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Supercritical fluid extraction of alkaloids from *Ilex paraguariensis* St. Hil.

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ABSTRACT

In this work a study about extracts obtained from *Ilex paraguariensis* St. Hil. leaves with supercritical CO_2 was carried out. Experiments were performed in a pilot-scale extractor with capacity of 1 L at pressure of 120, 150, 170 and 200 bar for four temperature conditions, 313.15, 323.15, 333.15 and 343.15 K. The experimental data were fitted using a mathematical model characterized by the existence of two distinct periods of extraction. Numerical results for the adjusted parameters correlated very well the supercritical extraction experimental data. The confidence of results obtained from mathematical model and experiments was assured through the chi-square test.

1. Introduction

Yerba mate (*Ilex paraguariensis* St. Hil.) contains methylxanthines, flavonoids, caffeoyl derivative compounds, vitamins and saponins (Cardozo et al., 2007). The pharmacological effects of caffeine have been extensively investigated. Consumption of caffeine containing products is contraindicated in the case of ulcers, severe cardiovascular diseases, insomnia and nervous tension. On the other hand caffeine is widely used in the pharmaceutical and cosmeceutic industry (Velasco et al., 2008) or as an ingredient in functional foods. Yerba mate, an important natural product in South America, are also known for their anti-inflammatory, therapeutic, anti-rheumatic, stimulant and diuretic properties (Esmelindro et al., 2004). This plant is extensively cultivated in South Brazil where it is a well appreciated non-alcoholic beverage. However, the extracts of yerba mate are also recognized as a rich source of antioxidant phenolic acids (Alikaridis, 1987; Lodovici et al., 2001; Bastos et al., 2006) used for pharmaceutical purposes.

Phenylpropanoids are phenolic compounds usually recognized as responsible for the antioxidant and choleretic activities of plant extracts (Wang et al., 1999; Lee, 2000). Recent findings also support the role of these compounds as protective agents against cardiovascular disease as well as breast, gastrointestinal and skin cancers (Carbonaro et al., 2001). Furthermore, polyphenols have been shown to be potent antioxidants, interfering with the oxidative/antioxidative potential of cells or acting as free radicals scavengers (Mazzafera, 1997). *I. paraguariensis* act as stimulants of the central nervous system, muscles and circulatory systems in humans (James, 1991), with examples such as caffeine, theophylline and theobromine being natural components of yerba mate (Saldaña et al., 1999; Clifford and Ramirez-Martinez, 1990). There are many reports on the identification of purine alkaloids from *I. paraguariensis* (Cardozo et al., 2007; Jacques et al., 2007; Saldaña et al., 2002). Extraction of these compounds is potentially attractive because of their financial value, as well as for the production of methylxanthine-free products for human consumption (Saldaña et al., 2002). Recently the yerba mate consumption has been publicized due its health benefits: hypocholesterolemic, hepatoprotective, diuretic (Heck and Mejia, 2007) and antioxidant (Gugliucci, 1996). On the other hand, the presence of alkaloids in yerba mate has been reported as a source of toxic effects (Saldaña and Mohamed, 2003). This fact can be attenuated with the use of supercritical fluid extraction to remove the alkaloids.

In this work, a supercritical extraction process was carried out to acquire extracts from *I. paraguariensis* using CO_2 as a solvent at diverse conditions of pressure and temperature. The extracts were obtained from leaves using a supercritical extraction pilot

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Nomenclature

e_∞	asymptotic yield equal to the initial solute content in the plant	S_2	equilibrium fluid phase concentration of the solute interacting with the matrix
e	extraction yield	YMa	yerba mate after supercritical extraction
e_1	extraction yield at the end of the first period	YMb	yerba mate before supercritical extraction
K	partition coefficient		
Q	solvent-to-feed ratio	*Greek symbols*	
q_1	solvent-to-feed ratio at the end of the first period	α	significance level
S_1	solute solubility in the solvent	χ^2	chi-square test

plant. New experimental data are presented about the effects of temperature and pressure on the yield of extracts from yerba mate, performed in an automated pilot-scale unit of supercritical extraction. Caffeine and theobromine were analyzed in the extracts resulting from the supercritical extraction under different conditions. A complete quali-quantitative analysis of the main bioactive compounds: polyphenols and methylxanthines was carried out on the extract resulting from the supercritical extraction, and a comparison made between that of yerba mate before supercritical extraction and after supercritical extraction by HPLC.

