

DETERMINATION OF THE GLOBAL YIELD FOR THE SYSTEM LIPPIA ALBA + CO₂ AND MODELING OF THE PHASE EQUILIBRIUM OF THE MAJOR CONSTITUENTS: LIMONENE AND CARVONE

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Lippia alba is used in medicine of the Central and South Americas and Tropical Africa for treatment of gastrointestinal disorders, because of its antiulcerogenic activity. The major constituents of the *L. alba* volatile oil are limonene and carvone. In order to identify the best process conditions, that is, the conditions that maximize the solubility of *L. alba* volatile oil in carbon dioxide, the phase equilibrium of the systems: limonene + CO₂ and carvone + CO₂ were studied. Literature data and the Peng-Robinson equation of state were used. Based on the predicted phase equilibrium the experimental conditions were chosen. The SFE yields and extract compositions were compared to that of the conventional processes: hydrodistillation and ethanol low pressure extraction. The effects of temperature and pressure on the global yield were evaluated by an analysis of variance (ANOVA). The largest SFE yield (6.5%, mass of extract/mass of dry solid) was obtained at the 45 °C and 100 bar.

Keywords: *Lippia alba*, SFE, limonene, carvone, phase equilibrium

INTRODUCTION

The species of the genus *Lippia* are mainly distributed throughout South and Central America countries, and Tropical Africa territories. This genus includes approximately 200 species of herbs, shrubs and small trees [1]. There are few studies published about the chemical composition and pharmacological aspects of the genus *Lippia*. There are many references about the traditional uses of these species, often these are contradictory activities. Few pharmacological studies have been made, among these studies are included the species *Lippia multiflora* Moldenke and *Lippia alba* (Mill) N.E. Brown [2]. The parts used are leaves or aerial parts, and flowers and they are prepared as an infusion or decoction, administered orally. The species *Lippia alba* is employed in Guatemala, Venezuela, and Brazil as a remedy for colds, gripe, bronchitis, coughs and asthma [3], stomach ache and indigestion [4], can act as a carminative [5], and in Central America and Brazilian Amazon it is administered to treat hepatic diseases [6], syphilis [7], diarrhea and dysentery [8]. There were found uses as anticonvulsant and effect cytostatic (López et al. (1979), cited by [2]). The behavioral effects (anxiolytic, locomotor activity, myorelaxant) of the essential oils (EO) from *Lippia alba* (Mill.) N.E. Brown in mice, were studied [10]. The antiulcerogenic activity of *Lippia alba*

[11] was studied by oral administration of an infusion; *L. alba* was found to be effective in preventing gastric ulceration induced by indomethacin. Carvone and limonene were [9] extracted from caraway seed (*Carum carvi* L.) with supercritical CO₂. The process conditions have been optimized with respect to pressure, temperature, CO₂ flow rate, and extraction time. The highest yield of carvone and limonene was obtained at 125 bar, 32°C, CO₂ flow rate of 4 kg/h, and extraction time above 45 min. Raeissi and Peters [12] studied the phenomenon of double retrograde vaporization in the binary system ethane-limonene. The work reported bubble, dew, and critical point data for the binary system at five different compositions ranging from 99.50 to 99.93 % (mol) of ethane, and temperatures of 280 to 330 K and pressure of 30 to 60 bar. The system exhibited triple- and quadruple-valued dew points at certain concentrations; the binary system also shown the phenomenon of double retrograde vaporization. The authors' explanation is that: Double retrograde vaporization occurs within very limited ranges of composition and temperature, and very close to the critical point of the more volatile component; it is the result of the continuous phase transition from the critical point of a pure volatile component to the critical point of the mixture, very highly concentrated with the more volatile component. The authors [12] believe, that this is a normal behavior, which always occurs in binary mixtures in which the two components differ largely in molecular size; accordingly with the authors, because the region that it is occur is so small, it is easily missed experimentally. Their results indicated that a very minor change in the concentration can vary the shape of the dew point curve significantly; they quantitatively showed how is small the temperature range for double retrograde vaporization.

