PHASE EQUILIBRIUM DATA OF *Casearia sylvestris* EXTRACT + ETHANOL + CO$_2$ SYSTEM

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Abstract. The interest in high purity compounds with biological properties has increased in the last years in food and pharmaceutical industries. *Casearia sylvestris* is a native medicinal plant in Brazil, which is rich in substances as flavonoids and diterpenes. In order to preserve their activity food and pharmaceutical industries are focusing on coating or encapsulating the active ingredient into biopolymers in a micrometer range. The Supercritical Anti-Solvent (SAS) process is a high pressure alternative for the micronization/encapsulation of bioactive extracts. The phase equilibrium behavior of the involved systems (extract, solvents and polymer) provides the knowledge of best operational pressure and temperature for the SAS technology assays. Therefore, the aim of this work is to investigate the phase equilibrium behavior of the system composed by *Casearia sylvestris* extract, ethanol and supercritical carbon dioxide (CO$_2$) to afford information for the encapsulation process. *C. sylvestris* extract used in this study was obtained by supercritical fluid extraction (SFE) with CO$_2$ at 300bar and 50°C, added of 5% (w/w) of ethanol. The phase equilibrium data was attained through the static synthetic method using *C. sylvestris* extract dissolved in ethanol (1:100, w/w) at temperatures from 35°C to 75°C and CO$_2$ mass contents from 60% to 90%. The system exhibits liquid-vapor equilibrium and a lower critical solution temperature behavior. The data obtained in this study will be used on the selection of an adequate condition for encapsulation of *C. sylvestris* extract in Pluronic F-127.

Keywords: particle formation, SAS, supercritical CO$_2$, Pluronic F-127.

1. Introduction

The knowledge of the phase equilibrium of natural extracts in supercritical fluids, obtained by experimental measurements, is fundamental for determination of optimal conditions for separation and precipitation processes, especially for food and pharmaceutical industries. The optimization of these conditions is an important field of study [1]. Phase equilibrium data comprise the basic support for many chemical processes and separation operations that are conducted at higher pressures [2].

Casearia sylvestris is a native medicinal plant in Brazil, which is rich in substances as flavonoids and diterpenes [3-6]. The major components of the essential oil of this plant are β-caryophyllene, α-humulene, bicyclogermacrene, germacrene B and D [7]. The extract of C. sylvestris presents biological activity as cytotoxic and antitumor potential and anti-inflammatory activity [5;8-10] These biological activities are not attributed only to the action of a single compound, but possibly to the synergistic effect among the compounds in the extract [11]. In order to preserve their activity food and pharmaceutical industries are focusing on coating or encapsulating the active ingredient into biopolymers in a micrometer range [12-16].

Considering this and the interest of pharmaceutical industries in substances with biological activity, usually present in complex mixtures, the phase behavior of a system plays a crucial role in understanding the precipitation mechanism, such as jet breakup, nucleation and growth kinetics, and mass transfer, as well as to determine the most satisfactory operating conditions during the precipitation. Several process using supercritical fluids have been proposed in order to obtain solid particles with characteristics such as particle size, morphology and crystalline structure, which are difficult to obtain using traditional methods [17-18].

The Supercritical Anti-Solvent (SAS) process is a high pressure alternative for the micronization/encapsulation of bioactive extracts. The phase equilibrium behavior of the involved systems (extract, solvents and polymer) provides the knowledge of best operational pressure and temperature for the SAS technology assays.

The aim of this work was to investigate the phase equilibrium behavior of the system composed by *Casearia sylvestris* extract, ethanol and supercritical carbon dioxide (CO_2) to afford information for the encapsulation process. For this purpose the phase equilibrium data was attained obtained by means of the static synthetic method.

2.   Material and Methods

2.1 Sample preparation and obtention of *Casearia sylvestris* extract

Leaves of *C. sylvestris*, provided by Brazervas Laboratório Fitoterápico Ltda., Osório/RS, Brazil, were air-dried at room temperature up to 13.72 ± 0.05 % (w/w) of moisture. The dried material was ground in a knife mill (De Leo, Porto Alegre/RS, Brazil). The sample was stored at -18 ^{o}C until the extractions were performed.