The experimental data were used to perform the mathematical simulation of the supercritical extraction from *I. paraguariensis*. The mathematical model used here, is characterized by the existence of two periods in the extraction process. The experimental data were fitted by two straight lines that describe the behavior of the yield as a function of the extraction time. The behavior with such characteristics is associated with the extraction process of compounds with low volatility. Numerical results are presented and discussed for the adjusted parameters, which were estimated by minimization of the sum of squares of errors between the experimental data and the prediction used in the model. A statistical analysis of the results is presented and it demonstrated confidence in the mathematical procedure performed here.

2. Materials and methods

2.1. Extraction process

Raw material was provides by Madrugada Alimentos Ltda. (RS, Brazil). The average content was 4.1% and the average particle diameter of 1×10^{-3} m. A sample (0.2 kg) was used for extraction in a Pilot Equipment (Cassel et al., 2008). Three replicates for each extraction condition were performed. The schematic diagram of the experimental apparatus can be seen in Fig. 1. It included a positive liquid displacement pump (Thar P-200, USA) for solvent delivery (20–200 g/min), a 1000 mL high-pressure extraction vessel (B1), and a separator flask (B2). The extraction vessel was supplied with a heating jacket and an automated temperature controller.

Heating tapes were used throughout the apparatus to maintain a constant temperature in the extraction section. To ensure a constant and steady solvent delivery the pump head was cooled by a circulating fluid, which passed through a chiller. Flow rates and accumulated gas volumes passing through the apparatus were measured using a flowmeter assay, 1–300 g/min (Thar 06618-2, USA). Ke (USA) micrometering valves (VC1) were used for flow control throughout the apparatus. Heating tapes with automated temperature control were also used around this valve to prevent both freezing of the solvents and solid solute precipitation following depressurization. Pressure in the extractor was monitored with a digital transducer system, Novus 8800021600, acquired from Novus Produtos Eletrônicos (Brazil) with a precision of ±1.0 bar. The temperature controller (TC) was connected to thermocouples (PT-100, with an accuracy of 0.5 K).

2.2. Chemical analysis

2.2.1. Samples preparation

Yerba mate leaves: 500 mg were extracted with methanol in a reflux condenser for the duration of 30 min (2×50 mL). Combined methanol extracts were filtered and the final volume adjusted to 100 mL with methanol.

Analysis of methylxanthines: To 2 mL of the methanolic solution, 8 mL of a mixture of water–acetic acid (98:2) (solvent A) was added 500 mg were extracted twice (2×50 mL) with methanol in a reflux condenser for 30 min. Combined methanol extracts were filtered and final volume adjusted to 100 mL with methanol.

Analysis of flavonoids and caffeoyl derivative compounds: 5 mL of the methanolic extract was diluted with methanol and the final volume adjusted to 10 mL.

Supercritical extract: 2.00 mg was dissolved in methanol under ultrasonic agitation and the final volume adjusted to10 mL. Then, to 2 mL of this solution, solvent A was added and the final volume adjusted to10 mL.

2.2.2. High performance liquid chromatography

A VarianTM series 9000 chromatograph equipped with a binary pump Varian 9012 was used. Quantitation of caffeoyl derivatives, flavonoids and methylxanthines was carried out using a validated HPLC external standard method (Filip et al., 2001; Kopcak and Mohamed, 2005). Reference compounds were purchased from SIGMA Company. A reverse phase Luna IB-SIL RP 18 (5 μ, 250×4.6 mm) Phenomenex column and a binary gradient system consisting of solvent A: water:acetic acid (98:2); solvent B: methanol:acetic acid (98:2) (caffeoyl derivatives and flavonoids) or methanol:acetic acid (96:6) (methylxanthines) were used. For caffeoyl derivatives and flavonoids the gradient was from 15% B to 40% B in 30 min, 40% B to 75% B in 10 min and 75% B to 85% B in 5 min. Flow rate was set at 1.2 mL/min. For caffeine and theobromine the gradient was from 17% B to 20% B in 10 min; 20% B (isocratic) for 5 min; 20% B to 23% B in 10 min and 23% B to 100% B in 5 min with a flow rate of 1.0 mL/min. Identification and quantitation was carried out by simultaneous detection with a UV Varian 9050 UV detector and a Varian 9065 DAD at 325 nm for caffeoyl derivatives, 255 nm for flavonoids and 273 nm for methylxanthines. The samples were injected with a Rheodyne injector fitted with a 20 μL loop.

