Attainment of non-volatile extracts of *Schinus molle* L with supercritical fluid and co-solvents for antimicrobial applications

Rodrigo S. Silva, Roberto Góes Neto, Manuel A. Falcão, Eduardo Cassel, Rubem M. F. Vargas*

Unit Operations Laboratory – Faculty of Engineering, PUCRS, Av. Ipiranga, 6681-Prédio 30 –Bloco F - Sala 277, 90619-900 Porto Alegre, RS, Brazil, e-mail: rvargas@pucrs.br – Fax +55-51-33203823

Abstract. The industrial processes must be in accord to current demands of the society, in this sense the search of natural bioactive compounds have demonstrated to be a world-wide trend. *Schinus molle* L., also known as pepper tree, has been reported to have antimicrobial, antifungal, anti-inflammatory, antispasmodic, antipyretic, antitumoural and cicatrizing properties. Thus, in this work an investigation about the power of extract obtained in supercritical conditions from leaves of the *Schinus molle* L. with carbon dioxide and co-solvents was performed to study its antimicrobial role. To reach the aim of this work the supercritical fluid extraction was carried out in a pilot scale equipment using carbon dioxide modified by addition of co-solvent, such as ethanol and water at 150 bar and 60^{o}C. In view of future industrial application, the mathematical modeling of the process was carried out. Qualitative analysis to verify the antimicrobial activities were carried out by means of bioautography technique for *Staphylococcus aureus*, *Pseudomonas aeruginosas*, *Escherichia coli*, *Micrococcus luteus*, and *Salmonella choleraesuis*

INTRODUCTION

The implantation of industrial processes needs forecasts to promote the adequate installation of the process; in this sense the mathematical modeling becomes a basic tool. Different modeling of the physical problem is regulated by mathematical equations which will describe the behavior of the system according to a set of hypotheses assumed in the step of the physical description of the process. Mathematical model enable us to generalize the experimental results, which can be applied to a new process conditions and to materials other than those investigated. Moreover, they are useful in the future scale-up process [1]. In the natural product industries, the characteristics associated to initial distribution of the solute in the vegetal matrix as well as the type of the raw material affects the selection of the possible mathematical model. Each different description corresponds to a different mathematical model [2].

In the literature, simple mathematical models were available as well as models more complexes due to the higher inclusion of information about the physical system. Different approaches have been proposed for mathematical modeling of the supercritical fluid extraction. The models based on mass transfer have been largely used.

In this work, a mathematical model was used to simulate the supercritical fluid extraction of *Schinus molle* L. using co-solvent (water and ethanol) and carbon dioxide as solvent. The mathematical model was based on the particle phase mass balance assuming that the intraparticle transport was the limiting factor of the supercritical fluid extraction. The diffusion was assumed in the particles with form of slab subjected to convection mechanism at their external surface. The thickness of the slab was determined experimentally and the hypotheses of the simetry in relation to central line of the slab were assumed.

Supercritical fluid extraction is a technique widely used in separation processes of natural products, where the solvent usually used is not toxic, such as carbon dioxide. The supercritical fluid has properties such as high diffusivity, low viscosity and low surface tension, which gives it attractive characteristics as a solvent for the extraction of components from the solid matrix [3].

One of the main parameters of supercritical extraction is the type of co-solvent that it will be used. A co-solvent is an organic substance having volatility intermediate to the supercritical fluid and the solute to be extracted, which is often added to the supercritical fluid solvent in order to change the solvent characteristics, such as polarity and specific interaction, without significantly changing the density and compressibility of the original supercritical fluid solvent [4]. The role of the co-solvent is to increase the polarity and solvent strength as well as also to promote the improvement of the selectivity of separation of the solute.

The pepper tree (*Schinus molle* L.) is a tree native to the Peruvian Andes [5] and is widely grown in tropical and subtropical countries [6]. The leaves of this tree are the source for extraction of the essential oil used in popular medicine and as a repellent and bioinsecticide [5,7]. The volatile oil is reported to have antimicrobial, antispasmodic, antipyretic, antifungal and cicatrizing properties [8, 9, 10]. With regard the nonvolatile compounds, reports there are in the literature [7, 5].

