Hypericum carinatum: supercritical fluid extraction, HPLC determination and mathematical modeling

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ABSTRACT. The genus *Hypericum* has been studied worldwide due to its pharmacological and biological effects. Uliginosin B, a dimeric phloroglucinol, and the benzophenones cariphenone A and B, for example, are the major responsible for the anti-proliferative and antimicrobial activities of *Hypericum carinatum*, a native species to southern Brazil. Although presenting therapeutic potential, these compounds are obtained in low yield with conventional extraction methods and can suffer thermal and oxidative decomposition. The use of supercritical carbon dioxide, an environmentally safe solvent, is a promising alternative. Thus, the aim of this study was to determine some extraction conditions in view of future procedures of scale-up. The aerial parts of plant were extracted under constant temperature (40 °C) and pressures of 90, 150 and 200 bar. HPLC analysis and mathematical modeling were used to quantify the metabolites and assess the unknown parameters of the extraction, respectively. The better extraction condition to obtain the bioactive compounds was 40 ºC and 90 bar. The mathematical model fitted very well the experimental data.

INTRODUCTION

The genus *Hypericum* has been investigated worldwide for its economical, chemical and biological importance. Several species are used in traditional medicine as antiseptic, diuretic, stomachic, wound healing agents and in the treatment of infectious diseases. *Hypericum perforatum* is one of the best-known members especially due to its therapeutic value as an antidepressant drug. The bioactive metabolites commonly described for the genus include naphthodianthrones, flavonoids, phloroglucinol derivatives and xanthones [1-3].

In the southern Brazil, there are about 20 native species. Pharmacological studies have shown that some of them present antidepressant, antinociceptive, MAOI, antiviral, antibacterial, antifungal and anti-proliferative activities. Uliginosin B, a dimeric phloroglucinol, and the benzophenones cariphenone A and B have been pointed out as the major responsible for the anti-proliferative and antibacterial activities of *Hypericum carinatum*, for example [1, 4, 5]. Although presenting therapeutic potential, these compounds are obtained in low yield with conventional extraction methods and can suffer thermal and oxidative decomposition [6, 7].

The use of supercritical fluids is a promising alternative. The supercritical fluids have properties such as high diffusivity, low viscosity and low surface tension conferring attractive characteristics as a solvent for extraction of components from a solid matrix [8]. Carbon dioxide (CO_2) is the most used solvent since it is non-toxic, non-explosive, readily available

and of low cost. Furthermore, at supercritical conditions it presents advantages such as selectivity for lipophilic compounds and non degradation of thermolabile substances [9-12], features very appropriate for the extraction of bioactive compounds from *Hypericum* species. Thus, the aim of this study was to assess the effect of extraction conditions (temperature, pressure and time) regarding the recovery of extracts and concentration of uliginosin B, cariphenone A and cariphenone B. In view of future procedures of scale-up, applications of mathematical modelling were also carried out.

MATERIALS AND METHODS

Plant material

The aerial parts of *Hypericum carinatum* Griseb. were collected during its flowering stage in Glorinha, Rio Grande do Sul, Brazil, in December, 2010. The plant material was identified by S. Bordignon (Departamento de Botânica, Universidade Luterana do Brasil). A voucher specimen was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul (ICN - Bordignon 1520). Plant material was dried at room temperature and powdered (1 mm mesh opening) in a cutting mill.

Extraction methods

Supercritical extractions were carried out on a pilot-scale automated equipment according to procedures previously described [13]. Powdered plant material (100 g DW) was extracted at temperature of the 40 ºC and pressures of 90, 150 and 200 bar. The extractions were performed following the experimental procedure defined by Cargnin et al. [14]. The samples were collected every 10 min in order to evaluate yield and composition of the extracts *versus* extraction time. The supercritical carbon dioxide flow rate was 6.7×10^{-4} kg.s^{-1} (through the extraction vessel) using a flowmeter assay (Sitraus F C Massflo 2100 - Siemens) with accuracy of < 0.1%. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with precision of ±1.0 bar. The temperature controller was connected to thermocouples (PT-100) with accuracy of < 0.5.

