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Modifier-Enhanced Supercritical CO_2 Extraction with GRAS Solvents of Coumarin from Cumaru Seeds (Dipteryx Odorata)

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Cumaru seeds (*Dipteryx odorata*) are commercialized as flavoring in cigarettes, chocolate, perfumes, cosmetics and naturopathy. *Dipteryx odorata* is the specie that has the highest content of the coumarin compound in the Dipteryx family. Furthermore, coumarin has a pharmacological potential against prostate cancer, prevention of metastasis, prevention and retardation of malignant cancer. Coumarin compounds are obtained by organic solvent conventional methods from cumaru seeds. However, the solvent organic extraction method has some inconvenient such as low selectivity, residual traces of organic solvent in extract and separation process demands high-energy expenditure. Supercritical extraction method has been widely used to obtain natural extracts with highly purity, cause it does not contain organic solvents, and so it is suitable for various pharmaceutical and food applications. In this work, coumarin was obtained from cumaru seeds by supercritical extraction using different experimental conditions of temperature, pressure, type and percentage of modifier. Soxhlet extraction with hexane and ethanol was used as a reference method. The content of coumarin on the extracts obtained by Soxhlet technical were 0.5 wt% for ethanol and 1.43 wt% for hexane, respectively. Modifier-enhanced supercritical CO_2 extraction of the coumarin showed a higher selectivity than extraction using Soxhlet technical. Particularly, to the extraction with 3 wt% ethanol as modifier, 353 K and 18 MPa, coumarin content was 20.9 wt% on extract. Termogravimetric analysis (TGA) showed no degradation of the coumarin in the temperatures of extractions carried out. Finally, it was possible to obtain coumarin with high purity directly from the cumaru seeds using the technical of the modifier-enhanced supercritical CO_2 extraction with GRAS solvents.
Keywords: Coumarin; Cumaru seed; Supercritical fluid extraction; modifier, Soxhlet.

1. Introduction

Dipteryx odorata, popularly known as cumaru, is an arboreal species, northern native of Brazil, Central America, Guyana and Venezuela. The seed is the major source of coumarin among the species of *Dipteryx* family (Allen & Allen 1981). Coumarin ($C_9H_6O_2$) is a compound formed by one aromatic ring fused to a lactone ring (Oliveros-bastidas et al. 2013). It showed satisfactory effect against prostate cancer, prevention of metastasis and retardation of malignant cancer (Mohler et al. 1994; Thornes et al. 1994). The antinociceptive, anti-inflammatories and bronchodilators of different plants are related to the presence of coumarin (Leal et al. 2000) and that is present in several groups of drugs (Venugopala et al. 2013). Maceration, Soxhlet extraction, ultrasound were performed in the past to obtain coumarin from cumaru seeds. However, there are some aspects that should be avoided in extractions of bioactive compounds, such as the toxicity of the solvents, the stability of the compounds and the selectivity of the process used. The supercritical fluids extraction (SFE) is an alternative method that allows the enhancement of the extraction (Ahangari & Sargolzaei 2012; Lemos et al. 2012). Among the various gases used in SFE, carbon dioxide is the most common, being highly soluble in nonpolar solutes and high molecular weight. To increase the interaction with polar solutes, there is the

