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Supercritical fluid extraction of 6-methoxyeugenol from *Piper hispidinervum*

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ABSTRACT

Long pepper, a vine that grows wild in Brazil, stands out among the aromatic varieties of the genus *Piperaceae*. The *Piper hispidinervum* essential oil presents a high concentration of safrole (85%) and terpinolene (8%), and numerous biological activities. On the other hand, the long pepper presents a high content of phenolic compounds in its nonvolatile extract, among which the 6-methoxyeugenol emerge for its antioxidant activity. In this context, this study aims to find the optimal conditions for the supercritical fluid extraction (SFE) of 6-methoxyeugenol from the aerial parts of long pepper, evaluating different extraction conditions. A factorial experimental design was used with 3 factors, 2 levels and triplicate central point, varying the pressure in the range of 80 to 110 bar, temperature from 40 to 60 °C and the solvent/co-solvent ratio between 0 and 5% (m/m) of H_2O. Firstly, the *P. hispidinervum* aerial parts were submitted to a steam distillation process, in order to extract its volatile compounds. The plant material was then dried, milled and submitted to the SFE. The extracts were analyzed by HPLC, using a calibration curve to quantify the 6-methoxyeugenol. A response surface in terms of $mass_{6\text{-}methoxyeugenol}/mass_{plant}$ was built along with a variance analysis (ANOVA). For the optimal condition, experimental curves (mass *versus* time) were built for the extract and the 6-methoxyeugenol, based in triplicate experiments. A mathematical model based on a differential mass balance was fitted to the experimental curves, obtaining relevant mass transfer parameters. The results indicate that the optimal conditions for the extraction of 6-methoxyeugenol, among the study parameters of this work are 110 bar, 40º C, 5% co-solvent/solvent ratio for a $1000 g.h^{-1}$ flow rate. The optimal condition was confirmed by the experimental results yielding 1.86 ± 0.21 mg/g_{plant}.

Keywords: 6-methoxyeugenol, long pepper, SFE, modeling.

INTRODUCTION

Antioxidants are compounds that have the ability to slow the oxidation rate of an oxidizable substrate, primarily through the inhibition of free radicals. This property of some compounds or extracts is used by various sectors such as food, cosmetic and pharmaceutical to delay food deterioration, fight skin aging and help prevent diseases, [1, 2], but the indiscriminate use of antioxidant compounds has been questioned about their safety, especially when they are synthetic compounds. As an alternative, studies carried out in the last years report numerous plants that present compounds with antioxidant action, mainly in their foliage. Among them are some species of the family Piperaceae [1-4].

The Piperaceae family is represented by herbaceous plants, shrubs and rarely trees. Among the aromatic species is *Piper hispidinervum*, popularly known as long pepper, found in wild conditions in the Rio Acre Valley [5, 6]. Recently, the cosmetic and insecticide industries had a great interest in this plant due to the high concentration of safrole present in its volatile extract, used in the synthesis of heliotropin (hair fixative), and piperonyl butoxide, synergetic agent. However, there are several studies on the volatile extract of *Piper hispidinervum* [7, 8], but the non-volatile extract has been little studied [3]. Among the compounds present in the non-volatile extract, we can highlight the presence of 6-methoxyeugenol (4-allyl-2,6-dimethoxyphenol), a phenolic compound with high antioxidant activity [9].

The volatile extracts of *Piper hispidinervum* are obtained by steam distillation and the non-volatile extracts are usually obtained by infusion and/or maceration of the plant leaves at low pressures in selected organic solvents according to the polarity of the compounds of desire [10]. Another extraction method used to obtain non-volatile compounds is the extraction with supercritical fluid, which has the advantages of low extraction temperature, thus avoiding the degradation of thermosensitive extracts, easy solvent/extract separation, and high selectivity when co-solvents are used. Studies show that the extraction with supercritical CO_2 combined with polar co-solvents is indicated to obtain phenolic compounds [3, 11]. Another important advantage of supercritical extraction is the number of variables that can be manipulated during the process, such as temperature, pressure, granulometry of the raw material, solvent flow rate, co-solvent/solvent ratio, bed porosity, among others.

Based on the work of Almeida et *al.* [12] and Garcez et *al.* [13], it was decided to evaluate in this study the effect of pressure, temperature, and co-solvent/solvent ratio on the yield of 6-methoxyeugenol extracted from the long pepper leaves, keeping the remaining variables constant. The experiments were performed following experimental surface response planning. The concentration of the compound of interest in the non-volatile extracts was determined by high performance liquid chromatography (HPLC) using the 6-methoxyeugenol standard as reference.