The supercritical fluid extraction (SFE) of *C. sylvestris* was held in a dynamic extraction unit previously described by Zetzl et al [19]. The extraction procedure was described by Michielin et al. [20]. Briefly, the extraction procedure consisted of placing 15 g of dried and milled material inside the column to form the particles fixed bed, followed by the control of the temperature, pressure and solvent flow rate. The extraction was performed and the solute collected in amber flasks and weighted in an analytical balance (OHAUS, Model AS200S, NJ, USA). The SFE assay was performed with CO_2 added with ethanol (ETOH) as a co-solvent. The extraction was done at 50 ^{o}C, 200 bar and CO_2 flow rate of 8.3 ± 2 g/min (3.5 h extraction), using 5 % (w/w) of ethanol. The SFE assay was performed with 99.9 % pure carbon dioxide, delivered at pressure up to 60 bar (White Martins, Brazil).

The resulting mixture from the SFE was separated by using reduced pressure to evaporate the solvents in a rotary evaporator (Fisatom, 802, Brazil). The *C. sylvestris* extract obtained was dissolved in etanol 99.5 % pure (1:100, w/w) and submitted to phase equilibrium experiments.

2.2 Phase equilibrium apparatus and experimental procedure

Phase equilibrium experiments were attained through the static synthetic method in a high-pressure variable-volume view cell. The experimental apparatus and procedure have been used in a variety of studies [18, 21-22]. Briefly, the experimental set-up consists of a variable volume view cell, with a maximum internal volume of 27 mL, with two sapphire windows (one for light entrance and other for visual observation), an absolute pressure transducer (Model 511, Huba Control, Würenlos/Denmark) and a syringe pump (260HP TELEDYNE ISCO, Lincon/NE/E.U.A), wih pressure range from 0.7 to 655.2 ± 0.5 bar. The equilibrium cell contains a movable piston, which permits the pressure control inside the cell. Phase transitions were recorded visually through manipulation of the pressure using the syringe pump and the solvent as pneumatic fluid. Initially, a precise amount of the *C. sylvestris* extract was weighted in a analytical balance (OHAUS, Model AS200S, NJ, USA) with $\pm$ 0.0001 g of precision and loaded into the equilibrium cell. The cell was then flushed with low-pressure CO_2 to remove any residual air. A known amount of solvent at 5 ^{o}C and 100 bar was loaded into the equilibrium cell using the syringe pump, resulting in an accuracy of $\pm$ 0.005 g in CO_2 loadings until a desired global composition was achieved. Then, the cell content was kept at continuous agitation with a magnetic stirrer and a Teflon-coated stirring bar. The temperature control was then turned on, and once the desired temperature was reached, controlled within $\pm$ 0.2 ^{o}C, the pressure system was increased until the visualization of a one-phase system in the cell. At this point the system was kept at least 30 min to allow stabilization, and then the pressure was slowly decreased (at a rate of 3.5 bar/min) until incipient formation of a new phase. This procedure was repeated at least two times for each temperature tested and

global composition. After the measurement at a given temperature, the cell temperature was established at a new value and the experimental procedure was repeated.

The phase equilibrium data was performed at temperatures from 35 ^{o}C to 75 ^{o}C and CO_2 mass contents from 60 % to 95 %. For CO_2 mass contents below 60 % it was not possible the visualization of phase equilibrium transitions due to the dark green color of the *C. sylvestris* extract.

3. Results and Discussion

Table 1 shows the phase equilibrium data obtained for the multicomponent system studied, *C. sylvestris* extract + ethanol + CO_2, covering the temperature range from 35 ^{o}C to 75 ^{o}C and the CO_2 mass content (*w*) from 60 % to 95 %. This table shows the equilibrium results in terms of transition pressure and presents the associated experimental error for each condition represented by the standard deviation of replicate measurements (σ). The pressure-composition (*P-w*) diagram for the results showed in Table 1 is presented in Figure 1.

