Annatto seed oil fractionation by sequential supercritical CO_2 extraction

Renata Vardanega[a*], Gislaine C. Nogueira[a], Carla D. O. Nascimento[a], Adelia F. Faria-Machado[b], M. Angela A. Meireles[a]

[a]LASEFI - Department of Food Engineering, School of Food Engineering, University of Campinas (UNICAMP), R. Monteiro Lobato 80, 13083-862 Campinas, SP, Brazil

[b]Embrapa Food Technology, Avenida das Américas 29501, 23020-470 Rio de Janeiro, RJ, Brazil

* renatavardanega@gmail.com

ABSTRACT

The interest for annatto seed (*Bixa Orellana* L.) products has increased due to its potential applications in the food, pharmaceutical and textile industries. Besides the well known natural colorant bixin, annatto seeds also contain a tocotrienols-rich lipid fraction and geranylgeraniol. Tocotrienols and tocopherols constitute the vitamin E, however, studies have been shown that tocotrienols are more effective than tocopherols regarding antioxidant and anticancer activity. Geranylgeraniol is the major terpenic constituent of annatto seed and it has several therapeutic properties such as anti-inflammatory activity, action against Chagas disease, inhibition of the microorganism that causes tuberculosis and anticancer activity. Thus, the fractionation of the annatto seed extract to obtain the tocotrienols-rich fraction and a geranylgeraniol-rich fraction can be a promising route to diversify and to improve the added-value of annatto seed products. Pressure and temperature conditions resulting CO_2 densities ranging from 289 kg/m^3 to 915 kg/m^3 were investigated to find extracts with different chemical compositions. Extracts obtained at 289 kg/m^3 presented the highest geranylgeraniol content (39 ± 9 g/100 g of extract) and the lowest tocotrienols content (0.95 ± 0.04 g/100 g of extract). To recover fractions enriched with tocotrienols it was necessary to achieve CO_2 density higher than 628 kg/m^3. Therefore, a sequential sepercritical CO_2 extraction process performed in two steps can be useful to obtain a geranylgeraniol-rich fraction at 289 kg/m^3 in the first step followed by a tocotrienols-rich fraction obtained at 840 kg/m^3 in the second step.

Keywords: Geranylgeraniol, tocotrienols, *Bixa Orellana*, fractionation.

INTRODUCTION

Annatto seed is mainly known for its high colorant power due to the presence of the carotenoid bixin. Most of the studies involving the annatto seed extraction are focused on obtaining this colorant, since it is largely used by the industry [1-4]. Recently, the oil obtained from annatto seed has also been gained popularity for containing other well known bioactive compounds such as tocotrienols and tochopherols (popularly known as vitamin E) and geranylgeraniol [5, 6]. Tocotrienols have been reported to possess anticancer activity by delaying the development of mammary tumors [7] and also to reduce the risk of diseases. Geranylgeraniol is the major terpenic constituent of annatto seed and present several therapeutic properties,

such as anti-inflammatory activity [8], action against Chagas disease [9] and anticancer activity [10].

Besides the therapeutic properties of annatto seed oil, studies have reported that the initial removal of the oily fraction via supercritical CO_2 extraction can favor the subsequent extraction of bixin [3, 11, 12]. Thus, an integrated process to firstly recover the annatto seed oil by supercritical CO_2 extraction followed by a solvent extraction to recover the bixin fraction comes out as a promising alternative to enhance the utilization of this plant material in a biorefinery concept.

Albuquerque and Meireles [13] patented a supercritical CO_2 extraction process for obtaining the crude oil and defatted annatto seeds. In light of the rich chemical composition of annatto seed oil, the fractionation of the crude oil to obtain tocotrienols- and geranylgeraniol-enriched fractions is a way to improve the quality of these extracts in terms of high concentration of the target compounds [14]. Recently, a process was developed aiming to fractionate the crude oil obtained by supercritical CO_2 in two separators vessels, i. e., the first separator operating at 39 °C and 9 MPa, and the second separator operating at 9 °C and 4 MPa. The fractions obtained in each separator where qualitatively evaluated and presented distinct chemical profile [15].

