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Supercritical fluid extraction of phyllanthin and niranthin from *Phyllanthus amarus* Schum. & Thonn

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ABSTRACT

The lignans phyllanthin and niranthin were extracted from *Phyllanthus amarus* using supercritical CO_2 as solvent (SFE1) at different pressures (10, 20, 30 MPa) and temperatures (30, 40, 50 °C). The addition of 10% (w/w) of cosolvent (SFE2) was investigated at the same SFE1 conditions. Economic evaluation was performed for both processes. Temperature and pressure have no significant effects on the lignan yield of SFE2 with ethanol:water 50:50 v/v. The use of cosolvent increased the lignan recovery *rus*, but decreased the selectivity of the process. The SFE2 extracts showed lignan concentrations between 2.5% and 4.0%, whereas in SFE1 the lignan concentration of the extracts varied between 25% (60 °C/30 MPa) and 35% (40 °C/10 MPa). SFE2 (40 °C/10 MPa) presented the lowest cost of manufacturing. However, the SFE1 extract (40 °C/20 MPa) is ten times purer, which is a remarkable advantage given that the subsequent purification processes are expensive.

1. Introduction

Phyllanthus amarus Schum & Thonn (*Euphorbiaceae*), a plant widely distributed in tropical and subtropical areas such as India, rainforests of the Amazon, the Bahamas, China and Malaysia [1], was selected for this work due to the bioactivity of its extracts. This plant has been largely studied with pharmacological and clinical purposes. Some activities are assigned to the extracts and the compounds isolated from *P. amarus*, such as: antioxidant [2,3], antiviral [4,5], antibacterial [6], anti-inflammatory [7,8], multi-drugs resistance reversing [9], anti-allodynic [10], anti-hyperglycemic [11], control of insulin resistance diabetes [12] and radioprotective properties [13].

Lignans are the major active components found in *P. amarus* [7,9,14–16]. The main lignans isolated from *P. amarus* are phyllanthin, hypophyllanthin, niranthin, filtetralin, nirtetralin and 5-demethoxynirantin. Phyllanthin and niranthin have been reported as responsible for antioxidant, hepatoprotective, anti-inflammatory, anti-allodynic, antileishmania and antischistosomal activities [7,9,14–16].

Given the importance of lignans as bioactive compounds with potential application in pharmacy and nutrition, appropriate techniques for their extraction are required [17]. Supercritical CO_2 has been applied to extract lignans from seeds, fruits, caulomas and leaves of *Schisandra chinensis* [18–20]. However, no reports were found on SFE of lignans from *P. amarus*.

About two decades ago the most reported drawback of SFE was the high initial investment cost of an industrial plant. Many companies and investors considered SFE too expensive because of the high investment costs in comparison with classical low-pressure equipment, thus restricting the use of this technology for high-added-value products [21]. However, operating costs of SFE are relatively low, justifying its use. Moreover, recent studies report that the cost of the raw material is a limiting factor when the waste of SFE is not reused. Albuquerque and Meireles [22], in their work on SFE from annatto seeds, found the cost of raw material as the most representative in the Cost of Manufacturing (COM), being up to 80% of COM. The major contribution of the CRM to the COM was also observed by other authors [23].

Therefore, the aim of this work was to obtain phyllanthin and niranthin by SFE, using CO_2 as solvent from *P. amarus*, evaluating the influence of pressure, temperature, ethanol as cosolvent and performing the cost analysis of the manufacturing processes.

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2. Material and methods

2.1. Chemicals

The phyllanthin and niranthin standards were isolated from *P. amarus* extracts and identified in previous studies of the Chemical, Biological and Agricultural Research Center (CPQBA/UNICAMP, Campinas-SP, Brazil) [7,14]. Water was obtained by a distillation system. Absolute ethanol (analytical grade) was purchased from Synth (Diadema-SP, Brazil). Carbon dioxide (99.9% purity) was purchased from White Martins (Campinas-SP, Brazil). The solvents used in the HPLC analyses were of HPLC grade (Tedia, Shanghai, China).

2.2. Plant material

Phyllanthus amarus Schum. & Thonn was identified by Prof. Dr. Grady L. Webster (University of Califórnia Davis, USA). The voucher specimen is deposited in the herbarium of the Department of Botany of the Biology Institute of the University of Campinas (IB-UNICAMP, Campinas-SP, Brazil) under the code UEC 127.411. In this work the aerial parts of *P. amarus* obtained from the cultivation at the experimental field of CPQBA were used, always selecting the seeds from the same access of *P. amarus* (code: PA14/05).

The dried aerial parts (moisture content of 6.41%) were milled (Marconi, model MA340, Piracicaba-SP, Brazil) and stored in glass bottles at room temperature (23 °C) until the extraction procedures.

2.3. Characterization of particle bed

The particle bed characterization consisted in the determination of the mean particle diameter [24], apparent density (ratio between sample mass and bed volume), real density by helium pycnometry (Quantachrome Ultrapyc 1200e, Boynton Beach, USA) and calculation of the bed porosity Eq. (1).

$$d_p = \frac{1}{\sum_i^n \left(\frac{x_i}{d_{pi}}\right)} \tag{1}$$

Where: d_p is the average diameter of particles (mm); d_{pi} is the sieve i opening (mm); x_i is the mass fraction of the particles retained on sieve i; n is the number of fractions.