The objectives of this work were to investigate the influence of temperature and pressure in the supercritical extraction of *Lippia alba*, to detect the operating conditions that maximize the global yield, to search for operating conditions that maximize the extraction of the target components carvone and limonene, and to compare the SFE extract's chemical composition with the chemical composition of the extracts obtained by hydrodistillation and low-pressure extraction ethanol.

MATERIALS AND METHODS

Prediction of phase equilibrium

In order to choose the operating temperature and pressure, phase equilibria diagrams were made for binary systems limonene-CO₂ and carvone-CO₂. The binary interaction parameters (k_{ij} and l_{ij}) of the Peng–Robinson equation of state (PR–EOS) were determined using the experimental data of Gamse and Marr [13] at 30°C; the date were fitted the using the software Edeflash v 1.0 [18]. The critical properties of the pure substances were calculated by the Joback's method using the software Termofis v 1.0 [14]. Employing the estimated binary interaction parameters the equilibria data were calculated for the isotherms of 40 and 50°C.

Raw material characterization

The raw material was cultivated at the Experimental Farm of Lageado (Plant Production Department, Agronomy Science College/UNESP, Botucatu, SP, Brazil). The *L. alba* was dried up to 8.6% (wet basis) determined by the xylol distillation method [14]. The material was comminuted in a mill (Tecnal, model TE-631, Piracicaba, Brazil) and the particle size distribution of the solid was determined using an agitator (Bertel, model magnético, Caieiras, Brazil), for 10 minutes, containing sieves of the Tyler series meshes 24, 32 and 48.

Extractions: SFE, hydrodistillation and ethanol low pressure extraction

The extractions were performed using a Spe-ed unit (Applied Separations, Inc., model

7071, Allentown, USA) with a 6.3mL extraction cell; the bed was formed with particle of meshes 24 to 48; the apparent bed density was kept constant at 318.4kg/m^3 , and the solvent was CO_2 (99.5%, White Martins Gases Industriais, Campinas, Brazil); the assays were duplicated. The global yield was calculated as the ratio of the total mass of extract to the mass of dry solid. The volatile oil (essential) was obtained by hydrodistillation using the AOAC 962.17 method [15] using 0,003 kg of *L. alba* of meshes 24 to 48 and the extraction continued for 120 minutes. The oleoresin (ethanol extract) was obtained as follows: 3grams of *L. alba* of meshes 24 to 48 and 150mL of ethanol (96%, Merck, São Paulo, Brazil) were placed inside a Soxhlet apparatus of 500mL and maintained under reflux for 3 hours. The ethanol was removed using a rotatory evaporator (Laborota, model 4001, Viertrieb, Germany), with vacuum control (Heidolph Instruments GMBH, model Rotavac control, Viertrieb, Germany).

Characterization of the extract: chemical composition

The extracts were fractionated by TLC comparing performed using silica gel plates (20×20 cm, 1 mm of height, Merck, lot 940378601, USA) and a mixture 80:20 of hexane (96% purity, P.A., Merck, lot K28659183 104, USA) and ethyl acetate (99.5% purity, P.A., Merck, lot K225488323 837, Germany) as the mobile phase. The plates were revealed with anisaldehyde solution. The chemical compositions of the extracts were evaluated using a Gas Chromatographer with a Flame Ionization Detector (GC/FID, Shimadzu, model 17A, Kyoto, Japan), equipped with a capillary column of fused silica DB-5 (30 m×0.25 mm×0.25 μm , J&W Scientific, Folsom, USA). The carrier gas was helium (1.7 mL/min, 99.99% purity, White Martins Gases Industriais). The injector and detector temperatures were 250°C and 230°C, respectively. The temperature programming was 50°C (5min), 50to 280°C at 5 °C/min, and kept at 280°C for 5min. The sample split ratio was 1/30. Samples of 1 μl of extract diluted in ethyl acetate (5×10^{-6} kg of extract diluted in 1×10^{-6} m³ ethyl acetate; chromatographic grade, EM Science, lot 3903991, USA) were injected. The identification of the substances was based on comparison of the extract's chromatogram of *L. alba* chromatogram from the literature [16] .

