Quaternary Phase Equilibria for $scCO_2$ + Biophenolic Compound + Water + Ethanol

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The possibility of recovering biologically active polyphenolic compounds from hydro-alcoholic mixtures, with supercritical carbon dioxide, was investigated. In order to evaluate the viability of this fractionation step, vapor−liquid equilibrium measurements on the quaternary systems (CO_2 + ethanol + water + phenolic compound = resveratrol, *p*-coumaric acid, quercetin-3-glucoside, and catechin) were performed at 40 °C and at (15 and 20) MPa. The separation factors for hydro-alcoholic mixtures of different compositions in water + ethanol were determined and compared.

Introduction

This work is part of a major research project designed for the development of green processes for the recovery of biologically active polyphenols, namely, antioxidants, from botanical matrices. Resveratrol, catechin, *p*-coumaric acid, and Quercetin-3-glucoside, present in several edible foods, are among the most investigated polyphenols for their relevant biological activity. These compounds are of much interest to pharmaceutical, cosmetic, and food industries, not only for their antioxidant capacity but also for their anti-inflammatory and anti-cancer properties.[1−4]

Resveratrol is a naturally occurring phytoalexin produced by some plants in response to injury or fungal infection. In vitro, in vivo, and ex vivo experiments have demonstrated the numerous biological activities of this metabolite. Recently, much attention has focused on its role in chemoprevention.[5−7]

Quercetin-3-glucoside is a flavonol that occurs widely in plants and is significantly present in apples. Several biological actions of quercetin including protection of low-density lipoprotein (LDL) cholesterol against oxidation and anti-carcinogenic effects have been reported.[8,9] Additionally diabetic status in rats was found to be ameliorated about 25 % in rats fed with quercetin.[10]

Catechin has essentially antimicrobial and antioxidant activity and is the main phenolic compound responsible for the health benefits of green tea. Catechins are found in blood and tissues following oral ingestion, prevent human plasma oxidation, and act as inhibitors of LDL oxidation.[11,12] *p*-Coumaric acid prevents lipid peroxidation, reduces serum cholesterol levels, and enhances the resistance of LDL oxidation.[13]

Green processes for the recovery of the above-mentioned compounds from natural matrices are of extreme importance and should be investigated. Supercritical fluid extraction is a separation process gaining importance over conventional techniques for the extraction of natural products.[14,15] An exhaustive review regarding the use of supercritical CO_2 ($scCO_2$) for the recover of phenolic compounds from botanical matrices was recently published.[16] Supercritical carbon dioxide is the solvent of choice for producing natural extracts for human consumption because it is "GRAS"—generally regarded as safe, nonflammable, noncorrosive, and inexpensive.[17]

Although the supercritical fluid extraction from solid matrices represents most of industrial uses, it is a semi-continuous process involving the manipulation of large quantities of solid materials under pressure, which constitutes a major drawback in generally establishing this technology in natural compounds extraction. Hence, future applications will favor the continuous fractionation of liquid matrices with $scCO_2$ in counter-current extraction columns, using much smaller volumes under pressure and leading to much lower operating costs.[17]

Since in most cases the target substances to be recovered from natural products are trapped in a solid material, a primary extraction must be performed using a liquid solvent that has also to be classified as GRAS. Thus, the development of a green process for the recovery of biologically active polyphenols from botanical matrices comprises a primary hydro-alcoholic extraction to recover the target compounds from the solid matrices, followed by a second fractionation step with $scCO_2$.

The viability of this last fractionation step is dependent on the equilibrium behavior of (CO_2 + ethanol + water + phenolic compound). Previous related work was published on the study of the phase behavior of the quaternary system (CO_2 + ethanol + water + capsaicin) in order to obtain the separation factor of the natural product from hydro-alcoholic mixtures. In this work, high-pressure vapor−liquid equilibrium (VLE) experiments were performed for quaternary mixtures of (CO_2 + ethanol + water + phenolic compound = catechin, quercetin-3-glucoside, reveratrol, and *p*-coumaric acid) at (15 and 19) MPa and 40 °C. High-pressure phase equilibrium experiments for these particular systems are reported for the first time.