The present study aims to determine the pressure and the operating temperature together with the co-solvent/solvent ratio that generate the highest yield of 6-methoxyeugenol from the aerial parts of the long pepper using the supercritical extraction process. For the optimized extraction condition, the extract yield curve versus extraction time was experimentally determined, as well as the mathematical modeling of the extraction curve.

MATERIALS AND METHODS

Pretreatment

The aerial parts of fresh long pepper (leaves and stems) were initially pretreated to standardize the sample. This pretreatment consisted in extract volatile compounds from the raw material by the steam distillation process [14], conducted in the pilot unit in the Laboratory of Unitary Operations (LOPE). Then, the aerial parts were submitted to a drying process with air recirculation and controlled temperature at 40º C for 12 h. The dry plant was milled and characterized in terms of its granulometric distribution through the screening process, in a set of five vibrating sieves with a mesh opening varying

between 0.500 and 0.106 mm. The sieving procedure was performed in triplicate with a vibration time of 15 min.

Supercritical Fluid Extraction

The supercritical fluid extractions were conducted in the supercritical extraction pilot unit located in the LOPE [15]. The pilot unit consists of two cylinders of carbon dioxide (99.9% purity - AirProducts) (C1), a high-pressure pump responsible for raising the pressure above the critical condition (P1), a co-solvent pump (MP1), a condenser located before the pump to ensure that CO_2 is in the liquid state, avoiding cavitation (HE1). In addition, a heat exchanger raises the temperature above the critical condition (HE2), an extraction vessel where the vegetable material (EV1) is deposited; a micrometric valve used for solute/solvent separation through system expansion (MV1), an extract collection system (VS) and a mass flowmeter (MFT). The flowchart of the process is shown in Figure 1.

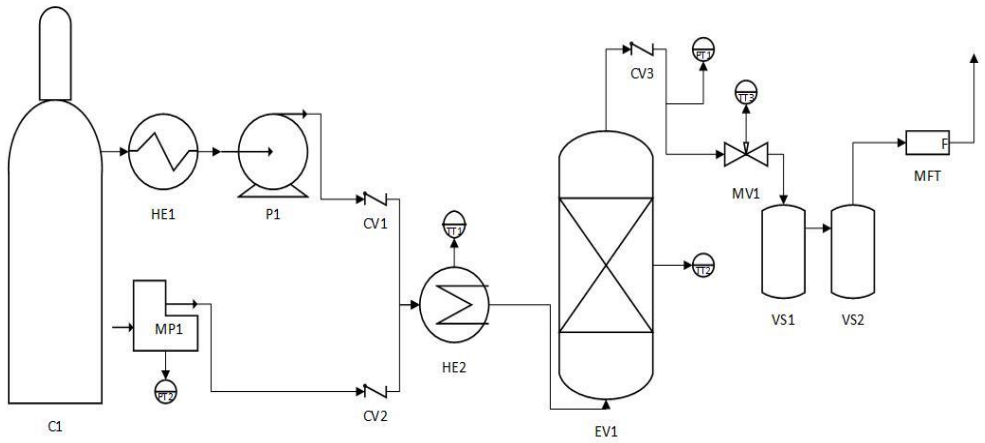

Figure 1 - Flowchart of the supercritical extraction pilot unit. Cylinder of CO2 (C1) - High pressure pump (P1) - Solvent pump (MP1) - Condenser (HE1) - Heat exchanger (HE2) - Extraction vessel (EV1) - Micrometric Valve (MV1) - Collection systems of extracts (VS) - Flowmeter (MFT).

In the first stage, the pretreated pepper was subjected to a supercritical fluid extraction for a previous analysis of the obtained extracts. For this, 80 g of vegetal material were used in sequential extractions, screening pressure at the following values: 90, 120, 150, 200, 250 and 300 bar, and the following variables were constant: solvent (1000 $g.h^{-1}$), co-solvent/solvent ratio (5% H_2O/CO_2), operating temperature (40° C) and extraction time (1 h) [12, 13]. The extracts obtained were analyzed in HPLC.

With the results of the 6-methoxyeugenol concentration, for the different pressure conditions analyzed in HPLC, the operating pressure at which the highest concentration of 6-methoxyeugenol was determined. From these results, a response surface plan was applied, following the Box-Behnken model, with the co-solvent/solvent ratio, pressure and operating temperature as variables, setting the flow variables of supercritical solvent mixture (1000 $g.h^{-1}$), extraction time (2 h) and specific granulometry.