Statistical analysis

The effects of temperature and pressure on the global yield were evaluated by an analysis of variance (ANOVA) performed using Statistica 5.0.

RESULTS AND DISCUSSION

The calculated phase equilibria data plotted as pressure-solubility graphics are in Figures 1 and 2. To avoid the retrograde vaporization region, but, still keep the system around the regions of increasing solubility the inspection of Figures 1 and 2 indicates that temperatures should be in the range of 40 and 50°C (higher temperatures can volatilize de compounds) and relatively low pressures (around 80 and 100 bar). Thus, the SFE assays were done accordingly with a factorial design with two levels of temperature (40 and 50 °C), two levels of pressure (80 and 120 bar) and a central point at 45°C and 100 bar; the assays were replicated at the central point.

Table 1 shows the global yields. The effects of temperature and pressure on the SFE global yields can be observed on Figure 3. The analysis of variance (ANOVA) shown that the effects of temperature and pressure were significant, and the effect of pressure was more important ($p = 0.018$) that that of temperature ($p = 0.098$), as can be observed in Figure 3.

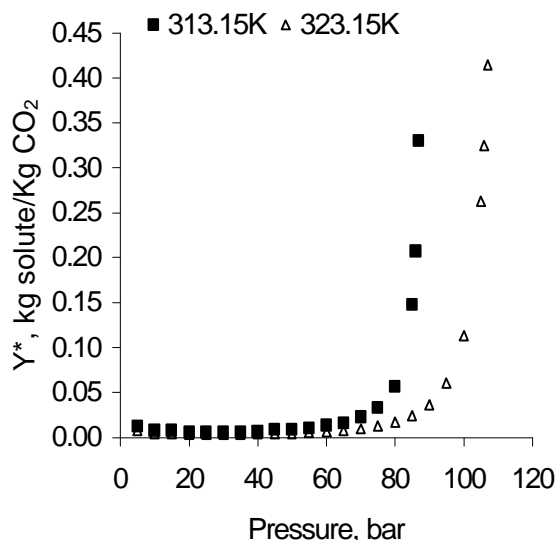
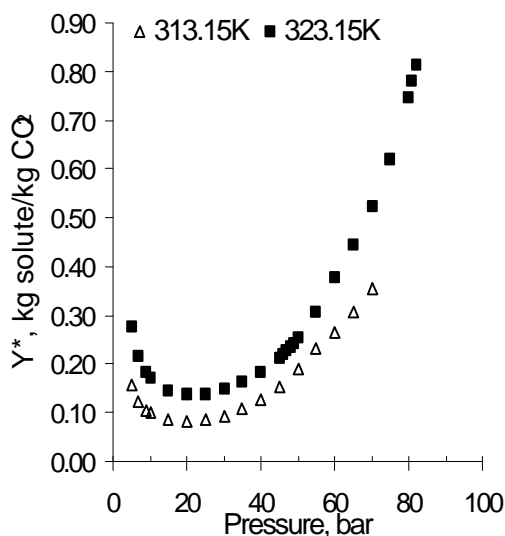


Figure 1: Solubility of limonene in CO₂ Figure 2: Solubility of carvone in CO₂ calculated by the PR-EOS.

Table 1: Operating conditions and extraction yields

Process	Operating conditions	Global yield (% , mass)
SFE	80bar/40°C	0.61
	80bar/50°C	0.29
	120bar/40°C	2.44
	120bar/50°C	3.76
	100bar/45°C	6.53 (standard deviation = 1.71)
Leaching	Ethanol at 0.95bar	16±3
Hydrodistillation	Steam at 0.95 bar	0.8± 0.1

The largest SFE global yield (6.5%, mass) was detected at 45°C and 100 bar that is approximately 40% of the yield obtained by the ethanol low-pressure extraction. Nonetheless, chemical compositions of the extracts were different. The SFE allowed extracting other compounds, thus, reducing the relative percent of carvone and limonene (Figure 4).