2.3. Standard compounds

Caffeine, theobromine, chlorogenic acid, caffeic acid, 1,5-dicaffeoylquinic acid (cynarin), rutin, quercetin and kaempferol standards of high purity, purchased from SIGMA, were used.

The amount of 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic isomer acids were calculated and expressed as cynarin.

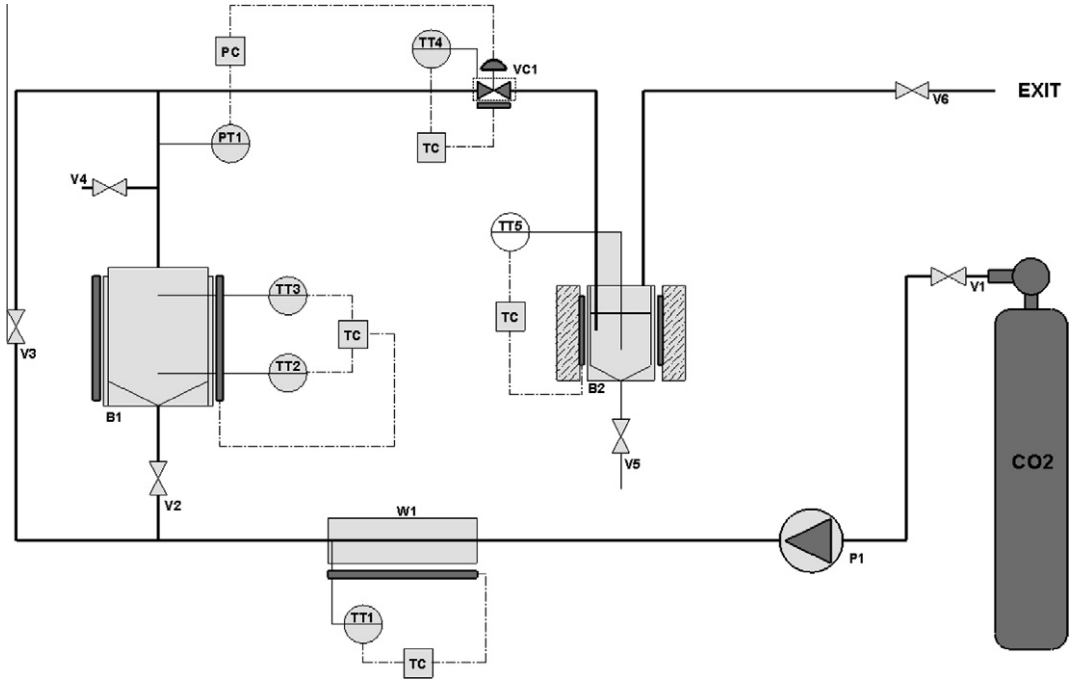

Fig. 1. Schematic diagram of the experimental apparatus: P1, high-pressure pump; W1, preheater; B1, extraction vessel; B2, separation vessel; VC1, micrometering; TC, temperature controllers; V1–V6, sphere valves.

3. Results and discussion

Caffeine and theobromine were analyzed in the extracts resulting from the supercritical extraction under different conditions (Table 1). The condition for decaffeination associated with higher caffeine concentration was 150 bar/323.15 K, and can be observed in Table 1. These data confirm the higher selectivity of CO_2 for caffeine followed by theobromine, pointed out by Saldaña et al. (1999).

On the other hand, the polyphenols (caffeoyl derivatives and flavonoids) and methylxanthines were analyzed in the extract obtained from the supercritical extraction and in yerba mate before and after extraction. HPLC–DAD was employed, the results being presented in Table 2. The results indicated that only caffeine (majority) and theobromine were extracted from the vegetal drug (yerba mate).

The results in Table 2 are expressed as % on dried wt. DCQ: dicaffeoylquinic acid. *3,4-DCQ; 3,5-DCQ and 4,5-DCQ were valorated and expressed as cynarin (1,5-DCQ). The detection limit was 0.2 ppm and the quantification limit was 1 ppm.

In the extraction process, the bed height (m), diameter (m), and volume (m^3) were, respectively, 0.13, 0.06 and 0.000367. Liquid CO_2, 99.99% purity (Air Products) was used for extractions and the flow rate of the solvent was 6.667×10^{-4} kg/s, for a sample a 0.2 kg of *I. paraguariensis*.