The chemical composition of extracts of *Schinus molle* L. as well as of other plants will go to depend on diverse factors such as the geographical localization of its plantation, the genetic variability and of the technology used in the extractive process [11]. Therefore, in this work, an investigation to verify the antimicrobial action of the *Schinus molle* L. extract was performed using different co-solvents in the supercritical fluid extraction.

This work report the accomplishment of experimental procedures for posterior mathematical modeling of the process of supercritical extraction of *Schinus molle* L. using carbon dioxide and co-solvents (water and ethanol). The experiments were performed in a pilot unit with the goal to generate information to future scale-up procedure associated to this technology. The antimicrobial action of the obtained extracts was investigated for *Staphylococcus aureus*, *Pseudomonas aeruginosas*, *Escherichia coli*, *Micrococcus luteus*, and *Salmonella choleraesuis* using the bioauthography indirect method [12]. This technique belongs to microbiological screening methods commonly used for the detection of antimicrobial activity.

MATERIAL AND METHODS

Material. The raw material, consisting of leaves and twigs, was collected in the state of Rio Grande do Sul in southern Brazil. The plant material was dried at 313.15 K during 48 h. A sample of 150 g of dried and milled material with an average particle thickness of 1.93×10^{-4} m was used for extraction.

Method of extraction. The extraction was performed in a supercritical extraction pilot plant [13, 14]. The solvent used was 99.9% carbon dioxide (Air Products) with a flow rate of 1.38×10^{-4} kg s^{-1} through the extraction vessel. The extractor temperature was 333.15 K and the pressure was 150 bar. The temperature was chosen on the basis of previous results on SFE of similar matrixes [8, 15]. The temperature of the separator vessel was 273.15 K. The equipment has as goal the passage of the extractor fluid pure (solvent) or dissolved (solvent and co-solvent) in supercritical condition through an extraction vase, where the vegetal material was deposited. The stages of the extraction process can be visualized in the Figure 1.

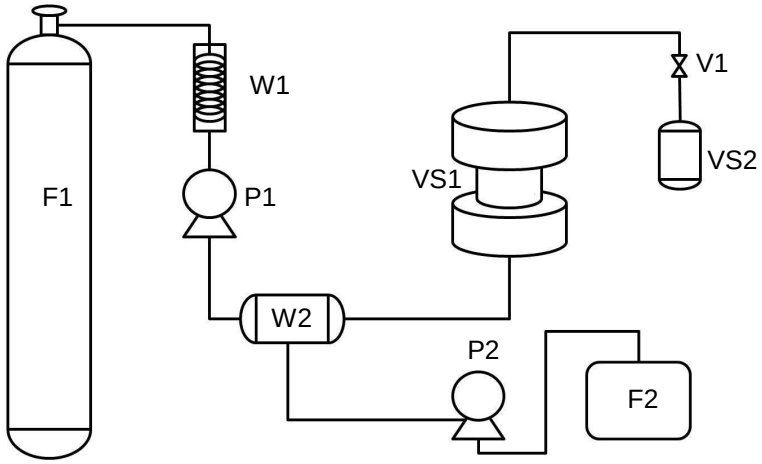

Figure 1. Schematic diagram of the experimental apparatus: P1 – high pressure pump; P2 – high pressure pump for co-solvent; W1 – preheater; W2 – heat exchanger; VS1 – extraction vessel; VS2 – separation vessel; V1– expansion valve; F1- CO_2 reservoir; F2 – co-solvent reservoir.

The co-solvent was added in the system using the pump Thar P-50 with flux of 5.0 g/min. The operation condition (150 bar and 60°C) was used due information available in the literature relative to extraction of non-volatile compounds from this plant [15].