Figure 4 shows the TLC for all extracts and the standards carvone and limonene; as shown, besides carvone and limonene the extracts contain other substances. The main compounds identified in the SFE extracts were carvone, γ -muurolene and bulnesol (Table 2). The largest content of carvone was detected at 50°C and 120 bar. Limonene was not detected in the SFE extracts, nonetheless, it was detected in the volatile oil (hydrodistillation), then, and it was probably volatilized in the separation vessel.

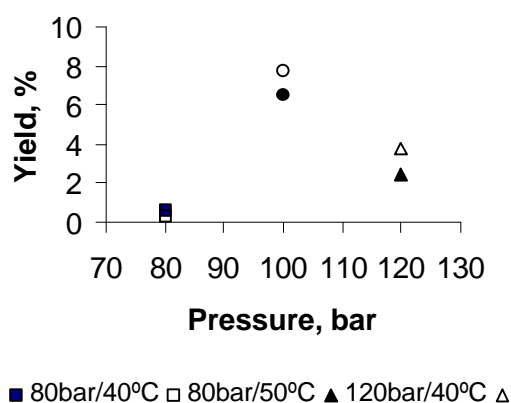
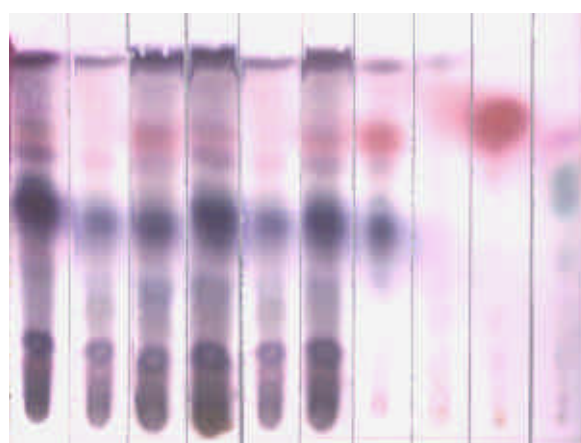


Figure 3: Temperature and pressure effects on the SFE global yields



1 2 3 4 5 6 7 8 9 10
 Figure 4: TLC of *Lippia alba* extracts. SFE - 1: 80 bar/40°C; 2: 80 bar/50°C; 3: 100 bar 45°C; 4: 100 bar/ 45°C; 5: 120 bar/40°C; 6: 120 bar/50°C; 7: Volatile oil ; 8: Leaching ; 9: Carvone; 10: Limonene

Table 2: Major compounds of *Lippia alba* extracts

Substance	Composition (relative proportion, area %)					
	Hydrodistillation	80bar		100 bar	120bar	
		40°C	50°C	45°C	40°C	50°C
Limonene	26.7	tr	-	-	tr	tr
Carvone	48.3	9.9	21.7	8.5 ± 2.6	9.0	24.1
?-muurolene	6.5	2.3	35.9	33.6 ± 41.6	39.5	37.0
Bulnesol	0.13	4.2	4.29	4.3 ± 0.6	2.5	4.1

tr: trace

CONCLUSIONS

The equilibria data of the major compounds of *L. Alba* extracts provided useful information to avoid operating the SFE system at regions near the double retrograde vaporization; the information was also helpful on identifying the conditions that solubility is expected to be at a maximum. The highest SFE global yield (6.5%, mass) was obtained at 45°C and 100 bar; nonetheless, leaching with ethanol resulted in yield of 16% (mass). In any case, the chemical composition of the extracts was different for the 3 processes studied.

ACKNOWLEDGMENTS

The authors are grateful to FAPESP (1999/01692-1) for the financial support. M. E. M. Braga and P. A. D. Ehlert thank FAPESP (02/01608-6 and 00/06848-1) for the Ph.D. assistantships.

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