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possibility of adding polar modifiers to CO_2, in small amounts, in order to overcome the disadvantages mentioned above in relation to organic solvent (Sullivan 1982). Ethanol, ethyl acetate and dichloromethane are considered GRAS solvents (Generally Regarded as Safe) and most widely used as modifiers for the extraction of antioxidants bioactive components (Kitzberger et al. 2007; Meireles 2009). Modifier-enhanced supercritical CO_2 extractions of the coumarin from cumaru seeds reports in the literature are still scar. There are many works in literature using carbon dioxide and co-solvents to extraction coumarin from guaco leaves, since guaco leaves are coumarin source most studied. Vilegas et al. (1997) extracted coumarin from guaco leaves using five methods for the extraction of low-polarity compounds from Mikania glomerata Spreng. The highest content of coumarin in extract was 7.7% with liquid pressure n-hexane. Lanças et al. (1997) used supercritical carbon dioxide (scCO2) and mixtures of CO_2 and ethanol, but a low perform in extraction of coumarin using pressure of up to 8.5 MPa was achieved. The extraction experiments of coumarin with supercritical CO_2 from the emburana seeds carried out by Rodrigues et al. (2008) did not reached good performs. Oliveira et al. (2013) used scCO2 without modifier for coumarin extractions from the two guaco leaves species (Mikania laevigata and Mikania glomerata) and it was obtained a low coumarin content. So, this work investigated the variables of processes to coumarin extraction using the technique of supercritical CO_2 extraction with modified GRAS solvents from the cumaru seeds. Extractions were carried out using a fractional factorial design known as Taguchi method (orthogonal array L9), which enables the evaluation of four variables and three levels (Ansari & Goodarznia, 2012; Desai & Parikh, 2012) through few experiments. The variables evaluated were temperature (333, 343 and 353 K), pressure (18, 20 and 22 MPa), modifier type (ethyl acetate, ethanol and dichloromethane) and modifier percentage (1, 3 e 5wt%), and constant flow of 3 mL min^{-1}. Thermogravimetric analysis was performed in order to check if any thermic degradation of components on extraction process temperatures could take place (Santos et al. 2012; Shadangi & Mohanty 2014). Analysis by by liquid chromatography of the chemical composition of the extracts from the cumaru seeds obtained by supercritical and Soxhlet extraction were aimed this work.

2. Experimental

2.1 Materials

Liquefied CO_2 of purity 99.9% was supplied by White Martins SA (Brazil), in pressurized deep tube cylinders. The entire modifier and solvents such as ethyl acetate (EAc), ethanol (EtOH), dichloromethane (DCM) and hexane, with analytical grade, and standard coumarin, were obtained from Synth Ltd. (Brazil). The total of 3 kg of cumaru seed were purchased in Municipal Market of Belém city, in the state of Pará, Brazil. The seeds were crushed and the average particle size was between 1 and 2 mm. Crushed seeds were stored under refrigeration until the starting extractions. To determine the moisture content, 5 g of the seeds were dried in an oven (Nova. Ética, model 400/4ND, Brazil) at 323 K and weighed periodically until constant weight. After that, a final moisture was determined, resulting in approximately 10%.

2.2 Supercritical fluids extraction

The modifier-enhanced supercritical CO_2 extractions with GRAS solvents were carried out in a self-home apparatus. The apparatus included two pumps modifier system (ISCO, Model 500D), two thermostatic baths and an extractor of 165 mL of capacity. The detailed description were given in previous work (Lemos et al. 2012; Gonçalves et al. 2013; Garcia et al. 2012). Initially, carbon dioxide and modifier were pressurized using SISCO pump system. In each extraction run, it was used 60 g of crushed cumaru seeds. After pressurization of extractor, cumaru seeds and scCO2-solvent mixture were kept in contact by 20 min. In all runs, the flow rate was 3 mL min^{-1}. The extract was collected into vials and the residue of organic solvent in extract was evaporated on a drying oven standard (Nova. Ética, model 400/4ND, Brazil) until a constant weight of sample. The extraction time was performed for 100 min. Taguchi design (L9) with three levels, resulting in nine experiments performed in duplicate was used to run experiments.

2.3 Soxhlet extraction

Since Soxhlet extraction is a conventional extraction technique, it was used for comparison purpose. Approximately 25 g of cumaru were extracted in Soxhlet for 48 h using hexane as solvent, at 333 K. Solvent excess was removed by rotary vacuum evaporator at 333 K. The mass obtained for each solvent extraction were expressed in mass percent yield. The same procedure was done using ethanol. The oil samples were stored in dark for further analysis.

2.4 Thermogravimetric analysis (TGA)

TGA were performed using DTG-60 H Shimadzu- with nitrogen flow of 100 $mL.min^{-1}$, from room temperature up to 873 K, with a heating ramp of 283 $K.min^{-1}$ using a sample weight of 5±0.5mg. This procedure was performed for the standard coumarin and extracts obtained by Soxhlet and SFE.