As observed in Table 1 and in the pressure-composition diagram (Figure 1), the experimental measurements revealed a complex phase behavior for the multicomponent system formed by *C. sylvestris* extract-ethanol-carbon dioxide, which consisted in the occurrence of biphasic equilibrium: liquid-vapor (LVE), liquid-liquid (LLE) and also three-phase equilibrium: liquid-liquid-vapor (LLVE). It can be observed from Table and Figure 1 that for CO_2 mass fraction (*w*) ranging from 60 % to 80 % only vapor-liquid transitions with bubble points were observed. In the area above the isotherms, a single phase is observed and a liquid-vapor equilibrium phase is observed in the region below the isotherms. In this range, if exists another kind of phase transition, it was not possible to be observed because the dark green color of the *C. sylvestris* extract From 87.5 % to 95 % of CO_2, liquid-liquid and liquid-liquid-vapor transitions were noticed. Above 95 % of CO_2 mass content, the system changed the appearance from transparent to cloudy, with no phase separation of phases for all temperatures and pressures tested (data not show). Also, for CO_2 mass content below 92.5 % solids particles were observed on the liquid phase for temperatures of 35 ^{o}C and 45 ^{o}C.

The system studied shows a liquid-liquid (LL) immiscibility gap for all temperatures tested. Such transition behavior is commonly observed in CO_2 systems with high molecular asymmetry between the compounds [23], which seem to be the case of the system evaluated. Differences between the chemical natures of the constituents of the system may cause the complex phase behavior (LLE and LLVE) detected by the experimental assays. This complexity could increase especially with the enhancement in the number of compounds present in the mixture tested [18].
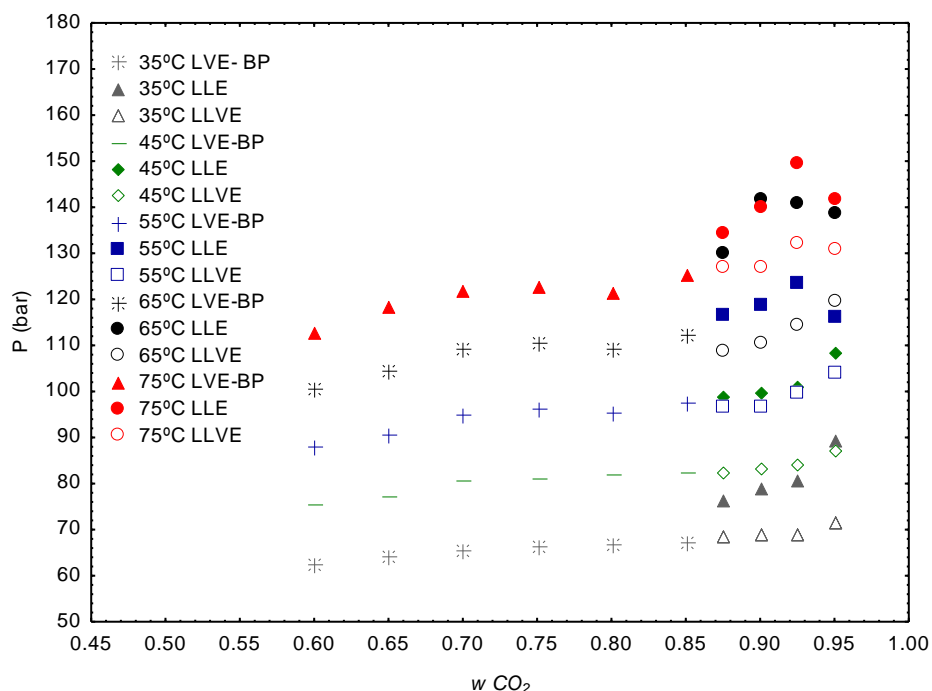

Figure 1. *P-w* phase equilibrium data for the multicomponent system *C. sylvestris* extract + ethanol + CO_2.