Experimental Section

Materials. These materials were used as received: 96 % (+)-catechin [CASRN 154-23-4], 90 % quercetin-3-glucoside [CASRN 776-86-3], and 98 % *p*-coumaric acid [CASRN 501-98-4] from Fluka; 99 % resveratrol [CASRN 501-36-0], 99.8 % ethanol [CASRN 64-17-5], and 99.8 % methanol [CASRN 675-67-1] from Riedel-de Häen CAS; and 99.998 mol % carbon dioxide from Air Liquide.

Apparatus and Procedures. VLE measurements were performed using the experimental apparatus schematically presented in Figure 1. The equilibrium cell is a stainless steel cylinder

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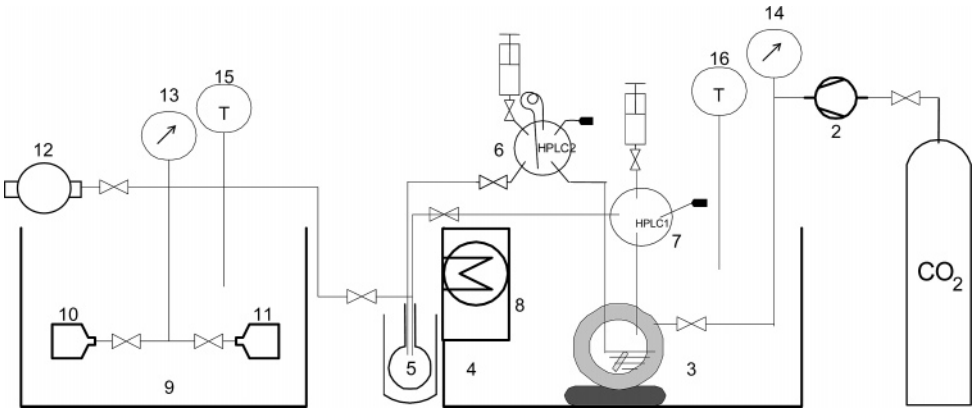

Figure 1. High-pressure apparatus for vapor−liquid equilibrium measurements: 1, CO_2 bottle; 2, CO_2 compressor; 3, equilibrium cell with sapphire windows; 4, thermostatic water bath; 5, glass trap; 6, six-port HPLC valve, liquid sampling; 7, four-way HPLC valve, gas sampling; 8, temperature controller; 9, water bath for low-pressure region; 10 and 11, expansion calibrated cylinders; 12, vacuum pump; 13 and 14, pressure transducer; 15 and 16, mercury thermometers.

with two sapphire windows and an internal capacity of approximately 30 cm^3. The cell is immersed in a thermostated water bath and has a magnetic internal stirring. While operating the CO_2 compressor, the desired pressure is brought into the cell. The pressure in the cell is measured with a pressure transducer 204 Setra calibrated between (0 and 343) MPa (precision: 0.1 %; accuracy: 0.15 % at the lowest pressure). The pressure in the low-pressure region is measured with a pressure transducer 204 Setra calibrated between (0 and 0.17) MPa with a precision of ± 0.05 %. The CO_2 is pumped using a pneumatic compressor NWA PM-101 75/97. The temperature is measured with mercury thermometers with a precision of ± 0.01 °C. The temperature is assumed to be homogeneous inside the thermostated water-bath, heated by means of a 2500 W resistance connected to a PID controller. The typical temperature stability during experiments is ± 0.01 K.