2.4. Extraction processes

The extraction experiments were performed in an experimental SFE unit assembled in the Laboratory of High Pressure on Food Engineering (LAPEA-DEA/FEA-UNICAMP) (Fig. 1, adapted from Pereira et al. [25] and Viganó et al. [26]).

2.4.1. Supercritical CO_2 extraction without cosolvent

Preliminary SFE experiments were performed without cosolvent (SFE1) to determine the working conditions to be used later, such as CO_2 flow rate (1.52×10^{-4} kg s^{-1}), dynamic and static times (50 and 20 min, respectively). In these tests, 3.0 g of sample were used and SFE was performed at 40 °C and 10 MPa, which are the mildest conditions among those to be evaluated. From these tests, the solvent/feed (S/F) mass ratio for the later experiments was defined.

The SFE1 experiments (Table 1) were arranged with two factors (temperature and pressure) and three levels of each factor with duplicate. The evaluated response variables were global yield (x_0), lignan yield in the sample (x_{ls}) and lignan yield in the extract (x_{le}), which were calculated using Eqs. (2)–(4), respectively. ANOVA and mean comparison tests of these results were performed using the software SAS 9.0.

$$x_0 = \left(\frac{m_{extract}}{m_{sample}}\right) x100 \tag{2}$$

$$x_{ls} = \frac{m_{lignan}}{m_{sample}} \tag{3}$$

$$x_{le} = \frac{m_{lignan}}{m_{extract}} \tag{4}$$

Where: $m_{extract}$ is the mass of extract (g); m_{sample} is the mass of sample used (g); m_{lignan} is the mass of lignan (mg).

The SFE procedure was as follows: i. the sample was placed in the extraction cell of 54.37 cm^{-3} (diameter of 3.03 cm and height of 7.54 cm); ii. The cell was heated for 10–30 min to reach the process temperature; iii. CO_2 was released from the recipient through the cooling bath to be liquified (−5 °C) and pumped to the heating bath, to reach the working temperature and finally enter the extraction cell, which already contained the sample; iv. The SFE bed was filled with supercritical CO_2 to achieve the working pressure; v. CO_2 was kept in contact with the sample during a static time (20 min) to ensure its saturation; vi. The outlet and micrometer valves were opened and the dynamic SFE was performed for 50 min; vii. After the extraction, the extract was weighed and x_0 was calculated; viii. The extracts were stored at 4 °C until the chromatographic analyses.

2.4.2. Supercritical CO_2 extraction with cosolvent

The SFE process with cosolvent (SFE2) was performed under the same temperatures and pressures of SFE1, and the cosolvent concentration in CO_2 was 10% (w/w). A 50% (v/v) mixture of ethanol and water (ρ = 909.85 kg m^{-3}) was chosen as cosolvent, based on the high lignan yields obtained by Pressurized Liquid Extraction (PLE) from the same plant [25].

The same S/F of SFE without cosolvent was used. Preliminary SFE2 experiments were performed to define the static time (5 min), dynamic time (25–28 min), CO_2 and cosolvent flow rates (1.65×10^{-4} kg s^{-1} and 1.06–1.17 mL min^{-1}, respectively), the amount of cosolvent added in static and dynamic times (0.0315 kg). In these tests, 1.7 g of sample was used and SFE was performed at 40 °C and 10 MPa. ANOVA and mean comparison tests of these results were performed using the software SAS 9.0.

The SFE2 procedure was the same as SFE1 with some modifications: i. A 10.9 cm^3 (diameter of 2.00 cm and height of 3.46 cm) extraction cell was used; ii. Before the static time, the required cosolvent amount was injected into the cell to achieve the required proportion, and then CO_2 was pumped into the cell until the working pressure of each experiment; iii. After the extractions, the solvent of each extract was evaporated in rotary evaporator (Marconi, MA120E, Piracicaba-SP, Brazil), and then x_0 was calculated.

2.5. Quantification of phyllanthin and niranthin by high-performance liquid chromatography (HPLC)

The extracts obtained by SFE were diluted in the mobile phase (1:1000 (v/v) for SFE1 and 1:15 (v/v) for SFE2) before injection into the chromatograph. The chromatographic analysis was performed for all the samples obtained in the SFE experiments.

The concentrations of phyllanthin and niranthin in the extracts were determined by a validated HPLC method, described by Pereira et al. [25], using an Alliance system (Waters, Milford, USA) with photodiode array detector (model 2996). The calibration curves of the lignans were obtained at the following concentrations: 1.09; 2.18; 3.26; 5.44; 7.62; 10.88; 16.32; 20.40; 32.64; 40.80; 65.28; 81.60 μg mL^{-1} for niranthin e 5.00; 10.00; 20.00; 40.00; 60.00; 80.00 e 100.00 μg mL^{-1} for phyllanthin.