HPLC analysis

The supercritical CO_2 extracts were treated with acetone and filtered off to remove waxes and insoluble impurities. Further, the enriched fractions were evaporated to dryness, dissolved in HPLC grade methanol, filtered (0.22 µm pore size, Merck) and analyzed by high-performance liquid chromatography.

Benzophenones determination

Cariphenone A analysis was performed using a Waters 600 pump and a Waters 2487 dual λ absorbance detector set to 270 nm. The separation was carried out with an isocratic solvent system (60% CH_3CN, 40% H_2O) through a Waters Nova-Pack C18 column (4 µm, 3.9 x 150 mm) adapted to a Waters Nova-Pack C18 60 A guard column (3.9 x 20 mm) and flow rate of 1 mL.min^{-1}. Metabolite determination was assessed by a calibration curve of pure standard isolated from *H. carinatum* and concentrations ranging from 5 to 1400 µg.mL^{-1} ($R^2 > 0.999$). The compound identification was made on the basis of the ultraviolet absorption spectra and retention time in comparison with the standard compound. Cariphenone B quantification was performed under the same conditions described above and the content was expressed as milligram of cariphenone A equivalents/100 g plant.

Uliginosin B determination

Uliginosin B yields were determined using the same equipment under isocratic solvent condition (95% CH_3CN, 5% H_2O, 0.01% TFA), flow rate of 1 $mL.min^{-1}$ and detection at 220 nm. Metabolite quantification was assessed by a calibration curve of pure standard and the concentrations ranged from 2 to 800 $\mu g.mL^{-1}$ ($R^2 > 0.999$). The identification was based on the ultraviolet absorption spectra and retention time in comparison with the standard compound.

Mathematical modeling

A model for supercritical-fluid extraction based on the concept of broken and intact cells was used to fit experimental data and simulates two extraction periods using the differential mass balances of the solute per unit volume extraction bed. The first period is guided by phase-equilibrium and the second by internal diffusion into the particles. The mathematical model was formulated by Xavier et al. [15] according to the discussion proposed by Sovová [16]. In this model, the mass of extracted compounds (solute) is assumed to be a *pseudo* component in terms of the mass balance. The solute mass balance in fluid and solid-phase was expressed by two partial differential equations that were analytically solved by Xavier et al. [15]. The extraction curve, written in terms of maximum extract yields, is expressed in two steps; the first one controlled by phase equilibrium and the second, by internal diffusion from particles, having the following expression

$$\frac{M(t)}{M(\infty)} = \frac{K_1\, t}{M(\infty)}. \tag{1}$$

for the first period, where

$$K_1 = \dot{m}\, Y^*, \tag{2}$$

being $\dot{m}$ the solvent flow rate and Y^* is weight fraction for the equilibrium fluid-phase; and

$$\frac{M(t)}{M(\infty)} = (1 - e^{-K_2\, t}). \tag{3}$$

for the second period, where K_2 is defined as follow

$$K_2 = \frac{k_s a_0}{(1-\varepsilon)}. \tag{4}$$

where k_s is the solid-phase mass transfer, and a_0 is the specific surface area per unit volume of extracted bed.

Summarizing, the extraction curve is linear in function of the time for the first step of the extraction and exponential for the second period [15].

RESULTS AND DISCUSSION

The results obtained are summarized in the tables 1, 2, 3 and 4. Uliginosin B, cariphenone A and cariphenone B extraction curves obtained by supercritical CO_2 extraction of *H. carinatum* under temperature (40 ºC) and pressure (90, 150 and 200 bar) constants are shown in figures 1, 2 and 3.

Table 1: Total supercritical *H. carinatum* extract yields and respective fractions obtained after treatment with acetone.