Mathematical modeling. The mathematical model is based on two periods to describe the extraction curve. The equations are based on mass balance of the solute. The extract (solute) is considered a single component in terms of mass balance. The mass transfer properties of extract recovered were considered the same throughout the process. The first part of the extraction curve is associated to the extraction of the free solute from broken cells. In this stage, it was assumed that the fluid phase is in equilibrium with the solid phase throughout the extractor. With these hypotheses the following expression can be written for the solute mass flow which migrates to fluid-phase

$$\dot{m}_A = C_A^* Q \tag{1}$$

where $C_A{}^*$ is equilibrium fluid-phase concentration and Q is the volumetric solvent flow. To evaluate the extraction curve for this step, the following quantity is calculated

$$M_A(t) = \int_0^t \dot{m}_A \, dt \tag{2}$$

The following result is obtained from the Eqs. (1) and (2)

$$\frac{M_A(t)}{M_\infty} = \frac{F\,t}{M_\infty} \tag{3}$$

where the parameter $F = C_A{}^* Q$ and M_∞ is the maximum value for the extract obtained in the extraction. It is important to mention that result presented in Eq. (3) is valid for $t \le t^*$, being t^* the time associated to beginning of the second step. The second period is controlled by the solute diffusion from inner cells of the vegetal structure, this step correspond to the diffusion from intact cells. The diffusion is modeled by the Fick second Law written for one-dimensional rectangular system

$$\frac{\partial^2 C_A}{\partial x^2} = \frac{1}{D}\frac{\partial C_A}{\partial t} \tag{4}$$

in $-a < x < a$ and $t > t^*$ subjected the following boundary conditions

$$in \quad x = 0, \quad \frac{\partial C_A}{\partial x} = 0 \tag{5}$$

$$in \quad x = a, \quad -D\frac{\partial C_A}{\partial x} = k_c(C_A - C_{A\infty}) \tag{6}$$

and the temporal condition for $t = t^*$, $C_A = C_{A0}$. Using the solution presented by Crank [16] for the diffusion equation in a slab subject to convective boundary condition, the following result can be written

$$\frac{M(t)}{M_\infty} = 1 - \sum_{n=1}^{\infty} \frac{2L^2\, exp(-\beta_n^2 Dt / a^2)}{\beta_n^2(\beta_n^2 + L^2 + L)} \tag{7}$$

where βn are zeros of the following equation

$$\beta\, tg\beta = L\,. \tag{8}$$

The parameter $L = \frac{a\,k_c}{D}$, being a, the half thickness of slab; k_c, is the superficial coefficient of mass transfer, D is the effective diffusion coefficient, $M(t)$ is the recovered mass at time, t, M_∞ is the maximum value for the extract obtained in the extraction.

Antimicrobial Test. The antimicrobial activity against *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosas* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella choleraesuis* (ATCC 10708) and *Micrococcus luteus* (ATCC 9341) was performed using a bioautographic method. First a thin-layer chromatography (TLC) of the supercritical extracts of S. molle was made in silica gel GF254 plates [17] and developed using toluene/ethyl acetate (93:7). After elution, the plates were overlaid with Mueller-Hinton agar inoculate in petri dishes, the microorganisms *S. aureus*, *P. aeruginosas*, *E. coli* and *M. luteus* were inoculated at $1{,}0x10^6$CFU/mL and *S. choleraesuis* at $2{,}0x10^6$CFU/mL. After 24 hours at 37°C, a solution of INT "p-iodonitrotetrazolium violet" was added, for a better visualization of inhibition halos [18, 19].

RESULTS AND DISCUSSION

Experimental Data for Extraction. The experimental results for the supercritical fluid extraction were presented in Table 1 in terms of the extract mass as function of the time.

Table 1. Experimental Data for supercritical fluid extraction of *Schinus molle* L. using co-solvents

Time (min)	CO_2 (g)	$CO_2 + H_2O$ (g)	$CO_2 + C_2H_6O$ (g)
0	0	0	0
10	0.0510	0.023	0.003
20	0.2270	0.0495	0.104
30	0.4490	0.087	0.178
40	0.8675	0.1275	0.328
50	1.3260	0.1785	0.403
60	1.8175	0.2655	0.649
70	2.1350	0.309	0.794
80	2.4675	0.368	1.181
90	2.8540	0.419	1.416
100	3.2795	0.451	1.583
110	3.4715	0.4965	1.875
120	3.8025	0.55	2.055
130	3.8910	0.5725	2.199
140	4.0380	0.5995	2.288
150	4.0520	0.6095	2.475
160	4.0610	0.6125	2.591
170	-	0.6125	2.68
180	-	-	2.755
190	-	-	2.823
200	-	-	2.834
210	-	-	2.842
220	-	-	2.842

The fitting of the experimental data presented in Table 1 was carried out by least square method. This procedure promotes the numerical determination of the unknown parameters of the mathematical model. The results for the parameters were presented in Table 2 and the compatibility among the experimental data and the mathematical model can be observed in Figure 2. The obtained R^2 was 0.9922 for only CO_2, 0.9821 for CO_2 with water, and 0.9582 for CO_2 with ethanol.