In order to fit the experimental data (optimized conditions) and obtain important mass transfer parameters, the mathematical model used in this work was based in the model developed by Reverchon [16]. The model consists of one-dimensional mass balance for the extract, assuming the hypothesis of a linear behavior for the solid-fluid phase equilibrium among other assumptions.

The mass balance is given below (equation 1 and 2).
Fluid phase mass balance:

$$\frac{\partial \mathrm{C(z,t)}}{\partial \mathrm{t}} = -\upsilon \frac{\partial \mathrm{C(z,t)}}{\partial \mathrm{z}} - \frac{1-\varepsilon}{\varepsilon}\rho_s \frac{\partial \mathrm{q(z,t)}}{\partial \mathrm{t}} \quad (1)$$

Mass balance in the solid phase:

$$\frac{\partial \mathrm{q(z,t)}}{\partial \mathrm{t}} = -\mathrm{k}_{TM}[q(z,t) - K \cdot C(z,t)] \quad (2)$$

The extract concentration in the vapor phase is given by the function $C(z,t)$ and the concentration in the aromatic plant is described by the $q(z,t)$ function. Where υ is the interstitial vapor velocity; ε is the porosity of the bed; k_{TM} is the internal mass transfer coefficient; ρ_s is the specific mass of the aromatic plant and *K* is the equilibrium constant between the phases. The model also considers some initial and boundary conditions: $q(z,0) = q_0$ and $C(z,0) = 0$, q_0 is defined by the total amount of extract contained in the solid phase and the $C(z,0) = 0$ as a boundary condition. The linear comportment for solid-fluid phase equilibrium is expressed by $q^*(z,t) = K \cdot C(z,t)$.

HPLC analysis

The supercritical extracts were diluted in acetonitrile at a concentration of 10% by volume and then analyzed by Agilent 1200 series UV detector system. The method used was based on the literature [10] for phenolic compounds. The mobile phase was composed of Milliq water (A) and acetonitrile (B) with 2% acetic acid. The gradient used was 20-65% B in 60 minutes at a flow rate of 1.0 ml/ml at a temperature of 40° C. The injection volume was 5 μL, the column used was a C18 (4.6 x 250 mm x 5 μm), and the wavelength used at the detector was λ = 345 nm.

The results were compared to a calibration curve of the 6-methoxyeugenol guanine standard (Sigma-Aldrich) with a purity of 95%. To generate the calibration curve, the standard was diluted in acetonitrile at four concentrations ranging from 1 to 0.025% v/v.

RESULTS

In order to establish the pressure range to be investigated in the experimental design, extracts were obtained from the extraction with supercritical CO_2, following the screening method. The extracts were analyzed by HPLC and compared with a standard calibration curve. The process pressure at which the highest 6-methoxyeugenol yield was obtained in the screening step was 90 bar. With this preliminary result, the pressure range for the multivariate analysis was defined between 80 and 110 bar.

A factorial experimental design was used with 3 factors, 2 levels and triplicate central point, varying the pressure in the range of 80 to 110 bar, temperature from 40 to 60° C and the solvent/co-solvent ratio between 0 and 5% (m/m) of H_2O. All extracts were analyzed in HPLC and compared to the calibration curve of the 6-methoxyeugenol standard.

The yields of the supercritical extracts obtained ranged from 36.21 to 218.92 ($mg_{extract}/g_{plant}$), while the 6-methoxyeugenol content ranged from 0.092 to 2.020 ($mg_{6\text{-}methoxyeugenol}/g_{plant}$), as shown in Table 1.

Table 1 - Results of the supercritical extraction applied to the long pepper, using the Box-Behnken experimental design

Pressure (bar)	**Temperature (°C)**	**Co-solvent/ solvent ratio (%)**	**Global yield ($mg_{extract}/g_{plant}$)**	**6-methoxyeugenol yield ($mg_{6\text{-}methoxyeugenol}/g_{plant}$)**
80	40	0	68.49	0.608
80	60	5	36.21	0.092
95	50	2.5	45.83	0.756
110	60	0	127.72	1.438
110	40	0	139.24	0.656
95	50	2.5	85.66	0.999
95	50	2.5	73.41	0.946
80	40	5	68.28	0.460
80	60	0	218.92	0.867
110	40	5	127.88	2.020
100	60	5	77.65	0.719