	Yield	
Pressure	40 ºC	
(bar)	% Extract[a]	% Fraction[b]
90	0.95	0.91
150	0.97	0.92
200	1.89	0.97

[a]g of total extract /100 g of plant
[b]g of fraction /100 g of plant

Table 2. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40 °C and 90 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	1.30 ± 0.07	15.08 ± 0.51	0.78 ± 0.06
20	1.41 ± 0.10	17.30 ± 0.54	0.84 ± 0.03
30	3.01 ± 0.11	30.59± 0.54	1.45 ± 0.02
40	2.48 ± 0.21	37.57 ± 0.90	1.77 ± 0.03
50	6.48 ± 0.48	44.70 ± 2.36	2.10 ± 0.00
60	1.19 ± 0.11	18.78 ± 0.67	0.91 ± 0.03
70	1.75 ± 0.06	28.10 ± 1.32	1.65 ± 0.07
80	1.28 ± 0.05	13.68 ± 1.00	0.71 ± 0.05
90	2.76 ± 0.26	39.21 ± 0.18	1.97 ± 0.02
100	1.96 ± 0.15	32.63 ± 0.16	1.74 ± 0.01
110	0.81 ± 0.00	5.98 ± 0.37	0.39 ± 0.01
120	1.92 ± 0.00	16.11 ± 0.50	0.99 ± 0.03
130	2.48 ± 0.15	28.13 ± 1.03	1.99 ± 0.16
140	0.65 ± 0.04	10.24 ± 0.56	0.76 ± 0.04
150	0.30 ± 0.02	6.61 ± 0.05	0.59 ± 0.01
160	5.10 ± 0.01	12.28 ± 0.01	1.06 ± 0.00
170	5.62 ± 0.06	11.99 ± 0.03	0.99 ± 0.00
180	2.40 ± 0.03	7.32 ± 0.02	0.70 ± 0.00
Total	**42.90 ± 0.66**	**376.33 ± 4.14**	**21,39 ± 0.28**

In general form, the modeling of the supercritical extraction for the conditions here studied as well as for the total time for the experiments allows to evidence that for 90 bar, the extraction of metabolites of interest is practically controlled for the first stage of the extraction process, in which the higher amounts of these metabolites had been observed. With the increase of the

pressure, the second stage of the process becomes present, where the diffusion from internal cells of the vegetal matrix is more predominant in the mass transfer process. The mathematical modeling represented well the experimental data as it can be seen in Figures 1, 2, and 3. The unknown parameters present in the mathematical model were estimated by minimization of the sum of squares of errors between the experimental data and the prediction using the model. The numerical results for these parameters were presented in Table 5.

Table 3. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40 °C and 150 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	1.85 ± 0.15	34.28 ± 0.66	3.11 ± 0.09
20	2.96 ± 0.17	61.81 ± 1.27	6.60 ± 0.22
30	3.13± 0.10	34.99± 1.31	4.98 ± 0.21
40	2.30 ± 0.12	25.27 ± 0.90	3.57 ± 0.22
50	1.16 ± 0.02	11.22 ± 0.43	1.76 ± 0.04
60	1.00 ± 0.05	6.38 ± 0.12	1.19 ± 0.06
70	1.10 ± 0.02	8.10 ± 0.29	1.53 ± 0.05
80	1.47 ± 0.05	14.06 ± 0.81	2.33 ± 0.07
90	0.09 ± 0.00	0.16 ± 0.00	0.08 ± 0.00
100	0.43 ± 0.00	1.12 ± 0.01	0.49 ± 0.00
Total	**15.48 ± 0.06**	**197.40 ± 0.07**	**25.64 ± 0.39**

Table 4. Mass quantities of uliginosin B, cariphenone A, and cariphenone B in fraction of supercritical extract of *H. carinatum* at 40 °C and 200 bar per 100 g of plant.