Another strategy to recover fractions with distinct chemical composition is the sequential extraction by exploring the selectivity of CO_2 in different operational conditions. The tunability of the solvent power of CO_2 with pressure allows isolating target compounds during the extraction or fractionation of products [16-18]. Fractionated extractions from elderberry pomace were also performed using supercritical CO_2 extraction followed by enhanced solvent extraction with CO_2/EtOH/H_2O mixtures as solvent to recover the lipid fraction and anthocyanin-rich extracts, respectively [18]. Viganó et al. [19] successfully obtained three extract fractions of passion fruit bagasse concentrated in different target compounds by applying a sequential supercritical CO_2 extraction process with different combinations of temperature and pressure. Thus, the objective of this work was to investigate the influence of operational conditions on the chemical composition of annatto seed extracts aiming the proposal of a sequential supercritical CO_2 process to recover fractions with different compositions.

MATERIAL AND METHODS

Plant material and reagents

Annatto seeds (*Bixa orellana*) were purchased from Grão Salutte Vida Saudável (Campinas, Brazil). The seeds were manually selected and cleaned to remove heavy dirt and impurities and stored at -10 °C until the extractions. The seeds were not comminuted because the lipid fraction is mainly located on the particle surface. Carbon dioxide (purity > 99.9%) was purchased from Gama Gases Especiais Ltda (São Bernardo do Campo, Brazil).

Supercritical CO_2 extractions

The experimental assays were performed in a commercial Spe-ed unit (Applied Separations, 7071, Allentown, USA). The 25-mL extraction vessel was filled with 17 g of whole annatto seeds, resulting a bed density of 680 kg/m^3 and a bed porosity of 0.50. In the sequence, CO_2 was pumped in the bed and the condition of pressure and temperature was reached after 20 min (static period). Subsequently, the micrometric

valve was opened at a constant CO_2 flow rate to collect the extract. The CO_2 flow rate used for all assays was 5 g/min until reach the solvent to feed ratio of 25 (approximately 85 min).

Chemical characterization

Geranylgeraniol quantification

Geranylgeraniol quantification was performed according to the method described by Silva et al. [6] using high-performance liquid chromatograph equipped with a photodiode array detector (HPLC-DAD, Waters, Alliance E2695, Milford, USA). The compounds separation was obtained in a fused-core column (Kinetex C_{18}, 100 × 4.6 mm × 2.6 µm, Phenomenex, Torrance, USA). The mobile phase was composed by a solution containing methanol: ammonium acetate 50 mM (90:10, v/v). The column was maintained at 40 °C and the flow rate was 1 mL/min for 10 min in isocratic mode. An aliquot of 10 µL of each sample diluted to 500 ppm in ethanol was injected. Geranylgeraniol was detected at 210 nm and its quantification was performed using external calibration curve obtained with geranylgeraniol standard (purity > 85%, Sigma-Aldrich, St. Louis, USA).

Tocotrienols quantification

The tocotrienols quantification was performed using HPLC equipped with fluorescence detector (HPLC-FLR, Waters, Alliance E2695, Milford, USA). The tocotrienols separation was obtained in a C_{30} column (250 × 4.6 mm, 5µm, YMC, Allentown, USA). The mobile phase was methanol (A) and methylic tert-butyl ether (B), using the following gradient: 0 min 90% A; 0.5 min 85% A; 14.5 min 15% A; 15 min 10% A; 16 min 10% A. The column was maintained at 35 °C and the flow rate was 0.8 mL/min. The excitation and emission wavelengths used in the detector were 290 and 330 nm, respectively. Tocotrienols were quantified using external calibration curve of δ-tocopherol (purity ≥ 90%, Sigma-Aldrich, St. Louis, USA) and γ-tocotrienol (purity ≥ 95%, Merck, Darmstadt, Germany).

Statistical analysis

The influence of temperature (40 and 60 °C) and pressure (10, 17, 24 and 31 MPa) conditions on the extraction yield and chemical composition was evaluated using a fully randomized, full factorial design in duplicate. The influence of the parameters was determined by analysis of variance (ANOVA) using Minitab 16® (Minitab Inc, State College, USA) with 95% of confidence level (p-value ≤ 0.05). The significant differences between the treatments were evaluated using Tukey's procedure.