The experimental procedure followed in this work is similar to the one described in a previous paper.[19] Briefly, the equilibrium cell, immersed in the thermostated water-bath is initially loaded with approximately 10 mL of a mixture of (water + ethanol + phenolic compound) of known composition, and the system is pressurized with fresh CO_2 until the desired pressure is brought into the cell. At fixed temperature, a typical equilibration time is 1 h. A magnetic bar activated by a magnet performs stirring inside the cell. Samples are withdrawn from both phases through a high-pressure six-position switching valve (liquid sample) and a four-way switching valve (gas sample) into the sample loops. The samples are collected by quick depressurization and expansion into a large calibrated volume. The expansion volume is composed by two calibrated cylinders (V1 = 169 cm^3, V2 = 153 cm^3) and a calibrated glass trap (Vt = 11.4 cm^3). The two calibrated cylinders are used in an alternate mode, exchanging from V1 for gas samples to V2 for liquid samples. In the depressurization, the CO_2 is separated from the other compounds that precipitate in the glass trap. The gas in the samples is then expanded into calibrated volumes, and the amount of CO_2 in each sample is calculated from the measurement of the resulting subatmospheric pressure increase at the working temperature. The sample loops are later flushed with an adequate solvent for each compound (deionized water for *p*-coumaric acid and catechin, methanol for resveratrol, and a Na_2CO_3 aqueous basic solution (pH 11) for quercetin-3-glucoside) to collect the solute precipitated during the large pressure drop that occurred with the expansion and cleaned with fresh CO_2 smoothly pressurized. The amount of collected solute was determined by spectrophotometric analysis in a Thermo Spectronic Genesys 10UV.

Analytical Method. In the VLE experiments, samples were collected and diluted to a convenient volume using an adequate solvent for each compound: deionized water for p-coumaric acid and catechin, methanol for resveratrol, and a Na_2CO_3 aqueous basic solution (pH 11) for quercetin-3-glucoside. The resulting solutions were analyzed by a Thermo Spectronic Genesys 10UV spectrophotometer, and the amount of phenolic compound in the sample was determined.

All the phenolic compounds in study absorb in the region of the ultraviolet with a maximum absorbance at 310 nm for p-coumaric acid, 280 nm for catechin, 289 for resveratrol, and 370 nm for quercetin-3-glucoside. Calibration was obtained via use of standard samples between (1.0×10^{-4} and 1.0×10^{-3}) $g \cdot L^{-1}$.

Results and Discussion

In this work the possibility of extracting biophenolic compounds with $scCO_2$ from a hydro-alcoholic mixture was explored. The design of the supercritical extraction process to be carry out as a second stage separation requires a detailed knowledge of the VLE compositions of the quaternary mixtures (CO_2 + ethanol + water + natural product). Since the volume of the hydro-alcoholic extract can be reduced by evaporation, increasing the mass fraction of water in the matrix feed, two different mixtures with different water + ethanol compositions were studied. High-pressure VLE experiments were performed for quaternary mixtures of (CO_2 + ethanol + water + phenolic compound) at (15 and 19) MPa and 40 °C. Eight hydro-alcoholic mixtures containing one of the following phenolic compounds: catechin (0.010 $g \cdot L^{-1}$), quercetin-3-glucoside (0.015 $g \cdot L^{-1}$), *p*-coumaric acid (0.025 $g \cdot L^{-1}$), or resveratrol (0.012 $g \cdot L^{-1}$) were studied. The concentrations of the phenolic compound in the solutions are of the order of magnitude of those obtained in hydro-alcoholic extracts from grapes. Two different water + ethanol compositions—60 % (v/v) water or 90 % (v/v) water—were used in order to study the effect of the water content in the selectivity of the extraction process.

Samples from the vapor (CO_2-rich) phase and liquid (water + ethanol-rich) phase were analyzed for the phenolic compound by UV spectrophotometry. The compositions of both phases in carbon dioxide, water, and ethanol were calculated from mass balances using the correlation of Duarte et al. of phase equilibrium data for the (CO_2 + water + ethanol) system.[20] It was assumed that the presence of the natural compound, due to the very small concentrations in both phases, did not significantly affect the equilibrium ratios for the other components.