Experimental results of the supercritical extraction were obtained at the following range of pressure: 120–200 bar. The investigated temperature conditions were: 313.15, 323.15, 333.15 and 343.15 K. The extract had caffeine as the majority compound.

The maximum global yield of yerba mate extract, expressed as a weight, is listed in Table 3, for the conditions proposed and it occurred at the lowest temperature (313.15 K) for all pressures. The yield increased with the decrease of the extraction temperature; the effect of extraction pressure was less pronounced.

These results (Table 3) can be explained by the fact that there is a strong variation in density in the vicinity of the critical point of

Table 1
Content of caffeine and theobromine. ND, no detection.

Conditions	Caffeine content % (w/w)	Theobromine content % (w/w)
120 bar/313.15 K	25.7 ± 0.2	0.271 ± 0.003
150 bar/323.15 K	33.6 ± 0.3	0.234 ± 0.003
170 bar/333.15 K	12.7 ± 0.1	0.144 ± 0.002
200 bar/343.15 K	10.1 ± 0.1	ND

Table 2
Content of methylxanthines, caffeoyl derivatives and flavonoids in the assayed samples. Where *E*, supercritical CO_2 extract obtained at 170 bar and 333.15 K; YMb, yerba mate before supercritical extraction; YMa, yerba mate after supercritical extraction; ND, no detection.

Compounds %	YMb	YMa	*E*
Caffeine	1.038 ± 0.009	0.686 ± 0.008	12.7 ± 0.1
Theo bromine	0.167 ± 0.002	0.127 ± 0.001	0.144 ± 0.002
Chlorogenic acid	1.659 ± 0.009	1.566 ± 0.009	ND
Caffeic acid	0.0296 ± 0.0003	0.0267 ± 0.0003	ND
3,4-DCQ*	0.423 ± 0.003	0.367 ± 0.003	ND
3,5-DCQ*	4.25 ± 0.02	2.97 ± 0.03	ND
4,5-DCQ*	1.23 ± 0.01	1.21 ± 0.01	ND
Rutin	1.07 ± 0.01	1.07 ± 0.01	ND
Quercetin	0.008 ± 0.002	0.004 ± 0.002	ND
Kaempferol	ND	ND	ND

Table 3
Maximum global yield.

Maximum global yield (% w/w)				
	313.15 K	323.15 K	333.15 K	343.15 K
120 bar	1.00	0.38	0.08	0.09
150 bar	0.79	0.44	0.13	0.17
170 bar	1.00	0.43	0.28	0.35
200 bar	1.00	0.33	0.26	0.12

CO_2 (Perrut et al., 1997). In general, in this region, the supercritical carbon dioxide has a liquid-like density and the solvation power is increased.

The experimental data showed the existence of two significant periods in the extraction runs. The free solute in open cavities was extracted in the first period and its equilibrium fluid phase concentration was equal to the solubility of pure solute in the solvent (Sovová et al., 2007). In the second period, the free solute was extracted but the solute interacting with the matrix was extracted with a lower equilibrium fluid phase concentration. Perrut et al. (1997) and Sovová et al. (2007) observed this situation in their extraction studies. In this work the same behavior has been observed for the *I. paraguariensis* supercritical extraction process. This fact permits the use of a simple mathematical model to fit the experimental data. Two straight lines were fitted to experimental data. The equilibrium fluid phase concentration can be estimated from the slopes of extraction curves in both extraction periods. The behavior of experimental data was well represented by a simple model proposed by Sovová et al. (2007).

Sovová et al. (2007) presented the following mathematical formulation to describe the extraction process.

$$e = S_1 q, \quad \text{for } q \leqslant q_1 = \frac{e_1}{S_1} \tag{1}$$

$$e = e_1 + S_2(q - q_1), \quad \text{for } q_1 < q \leqslant q_1 + \frac{(e_\infty - e_1)}{S_2} \tag{2}$$

where e is the extraction yield, q is the solvent-to-feed ratio, e_1 is the extraction yield at the end of the first period, q_1 is the solvent-to-feed ratio at the end of the first period, S_1 is the solute solubility in the solvent, S_2 is the equilibrium fluid phase concentration of the solute interacting with the matrix and e_∞ is the asymptotic yield equal to the initial solute content in the plant (Sovová et al., 2007). The solute interaction with matrix is usually described by linear behavior characterized by partition coefficient K defined by Sovová et al. (2007). This mathematical approach requires two parameters to be adjusted, and it is used in this work.

$$K = \frac{S_2}{x_1} = \frac{S_2}{(e_\infty - e_1)} \tag{3}$$

The values of the parameters S_1, S_2, e_1, and K used in the simulation of the *I. paraguariensis* extraction process at 120, 150, 170 and 200 bar are presented in Tables 4–7, respectively, at different temperature conditions.