Table 1. Phase equilibrium experimental data for the system *C. sylvestris* extract + ethanol + CO_2.

T (°C)	P (bar)	σ (bar)	Transition type	T (°C)	P (bar)	σ (bar)	Transition type
	w_{CO2}=0.60				w_{CO2}=0.85		
35	62.8	0	LVE-BP	35	67.3	0.2	LVE-BP
45	75.75	0.07	LVE-BP	45	82.5	0.3	LVE-BP
55	88.1	0.1	LVE-BP	55	97.9	0.3	LVE-BP
65	100.8	0.2	LVE-BP	65	112.4	0.7	LVE-BP
75	113.0	0.4	LVE-BP	75	125.6	0.2	LVE-BP
	w_{CO2}=0.65				w_{CO2}=0.875		
35	64.2	0.1	LVE-BP	35	77	2	LLE
					68.7	0.4	LLVE
45	77.45	0.07	LVE-BP	45	99	0.6	LLE
					82.6	0.2	LLVE
55	90.7	0.4	LVE-BP	55	117	2	LLE
					96.6	0.5	LLVE
65	104.8	0.2	LVE-BP	65	130	6	LLE
					109.2	0.9	LLVE
75	118.3	0.3	LVE-BP	75	134	3	LLE
					127	8	LLVE
	w_{CO2}=0.70				w_{CO2}=0.90		
35	66	1	LVE-BP	35	79	6	LLE
					68.7	0.8	LLVE
45	80.6	0.4	LVE-BP	45	99.9	2	LLE
					83.6	0.4	LLVE
55	94.9	0	LVE-BP	55	119	3	LLE
					97	1	LLVE
65	109.2	0.3	LVE-BP	65	152.0	0.4	LLE
					111	2	LLVE
75	122	0.2	LVE-BP	75	140.1	0.1	LLE
					125	2	LLVE
	w_{CO2}=0.75				w_{CO2}=0.925		
35	66.4	0.4	LVE-BP	35	80.9	5	LLE
					68.95	0.07	LLVE
45	81.3	0.6	LVE-BP	45	101.2	0.1	LLE
					84	1	LLVE
55	96.3	0.2	LVE-BP	55	123.8	0.4	LLE
					99.8	0.4	LLVE
65	110.6	0.3	LVE-BP	65	141	2	LLE
					114.5	0.8	LLVE
75	123.0	0.2	LVE-BP	75	150	2	LLE
					132.3	0.4	LLVE
	w_{CO2}=0.80				w_{CO2}=0.95		
35	67	1	LVE-BP	35	89.3	9	LLE
					72	3	LLVE
45	82.2	0.2	LVE-BP	45	109	10	LLE
					87	4	LLVE
55	95.6	0.2	LVE-BP	55	116.6	0.5	LLE
					104	2	LLVE
65	109.4	0.4	LVE-BP	65	139	9	LLE
					120	2	LLVE
75	121.65	0.07	LVE-BP	75	142	5	LLE
					131.7	5	LLVE

T: Temperature (°C); P: Pressure (bar); σ: standard deviation (bar); V: vapour phase; L: liquid phase; E: equilibrium; BP: bubble point; w_{CO2}: mass fraction of carbon dioxide (CO_2).

4. Conclusions

Phase behavior of the system *C. sylvestris* extract + ethanol+ CO_2 was investigated for temperature range from 35 ^{o}C to 75 ^{o}C, covering carbon dioxide mass fraction from 0.60 to 0.95. Phase transition points were recorded for pressures up to 152 bar. Liquid-vapor, liquid-liquid and liquid-liquid-vapor transitions were observed. The phase equilibrium presented in this work will be helpful to define the operational conditions for precipitation and/or encapsulation processes of the *C. sylvestris* extract applying the supercritical technology, such as SAS process. Phase equilibrium data on the system Pluronic F-127+ ethanol + CO_2 is also important in order to define the SAS operational conditions for polymer encapsulation of *C. sylvestris* extract. Therefore it will be the focus of our further studies.

Acknowledgements

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