RESULTS AND DISCUSSION

Influence of the supercritical CO_2 parameters on the global yield

The influence of the supercritical CO_2 parameters was evaluated in a single-stage process in order to understand the effect of temperature and pressure on the global yield and composition of the annatto seed oil. The global yields ranged from 0.92 ± 0.03 to 2.6 ± 0.02 g/ 100 g of annatto seed (Table 1). Temperature and pressure had

significant effect on the global yield (p-value < 0.001). It can be verified that the increase in the operational pressure at a constant temperature resulted in the enhancement of global yield, which is mainly related to the increase of CO_2 density. The great increase of global yield was mainly observed with the CO_2 density increases from 289.95 to 628.61 kg/m^3 (0.92 ± 0.03 to 1.88 ± 0.04 g/ 100 g of annatto seed). The further CO_2 density increases from 664.59 to 915.24 kg/m^3 presented a slight difference in the global yield.

Table 1: Global yield, geranylgeraniol and tocotrienols contents of annatto seed oil obtained in different temperature and pressure conditions.

Pressure (MPa)	CO_2 density (kg/m^3)	Global yield (g/100 g annatto seed)	Geranylgeraniol (g/ 100 g oil)	Tocotrienols (g/ 100 g oil)
		40 °C		
10	628.61	1.88 ± 0.04 ^{e}	32.9 ± 0.4 ^{a,b}	8.5 ± 0.2 ^{b}
17	807.87	2.2 ± 0.1 ^{c,d}	28 ± 1 ^{a,b}	11 ± 1 ^{a}
24	872.48	2.3 ± 0.1 ^{b,c,d}	26.8 ± 0.3 ^{a,b}	10.8 ± 0.1 ^{a}
31	915.24	2.5 ± 0.1 ^{a,b}	26 ± 0.1 ^{a,b}	10.9 ± 0.2 ^{a}
		60 °C		
10	289.95	0.92 ± 0.03 ^{f}	39 ± 9 ^{a}	0.95 ± 0.04 ^{c}
17	664.59	2.14 ± 0.02 ^{d}	30 ± 2 ^{a,b}	10.44 ± 0.04 ^{a}
24	776.04	2.4 ± 0.1 ^{a,b,c}	26 ± 1 ^{a,b}	11.7 ± 0.4 ^{a}
31	836.98	2.6 ± 0.02 ^{a}	25 ± 1 ^{b}	11.4 ± 0.1 ^{a}

Influence of the supercritical CO_2 parameters on the geranylgeraniol content

The geranylgeraniol content of the annatto seed oils obtained in different extraction conditions are presented in Table 1, which ranged from 25 ± 1 to 39 ± 9 g/ 100 g of oil. This is the first time that the geranylgeraniol content of annatto seed oils was evaluated in different temperature and pressure conditions, since the previous works reporting geranylgeraniol content of annatto seed oils applied 40 °C and 20 MPa, as optimized by Albuquerque and Meireles [12] which presented geranylgeraniol contents of 18.9 - 25.0 g/ 100 g of oil [6, 20].

In the present work, it was observed that only pressure had a significant effect on geranylgeraniol content (p-value = 0.010). It can be seen in Table 1 that the highest geranylgeraniol content as well as the lowest global yield were obtained at CO_2 density of 289.95 kg/m^3 (60 °C and 10 MPa), the lowest density among the studied conditions. CO_2 densities higher than 628.61 kg/m^3 produced oils with geranylgeraniol content similar to those reported for annatto seed oils obtained by supercritical CO_2 extraction at 40 °C and 20 MPa [6, 20]. These results suggest that extractions performed at low CO_2 densities can be more selective for terpenes compounds such as geranylgeraniol. Xynos et al. [21] also observed that lower CO_2 densities improved the selectivity for recovering the volatile fraction from mastic gum, mainly constituted by terpenes.

Influence of the supercritical CO_2 parameters on the tocotrienols content

The tocotrienols content presented in Table 1 corresponds to the sum of δ- and γ-tocotrienols isomers, which are the major among the tocotrienols isomers found in annatto seed oil [12, 20]. Table 2 present the content of each δ- and γ-tocotrienols of the oil obtained in different extraction conditions, where it can be seen that δ-tocotrienol was the predominant isomer. The total tocotrienols content of annatto seed oil reported

in the literature (12.9 – 13.7 g/ 100 g of extract) [12, 20] are similar to those reported in this work (Table 1).

Table 2: Tocotrienols content of annatto seed oil obtained in different temperature and pressure conditions.