The temperature dependence of extraction yields was evident from experimental data in the four conditions of pressure investigated in this work. The average and standard deviation results of experimental extraction are reported in Figs. 2–5. The yield is given as a function of the solvent-to-feed ratio, q.

The mathematical modeling was successful and the values obtained for the parameters S_1 and S_2 are according to the hypotheses assumed in the approach used in this work. The two extraction periods were well identified, and the asymptotic behavior of the second period can be clearly observed in the Figs. 2–5. The results obtained with the applied model showed that it is in agreement with this expected findings. The chi-square test (χ^2) (Montgomery and Runger, 2007) was performed with 5% of significance level ($\alpha = 0.05$), where it was observed that the calculated value of χ^2 is less than the value tabulated of χ^2 for extraction's curves for each condition of pressure and temperature. So it is not rejected at the significance level of 5%, the hypothesis that the relationship between the yield of extraction and the ratio of accumulated solvent (q) was in accordance with the proposed model. Such a finding indicates that the predicted model is congruent with the observed data.

The extraction leads to the development of a yerba mate product with lower caffeine amounts, but without altering the content of potential antioxidant compounds. The supercritical extraction

Table 4
Parameters of extraction curves fitted to global yields from yerba mate leaves at 120 bar.

Pressure (bar) 120				
Temperature (K)	313.15	323.15	333.15	343.15
S_1 (g/g CO_2)	0.00095	0.0003	0.00008	0.0002
S_2 (g/g CO_2)	0.00024	0.00008	0.00001	0.000005
e_1 (%)	0.0076	0.0030	0.00064	0.00085
K (g/g)	0.100	0.100	0.071	0.062

Table 5
Parameters of extraction curves fitted to global yields from yerba mate leaves at 150 bar.

Pressure (bar) 150				
Temperature (K)	313.15	323.15	333.15	343.15
S_1 (g/g CO_2)	0.0006	0.0008	0.00013	0.000165
S_2 (g/g CO_2)	0.00009	0.00008	0.000036	0.000022
e_1 (%)	0.0072	0.0035	0.00104	0.0013
K (g/g)	0.099	0.062	0.101	0.062

Table 6
Parameters of extraction curves fitted to global yields from yerba mate leaves at 170 bar.

Pressure (bar) 170				
Temperature (K)	313.15	323.15	333.15	343.15
S_1 (g/g CO_2)	0.0008	0.00018	0.0002	0.0005
S_2 (g/g CO_2)	0.0001	0.00003	0.000056	0.000054
E_1 (%)	0.0094	0.003978	0.00241	0.003
K (g/g)	0.124	0.083	0.125	0.099

Table 7
Parameters of extraction curves fitted to global yields from yerba mate leaves at 200 bar.

Pressure(bar) 200				
Temperature (K)	313.15	323.15	333.15	343.15
S_1 (g/g CO_2)	0.0007	0.00009	0.00007	0.000052
S_2 (g/g CO_2)	0.00018	0.000026	0.000005	0.000024
e_1 (%)	0.0082	0.0030	0.0025	0.00088
K (g/g)	0.071	0.083	0.150	0.083

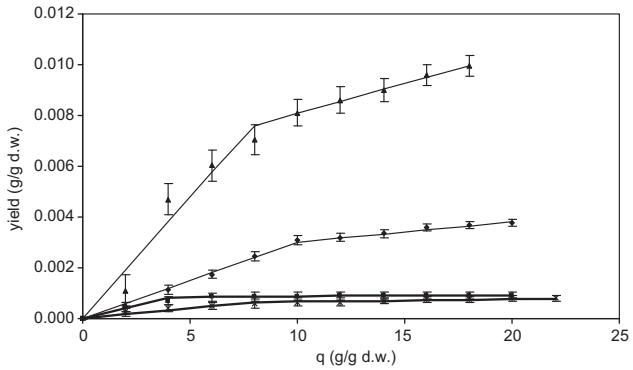

Fig. 2. Global extraction yields from yerba mate leaves with CO_2 at 120 bar. Extraction conditions: (▲) 313.15 K; (♦) 323.15 K; (×) 333.15 K; (■) 343.15 K; (–) Eqs. (1) and (2) with parameters from Table 3.