2.5 Coumarin analyses

Analyzes of the extracts was developed using liquid chromatography equipment Varian Polaris Model 210. A Luna 5μm column, stationary phase C18 (2) 100A (150 x 4.6mm) was employed at 303 K. Samples were manually injected in a volume of 2 μL. The mobile phase was 5% of acetic acid aqueous phase (Phase A) and a mixture of 60% acetonitrile and 40% methanol (Phase B) used in gradient mode, using a flow rate of 1.0 mL min-1. The gradient programs was used according to the following profile: 60% A (0 – 5 min), 60 – 40% A (5 - 10 min), 40 - 10% A (10 –20 min), 10% A (20 – 25 min), 10 – 60% A (25 – 30 min). The detection was at a 274 nm wavelength using a UV-VIS detector. The data were processed through the Galaxie software. A solution with standard sample Coumarin (Synth 99.5%) was also injected in order to compare the retention time. For the quantification of the compound in the extract, it was made a standard calibration curve from Coumarin concentrations of 20, 25, 30, 35 and 40 ppm.

3. Results

3.1. Supercritical Fluid Extract

Results obtained in the supercritical extraction and organic solvents extraction are shown in Table 1. Maximum yield achieved using the SFE was of 4.8% on higher pressure condition. High pressures results in higher CO_2 density and influence in its dissolution power, specially near the critical point mixture (Lemos et al. 2012). Soxhlet extractions resulted in higher yields (19.1 and 46.6%) than SFE, particularly using ethanol as solvent. This behavior may be related to the time of extraction, solvent recycling, and solvent polarity (Gonçalves et al. 2013; Benelli et al. 2010). The samples obtained by extraction with Soxhlet had a visual aspect very different than obtained by SFE.

Table 1 Experimental design (L9), Soxhlet extractions, mass percent yields and coumarin content on each extraction runs.

Exp	Organic solvent	T (K)	P (MPa)	% Mod	yield (%)	coumarin in extract (%)
1	EAc	333	18	1	3.4±0.2	7.2
2	EAc	343	20	3	3.3±0.2	5.3
3	EAc	353	22	5	3.9±0.2	2.1
4	EtOH	333	20	5	4.2±0.2	11.6
5	EtOH	343	22	1	2.9±0.1	8.2
6	EtOH	353	18	3	1.9±0.1	20.9
7	DCM	333	22	3	4.8±0,2	4.0
8	DCM	343	18	5	2.6±0.2	11.8
9	DCM	353	20	1	2.3±0.1	13.1
10	Soxhlet - Ethanol	318	-	-	46.5±0.3	0.5
11	Soxhlet - Hexane	318	-	-	19.1±0.9	1.4

The analysis of variance (ANOVA) of the effects of the factors for the mean are shown in Table 2. According to the p-value, all factors evaluated are significant (<0.05). The experimental error was obtained as a function of the replicate with 9 degrees of freedom.
The values of the effects of the factors on the average for each physical level are shown in Figure 1. Temperature and pressure factors have more influence than modifier type and percentage. The same results can be observed in Table 2.

Table 2. ANOVA for the investigated values. Regression coefficients individually estimated and their interactions for cumaru extract yields and coumarin concentrations in these extracts

Effect	SS	DF	MS	F	p-value
Modifier type	0.701111	2	0.350556	11	0.003
Temperature (K)	6.537778	2	3.268889	107	0.000001
Pressure (MPa)	3.967778	2	1.983889	65	0000004
Modifier (%)	1.621111	2	0.810556	26	0.0002
Error	0.275000	9	0.030556		

SS: Sums of Squares; DF: Degrees of Freedom; MS: Mean Squares; F: F – Ratio; p-value: probability.

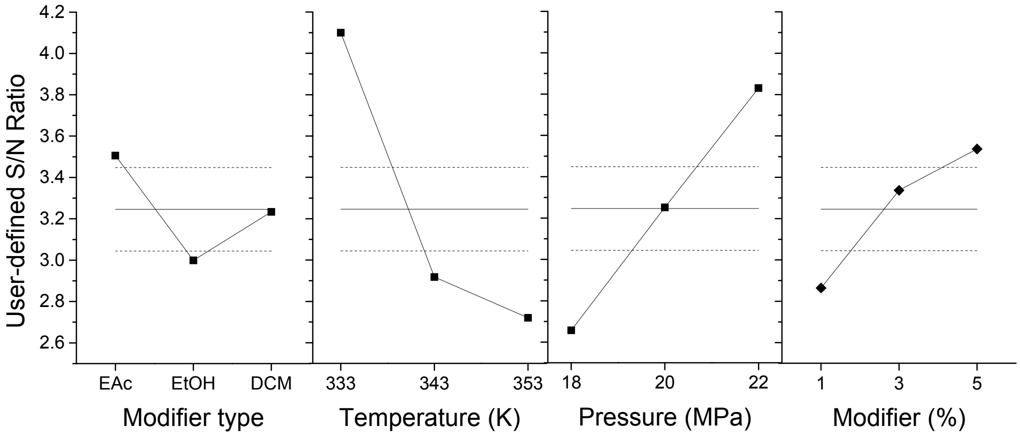

Figure 1: Effects of factors on the mean each variable of the supercritical extraction of cumaru seeds: modifier type (Ethyl acetate, ethanol and dichloromethane), temperature (333 – 353 K), pressure (18 – 22MPa) and modifier percentage (1 – 5%).