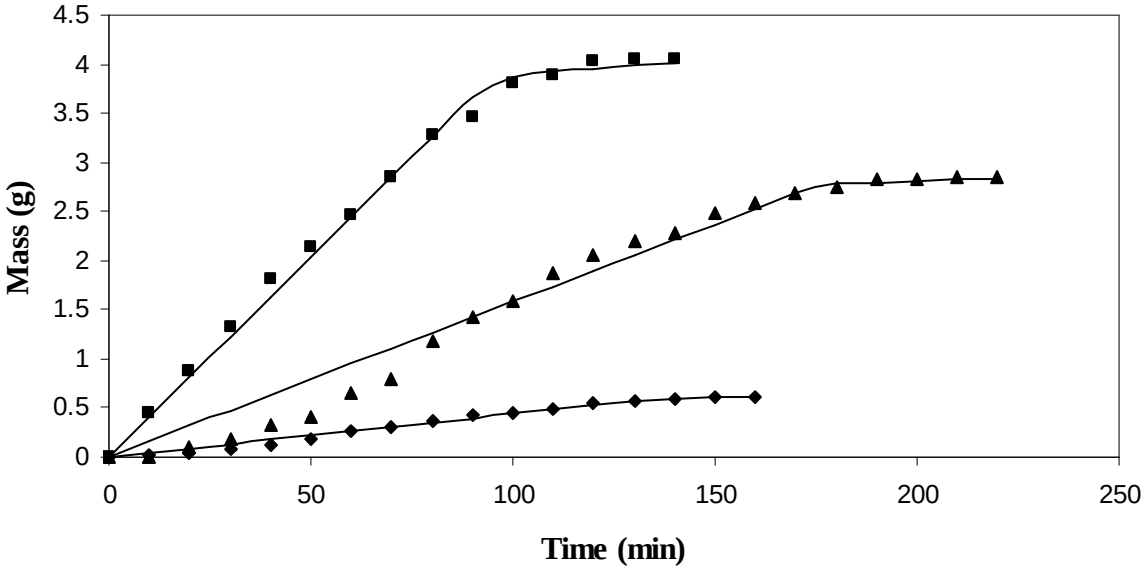

Figure 2. Curves of extraction by supercritical fluid extraction for *Schinus molle* L.: the continuous line is for the mathematical modelling and the symbols are for experimental data (CO_2 - ■ , CO_2 / ethanol - ▲, and CO_2 / water ♦).

The unknown parameters present in the mathematical model are the effective diffusivity, the convective mass transfer coefficient and the parameter F, which is associated with the solubility of the extracts. These parameters were estimated by minimization of the sum of squares of errors between the experimental data and the prediction using the model.

Table 2 – Parameter values for the mathematical model.

	F (g/s)	D (m²/s)	k_c (m/s)
CO_2	6.80×10^{-4}	1.72×10^{-9}	7.68×10^{-6}
CO_2 + ethanol	2.63×10^{-4}	4.76×10^{-10}	7.58×10^{-06}
CO_2 + water	7.33×10^{-05}	4.19×10^{-09}	4.87×10^{-06}

Results for antimicrobial assays. The detection of antimicrobial activity was not verified to *S. choleraesuis*, *S. aureus*, *P. aeruginosas* by bioautography procedures using extracts of *Schinus molle* L. The experiments performed to *E. coli* and *M. luteus* showed antimibrocial action of the obtained extracts of *Schinus molle* L. The qualitative results are presented in Figures 3, 4, and 5. Each figure was constructed according to following sequence: the first result is the bioautography for *Schinus molle* L. extract obtained with carbon dioxide and water, the second one for extract obtained with only carbon dioxide, and the third one for extract obtained with carbon dioxide and ethanol as co-solvent.