The variance analysis of the experimental data (ANOVA) was performed in the statistical software Minitab. According to the ANOVA data, considering the significance level of 90% ($\alpha = 0.1$), the only statistically significant variables are the quadratic temperature effect (T*T) and the interaction between pressure and temperature (P*T), while all factors and their interactions are not statistically significant. The analysis also indicates that the regression is statistically significant and thus the model can be applied to describe the variation of the amount of 6-methoxyeugenol in the final extract as a function of the studied variables. Thus, the model 2^3 allows estimating the response surface coefficients. The adjusted model is expressed by Equation 3, where the P is the pressure in bar, the temperature T, in Celsius (°C) and the co-solvent/solvent ratio in mass percentage.

$$C\left(\frac{mg_{6-methoxyeugenol}}{g_{plant}}\right) = 2.74803 - 0.0332285 * P - 0.00566985 * T - 2.13778 * RC + 0.00087069 * P * T + 0.0294895 * P * RC + 0.0325409 * T * RC - 0.000485 * P * T * RC \quad (3)$$

The adjusted model has a coefficient of determination (R^2) equal to 0.9874, which means that the model represents the experimental data well.

To find the optimal point, the Frank-Wolfe algorithm, a first-order iterative optimization algorithm for constrained optimization, also known as the conventional gradient method [17] was used. The optimum point obtained from the response surface was 110 bar, 40° C, 5% H_2O/CO_2, at a flow rate of 1000 $g.h^{-1}$ solvent and a total extraction time of 2 h. For this optimized condition, the expected yield using the response surface equation is 2.021 $mg_{6\text{-methoxyeugenol}}/g_{plant}$, and in order to validate it, an experiment was performed in the optimized conditions, in triplicate, resulting a 1.86±0.21 $mg_{6\text{-methoxyeugenol}}/g_{plant}$. The mass transfer parameters were fitted to the experimental data obtained at the optimized condition. The experimental and calculated extract yield (extract mass/plant mass) are presented in Figure 2. The fitted mass transfer coefficient (k_{TM}) is 5.82E-04 s^{-1} and the partition coefficient (K) is 2.73E-03 m^3/kg, with a determination coefficient (R^2) of 0.9821.

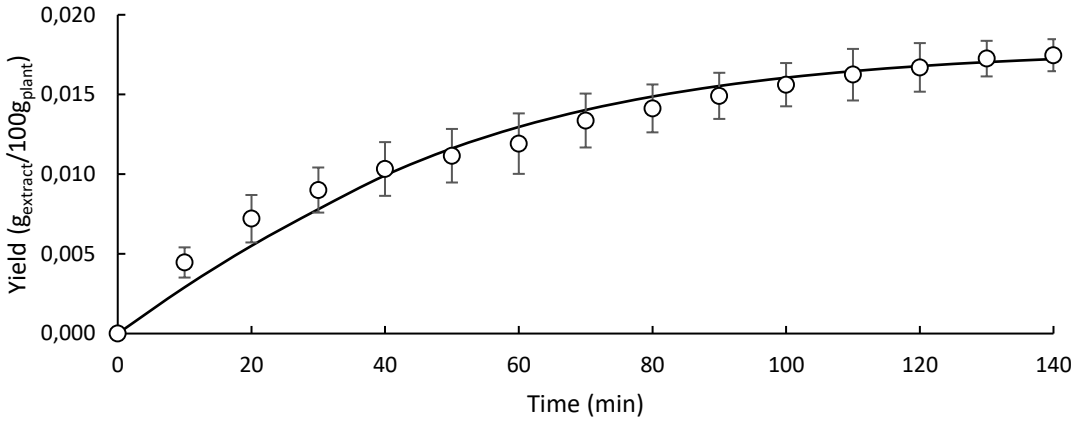

Figure 2 - Yield *versus* time for the experimental and model curves of the *Piper hispidinervum* supercritical fluid extract obtained at 110 bar, 40° C, 5% co-solvent/solvent ratio.

CONCLUSIONS

Through an initial evaluation (screening) for the design of experiment planning through Box-Behnken, the pressure ranges (80 - 110 bar), temperature (40 – 60 ºC) and co-solvent/solvent ratio (0-5% H_2O/CO_2) were determined from the supercritical extraction experiments. The results of the experiments with supercritical fluid indicate that the optimum conditions of the extraction of 6-methoxyeugenol from the long pepper, among the parameters of study of that work, are of 110 bar, 40º C, and 5% of co-solvent/solvent ratio for a flow of 1000 $g.h^{-1}$, water being used as co-solvent

It is suggested to continue this work in order to test the optimal condition, and then fractionate and isolate the 6-methoxyeugenol from the supercritical extract produced by the plant matrix.

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