Pressure (MPa)	δ-tocotrienol (mg/ g of oil)	γ-tocotrienol (mg/ g of oil)	Total tocotrienols (mg/ g of oil)
40 °C			
10	63 ± 2	21.5 ± 0.3	85 ± 2
17	80 ± 4	26 ± 1	107 ± 5
24	82 ± 1	26.2 ± 0.5	108 ± 1
31	83 ± 1	26 ± 2	109 ± 2
60 °C			
10	7.3 ± 0.3	2.2 ± 0.2	9.5 ± 0.3
17	78.8 ± 0.4	25.62 ± 0.05	104.4 ± 0.3
24	89 ± 3	29 ± 0.2	117 ± 3
31	86.3 ± 0.7	27.5 ± 0.7	114 ± 1

It can be observed that the lowest total tocotrienols content of 0.95 ± 0.04 g/ 100 g oil was obtained at the lowest CO_2 density studied (289.95 kg/m^3) while CO_2 densities higher than 628.61 kg/m^3 resulted oils with very similar tocotrienols content. It suggests that the solubility of tocotrienols is reduced in CO_2 densities lower than 664.59 kg/m^3. Viganó et al. [19] reported that passion fruit bagasse extract obtained by SFE using CO_2 densities lower than 748 kg/m^3 were more concentrated in tocols than those obtained at higher CO_2 densities and suggested that lower CO_2 densities are more selective for tocols. However, it is important to mention that the lowest CO_2 density studied by these authors was 669 kg/m^3.

These findings are import to stablish the operating conditions that allow obtaining annatto seed oils with different chemical compositions by varying temperature and pressure. The results of the present work demonstrated that at CO_2 density of 289.95 kg/m^3 it was possible to obtain an annatto seed oil with the highest geranylgeraniol content and the lowest tocotrienols content. Thus, a two-steps sequential CO_2 extraction can be useful to obtain fractions with different compositions, where a geranylgeraniol-rich fraction with low tocotrienols content is recovered at 60 °C and 10 MPa; and a tocotrienols-rich fraction is subsequently recovered at 40 °C and 24 MPa. Further experiments are needed in order to stablish the appropriate amount of solvent and time necessary for each step of the process. This information can be provided by kinetic experiments and analysis of the overall extraction curve.

CONCLUSION

The results demonstrated that the supercritical CO_2 extraction performed at different operational conditions in terms of temperature and pressure resulted annatto seed oils with different chemical compositions. The oil obtained at low CO_2 density (289.95 kg/m^3) resulted a fraction enriched in geranylgeraniol with a low tocotrienols content. This finding enables to stablish a two steps-sequential supercritical CO_2 extraction process to obtain a geranylgeraniol-rich fraction followed by a tocotrienols-rich fraction. From a biorefinery point of view, these results are a feasible strategy to diversify the annatto seed products as well as to improve its economics.