Time (min)	Uliginosin B (mg)	Cariphenone A (mg)	Cariphenone B (mg)
10	0.23 ± 0.01	15.65 ± 0.25	1.15 ± 0.05
20	1.45 ± 0.04	69.95 ± 2.20	5.17 ± 0.12
30	2.43± 0.10	85.77 ± 2.07	9.13 ± 0.27
40	1.37 ± 0.02	7.38 ± 0.21	1.98 ± 0.05
50	3.94 ± 0.15	5.19 ± 0.17	3.18± 0.03
60	1.93 ± 0.17	1.10 ± 0.13	1.10 ± 0.03
70	1.92 ± 0.18	0.78 ± 0.03	0.66 ± 0.04
80	0.09 ± 0.00	0.09 ± 0.01	0.05 ± 0.00
90	0.05 ± 0.00	n.d	n.d
100	0.10 ± 0.00	n.d	0.01 ± 0.00
110	0.86 ± 0.04	0.19 ± 0.01	0.23 ± 0.02
120	0.16 ± 0.00	0.09 ± 0.00	0.04 ± 0.00
130	n.d	n.d	n.d
140	n.d	n.d	n.d
Total	**14.53 ± 0.51**	**186.21 ± 1.99**	**22.71 ± 0.32**

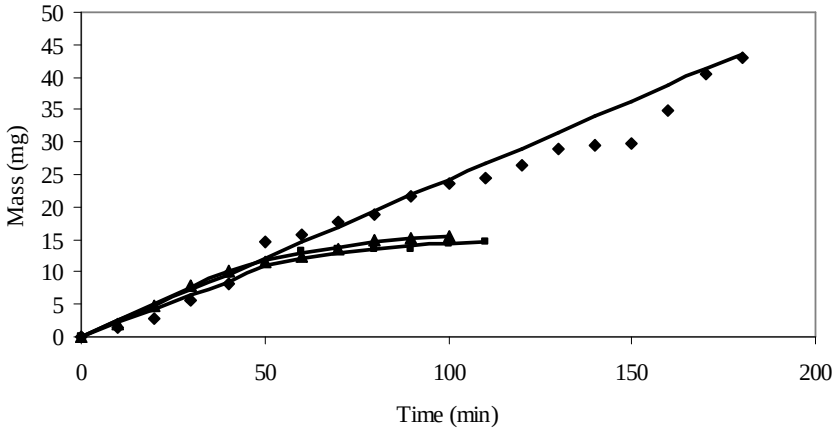

Figure 1. Mass of uliginosin B obtained by supercritical fluid extraction at 40 ^{o}C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

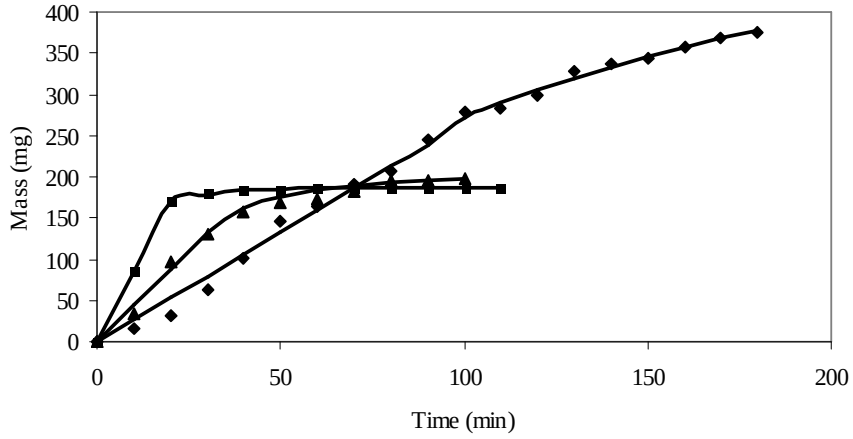

Figure 2. Mass of cariphenone A obtained by supercritical fluid extraction at 40 ^{o}C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