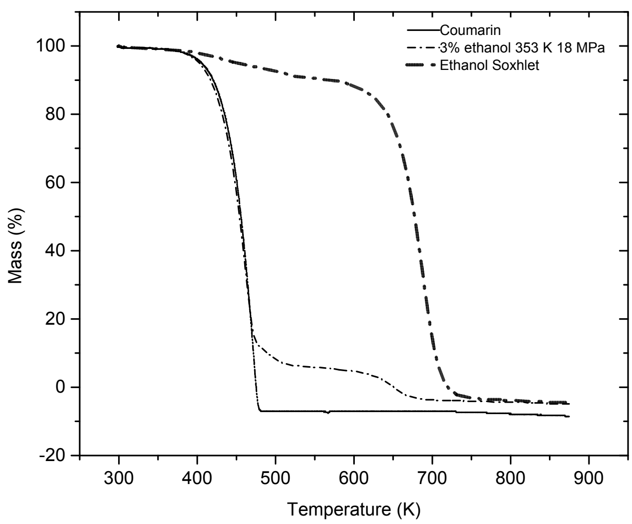

Figure 2: Thermogravimetric analysis coumarin standard, and samples obtained by 353 K, 18 MPa, 3% ethanol and via Soxhlet with ethanol.

3.2 Thermogravimetric Analysis (TGA)

Figure 2 shows the data obtained by TGA to the degradation of coumarin between 364 K and 475 K. Curves of TGA show that coumarin degrade on temperature up the 353 K. Thermal degradation was not observed for coumarin at temperatures used in extractions.
The high content coumarin on the extraction condition of 353 K, 18 MPa and 3% ethanol was evidencied by first decay zone of the curve that is higher than the second one zone. However, Soxhlet extract using ethanol as solvent had an opposite behaviour, which indicated a lower selectivity.

3.4 Coumarin extraction

The highest coumarin content was obtained on the lower pressure (18 MPa) and higher temperature (353 K) with 3% ethanol as modifier. Probably, mixture density played a major role in selectivity. On the other hand, larger amount of modifier (5%) can promote a drag on the target component. This behavior can explain the second higher yield (11.6%). The Soxhlet extraction using ethanol as solvent was less selective. HPLC and thermogravimetric analysis were convergent.

Conclusion

According to the total extraction yield results, ethanol and hexane conventional soxhlet extraction (19.1% and 46.5%) had a higher yield comparing to the scCO2 extraction (2.1% and 5.3%). However, all extracts were analyzed by HPLC to identify and quantify coumarin compounds and supercritical carbon dioxide extract (optimum condition) was found to have more coumarin content (2.1 % to 20.9%) comparing to the conventional soxhlet extractions (0.5% and 1.4%). The addition of modifiers had a positive effect on the extraction yield of coumarin content obtained from cumaru seeds. According with the results, it was possible to affirm that the most efficient process to obtain coumarin was supercritical fluid extraction (SFE) using 3% of ethanol as modifier, 353 K and 18 MPa. All parameters (temperature, pressure, modifier type and modifier percentage evaluated on extraction from cumaru seeds using SFE had statistical significant effect. Despite the good results in total yield obtained with the conventional extraction of soxhlet, the supercritical CO_2 extraction was tested to search for a better extraction method, consuming less solvent, specially those that are undesirable in the food industry. Conventional soxhlet extraction is not always acceptable for industrial applications due to the long extraction time, the high consumption of hazardous solvents and some other disadvantages. All works found in literature, using supercritical technology to extract coumarin from guaco leaves showed coumarin content lower than those obtained in this work. Therefore, cumaru seeds is a better source of coumarin than guaco leaves and supercritical technology is an adequate method to obtained high purity coumarin.

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