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REREFENCES

[1] T. Taham, F.A. Cabral, M.A.S. Barrozo, Extraction of bixin from annatto seeds using combined technologies, The Journal of Supercritical Fluids, 100 (2015) 175-183.
[2] T. Taham, D.O. Silva, M.A.S. Barrozo, Improvement of bixin extraction from annatto seeds using a screen-topped spouted bed, Separation and Purification Technology, 158 (2016) 313-321.
[3] L.M. Rodrigues, S.C. Alcázar-Alay, A.J. Petenate, M.A.A. Meireles, Bixin extraction from defatted annatto seeds, Comptes Rendus Chimie, 17 (2014) 268-283.
[4] S.C. Alcázar-Alay, J.F. Osorio-Tobón, T. Forster-Carneiro, M.A.A. Meireles, Obtaining bixin from semi-defatted annatto seeds by a mechanical method and solvent extraction: Process integration and economic evaluation, Food Research International, 99 (2017) 393-402.
[5] E.K. Silva, M.T.M.S. Gomes, M.D. Hubinger, R.L. Cunha, M.A.A. Meireles, Ultrasound-assisted formation of annatto seed oil emulsions stabilized by biopolymers, Food Hydrocolloids, 47 (2015) 1-13.
[6] E.K. Silva, G.L. Zabot, M.A. A. Meireles, Ultrasound-assisted encapsulation of annatto seed oil: Retention and release of a bioactive compound with functional activities, Food Research International, 78 (2015) 159-168.
[7] E. Pierpaoli, V. Viola, A. Barucca, F. Orlando, F. Galli, M. Provinciali, Effect of annatto-tocotrienols supplementation on the development of mammary tumors in HER-2/neu transgenic mice, Carcinogenesis, 34 (2013) 1352-1360.
[8] P. Giriwono, H. Shirakawa, Y. Ohsaki, S. Hata, H. Kuriyama, S. Sato, T. Goto, M. Komai, Dietary supplementation with geranylgeraniol suppresses lipopolysaccharide-induced inflammation via inhibition of nuclear factor-κB activation in rats, Eur J Nutr, 52 (2013) 1191-1199.
[9] R. Menna-Barreto, G. Laranja, M. Silva, M. Coelho, M. Paes, M. Oliveira, S. De Castro, Anti-Trypanosoma cruzi activity of Pterodon pubescens seed oil: geranylgeraniol as the major bioactive component, Parasitology research, 103 (2008) 111-117.
[10] R. Katuru, N.V. Fernandes, M. Elfakhani, D. Dutta, N. Mills, D.L. Hynds, C. King, H. Mo, Mevalonate depletion mediates the suppressive impact of geranylgeraniol on murine B16 melanoma cells, Experimental biology and medicine, 236 (2011) 604-613.
[11] M.N. Moraes, G.L. Zabot, M.A.A. Meireles, Extraction of tocotrienols from annatto seeds by a pseudo continuously operated SFE process integrated with low-pressure solvent extraction for bixin production, The Journal of Supercritical Fluids, 96 (2015) 262-271.
[12] C.L.C. Albuquerque, M.A.A. Meireles, Defatting of annatto seeds using supercritical carbon dioxide as a pretreatment for the production of bixin: Experimental, modeling and economic evaluation of the process, The Journal of Supercritical Fluids, 66 (2012) 86-95.
[13] M.A.A. Meireles, C.L.C. Albuquerque, Processo de obtenção de óleo de urucum e sementes desengorduradas, in: INPI (Ed.), Brasil, 2013.

[14] N.A. Gañán, J.S. Dambolena, R.E. Martini, S.B. Bottini, Supercritical carbon dioxide fractionation of peppermint oil with low menthol content – Experimental study and simulation analysis for the recovery of piperitenone, The Journal of Supercritical Fluids, 98 (2015) 1-11.
[15] A.L. Santana, J.C.F. Johner, M.A.A. Meireles, Thin-layer chromatography profle of annatto extracts obtained with supercritical carbon dioxide and subsequently high-pressure pahse equilibrium data, in: XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos, Gramado, RS, 2016, pp. 1-6.
[16] F. Temelli, O.N. Ciftci, Developing an integrated supercritical fluid biorefinery for the processing of grains, The Journal of Supercritical Fluids, 96 (2015) 77-85.
[17] J. Viganó, A.P.d.F. Machado, J. Martínez, Sub- and supercritical fluid technology applied to food waste processing, The Journal of Supercritical Fluids, 96 (2015) 272-286.
[18] I.J. Seabra, M.E.M. Braga, M.T. Batista, H.C. Sousa, Effect of solvent (CO_2/ethanol/H_2O) on the fracionated enhanced solvent extraction of anthocianins from elder berry pomace, The Journal of Supercritical Fluids, 54 (2010) 145-152.
[19] J. Viganó, J.P. Coutinho, D.S. Souza, N.A.F. Baroni, H.T. Godoy, J.A. Macedo, J. Martínez, Exploring the selectivity of supercritical CO2 to obtain nonpolar fractions of passion fruit bagasse extracts, The Journal of Supercritical Fluids, 110 (2016) 1-10.
[20] R. Vardanega, I.A. Dalmolin, G.C. Nogueira, T. Hatami, M.A.A. Meireles, Phase behaviour and mathematical modelling for the system annatto seed oil in compressed carbon dioxide+ethanol as co-solvent, The Journal of Supercritical Fluids, 130 (2017) 56-62.
[21] N. Xynos, A. Termentzi, N. Fokialakis, L.A. Skaltsounis, N. Aligiannis, Supercritical CO2 extraction of mastic gum and chemical characterization of bioactive fractions using LC-HRMS/MS and GC–MS, The Journal of Supercritical Fluids, 133 (2018) 349-356.