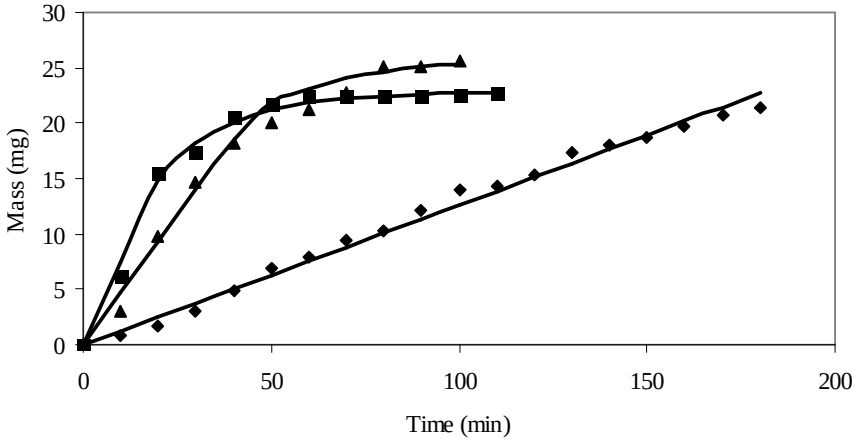

Figure 3. Mass of cariphenone B obtained by supercritical fluid extraction at 40 ^{o}C and different pressures. The continuous line is for the mathematical fitting and the symbols were used for the experimental data (♦ - 90 bar, ▲- 150 bar, and ■ - 200 bar).

Table 5. Numerical values for mathematical parameters

Pressure	Metabolites	K_1	K_2
90 bar	Cariphenone A	2.6588	0.0078
	Cariphenone B	0.1262	-
	Uliginosin B	0.2421	-
150 bar	Cariphenone A	4.4264	0.0418
	Cariphenone B	0.4655	0.0369
	Uliginosin B	0.2542	0.0239
200 bar	Cariphenone A	8.5668	0.1063
	Cariphenone B	0.7444	0.0534
	Uliginosin B	0.2134	0.0230

Especially in pharmaceutical, food and cosmetics industries, the use of supercritical fluids represents an alternative to the conventional methods of extraction since extracts of high quality are achieved. Nevertheless, satisfactory results are dependent on the optimization of process parameters such as pressure, temperature, fluid extraction, modifiers, flow rate, extraction time, among others [7]. According to the results of Table 1, the increased pressure produced higher yields of extract. In addition, in the pressures of 90 and 150 bar the percentage of acetone-soluble fraction represented about 95% of the total extract. At 200 bar of pressure, however, this value was 51%, indicating an elevated co-extraction of undesirable compounds such as epicuticular waxes. As expected, at constant temperature, the elevation of pressure results higher fluid density and consequently superior power of solvation [8].

HPLC analyses were performed aiming to evaluate the recovery of uliginosin B, cariphenone A and B in the fractions of supercritical extract. As it can be observed in the table 2, 3 and 4, the composition of extract varied according to the conditions employed. According to Pourmortazavi and Hajimirsadegh [17], four parameters are extremely helpful in the understanding the solute behavior in supercritical media: (i) the miscibility or pressure in which the solute migrates into the supercritical fluid; (ii) the pressure in which the solute reaches its maximum solubility; (iii) the pressure range of selective fractionation and (iv) a knowledge of the physical properties of the solute. Regarding the conditions employed in this study, the temperature of 40 ºC and 90 bar of pressure produced the higher quantity of the three compounds investigated. Moreover, at 40 ºC and 150 bar high concentrations of cariphenone B were obtained. It is also important to note that, in comparison to the conventional methods, the time of extraction is reduced to a few hours. Thus, higher selectivity and shorter time are factors that can compensate the costs of investment in the processes of supercritical extraction.

CONCLUSION

The composition of supercritical fluid extract of *H. carinatum* varied in according to temperature and pressure, being 40 ºC and 90 bar the better condition for extraction of the bioactive compounds: uliginosin B, cariphenone A and B. Additionally, the condition of 40 ºC and 150 bar also represents a great alternative for selective recuperation of cariphenone B. Besides, the time economy is notable especially when compared with the conventional methods of extraction. Depending of the employed condition, it is necessary only 90 to 180 minutes.

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