30±2 g/100 g of extract) and 1.2% (1.2±0.3 g/100 g of extract) when using ethanol and water, respectively.

Bixin is the major colorant compound present in annatto seeds, being found as cis-bixin. Trans-bixin, trans-norbixin and cis-norbixin are also found [26]. At specific conditions of temperature and pH, cis-bixin can be transformed in cis-norbixin, which is a water-soluble compound [27]. Therefore, obtaining an expressive amount of bixin using ethanol represents that bixin isomers are more soluble in ethanol than in water. Bixin-rich extract can be applied as a natural colorant in textile [28] and food [27, 29-33] industries. Some studies also mention the use of bixin as antioxidant agent for meat processing [34] and for preventing retinal degeneration [35], strengthening the existence of several applications for bixin-rich extract.

Process integration brings forward a recent trend for obtaining bioactive compounds involving supercritical technology and LPSE. The target substance (bixin) is extracted from defatted seeds; likewise, defatted seeds come from the extraction of tocotrienols-rich oil. In this sense, it is possible to improve the processes by achieving high yields of desirable components using a low-cost raw material.

CONCLUSION

The results shown in this study allow us to conclude that it is possible to obtain tocotrienols-rich oil from annatto seeds by SFE-CO_2 in continuous mode using the adopted criterion for scale up (equal S/F ratio and constant extraction time). Furthermore, process integration was succeeded by integrating SFE plus LPSE to obtain tocotrienols and bixin, respectively. So, the future goal is to develop studies about economic aspects for encouraging companies to implement industrial plants for producing the cited natural products in large scale, emphasizing the use of green technologies.

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