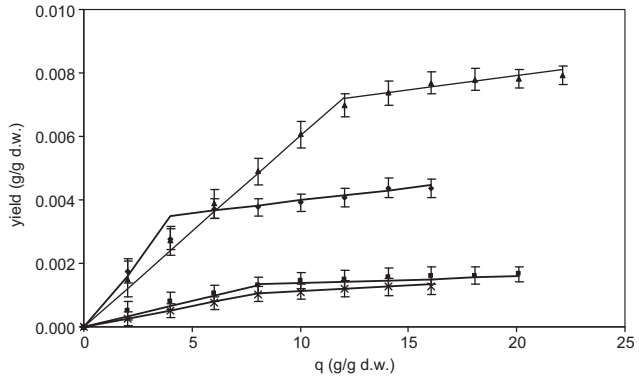

Fig. 3. Global extraction yields from yerba mate leaves with CO_2 at 150 bar. Extraction conditions: (▲) 313.15 K; (♦) 323.15 K; (×) 333.15 K; (■) 343.15 K; (–) Eqs. (1) and (2) with parameters from Table 4.

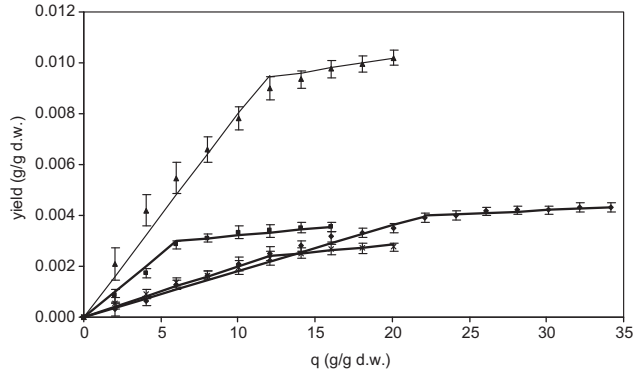

Fig. 4. Global extraction yields from yerba mate leaves with CO_2 at 170 bar. Extraction conditions: (▲) 313.15 K; (♦) 323.15 K; (×) 333.15 K; (■) 343.15 K; (–) Eqs. (1) and (2) with parameters from Table 5.

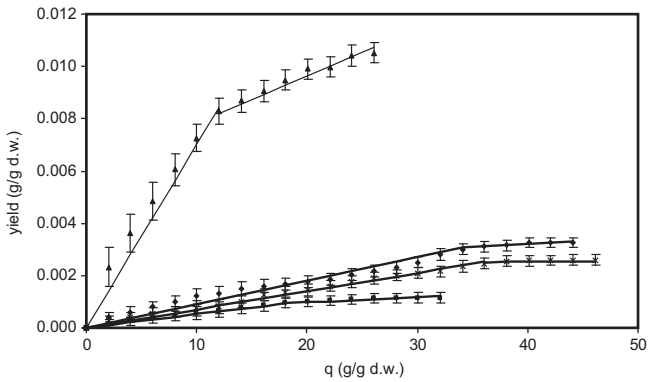

Fig. 5. Global extraction yields from yerba mate leaves with CO_2 at 200 bar. Extraction conditions: (▲) 313.15 K; (♦) 323.15 K; (×) 333.15 K; (■) 343.15 K; (–) Eqs. (1) and (2) with parameters from Table 6.

process from *I. paraguariensis* with CO_2 consisted of two different periods, both controlled by phase equilibrium for the pressure conditions investigated. The experimental data were well fitted using two straight lines described by the mathematical model. The simplicity of the mathematical model and the small quantity of the parameters to be adjusted make this procedure very attractive to simulate the supercritical extraction process. The initial part of all extract curves is linear and the second part presents an asymptotic yield behavior. The maximum yield observed by the extraction was at a temperature of 313.15 K for all pressures investigated. The experiments show an inverse behavior of global yield in comparison to augment of temperature for a fixed pressure condition. A correlation between the maximum global yield obtained and the caffeine content was observed. From the experimental results, the condition where the higher value for the selectivity for caffeine was observed at 150 bar/323.15 K. On the other hand, for the global yield, this situation occurred at 120 bar/313.15 K.

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