The result of bioautography for *S. choleraesuis* can be observed in Figure 3 and it indicated the non-presence of antimicrobial activity for all extracts of *Schinus molle* L.

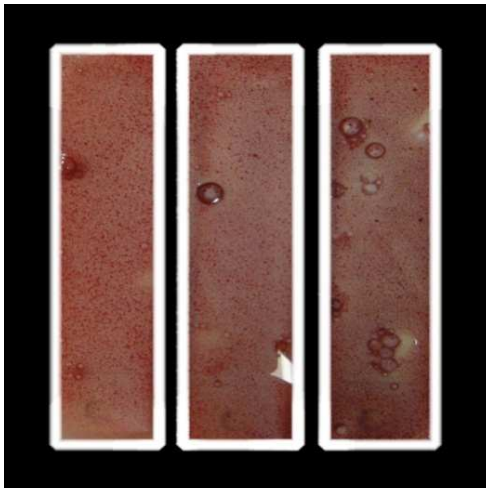

Figure 3 – Chromatographic plate in medium with *S. choleraesuis*.

The Figure 4 is referring to the *Escherichia coli* submitted to the bioautography test. It can be affirmed that all the extracts, that is, the obtained ones with only CO_2 and also the obtained extracts using co-solvent (water and ethanol) had presented effect antimicrobials due all plates show zones of inhibition growth.

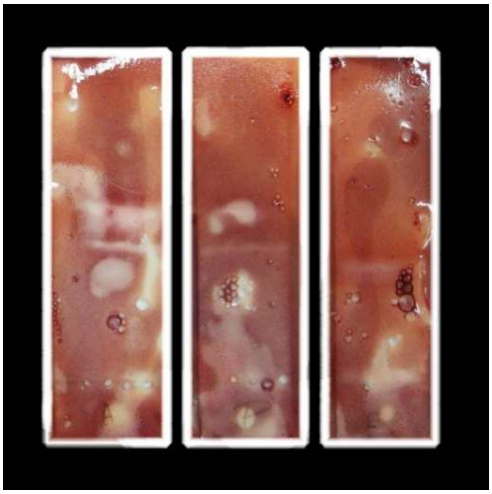

Figure 4 – Chromatographic plate in medium with *Escherichia coli*.

The Figure 5 is referring to *Micrococcus luteus* incubated in chromatography plate. The only assay that exhibited zones of inhibition growth was the plate with obtained extract using water as co-solvent.

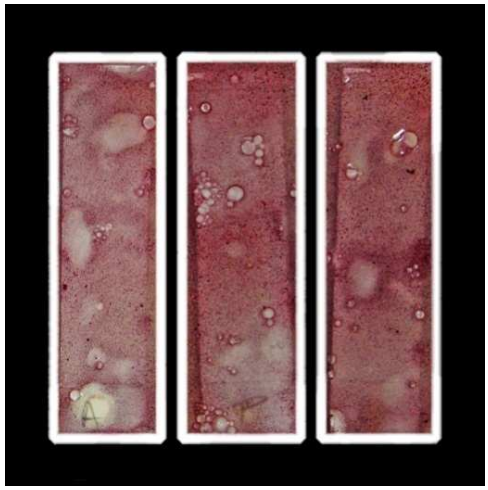

Figure 5 – Chromatographic plate in medium with *Micrococcus luteus*.

CONCLUSION

The used mathematical model showed feasibility to fitting the experimental data for the process of extraction in supercritical conditions, as much for the use of carbon dioxide being the solvent as for the situation where the co-solvents were used. The provided results for the parameters of the model can be subsidize futures works, also facilitating future process of scale-up. On the microbiological aspects, the results show that the obtained extracts of *Schinus molle* L. with carbon dioxide had presented antimicrobial action for *M. luteous* and *E. coli*. The bioautography procedures for *E. coli* showed antimicrobial action for the obtained extracts using CO_2, CO_2+water and CO_2+ethanol whereas for *M. luteus* bacterium the antimicrobial action was only verified for the extract for *Schinus molle* L. using supercritical CO_2+water. The bioautography tests for *P. aeruginosas*, *S. choleraesuis*, and *S. aureus* had not inhibited the growth of the respective bacteria.

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