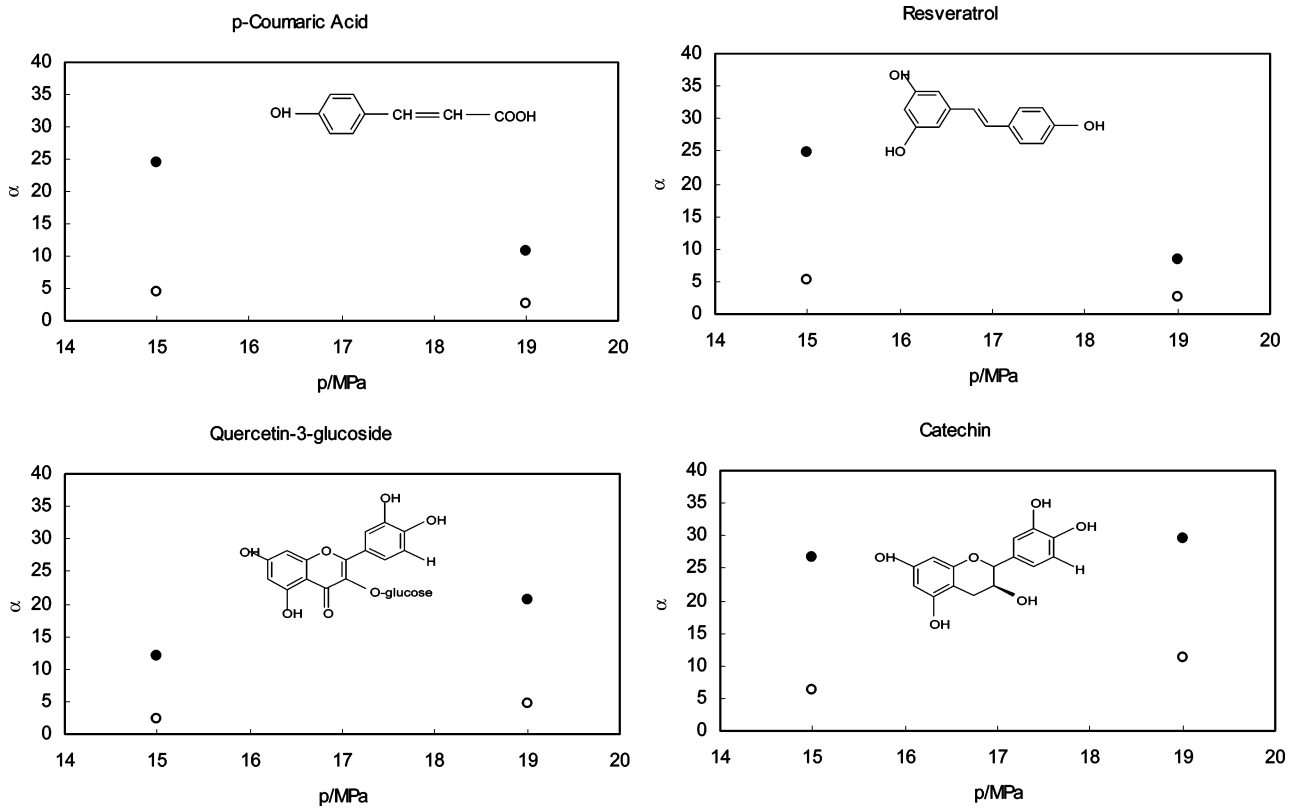

Figure 2. Separation factors of the antioxidant compounds between the coexisting phases, as a function of pressure: ●, 90 % water; ○, 60 % water.

Table 1. Solubility of Antioxidant Compound[a] and the Separation Factors of the Antioxidant Compounds between the Gaseous and Liquid Phases[b]

bioactive compound	water %	p MPa	solubility in CO_2 $\times 10^{-5}$ g·g^{-1}	$\alpha_{compound}$
p-coumaric acid	60	15	1.50 ± 0.09	4.6 ± 0.4
		19	1.32 ± 0.09	2.6 ± 0.2
	90	15	0.73 ± 0.04	24.4 ± 2.1
		19	0.64 ± 0.04	10.7 ± 1.1
resveratrol	60	15	0.82 ± 0.06	5.1 ± 0.6
		19	0.21 ± 0.01	2.7 ± 0.3
	90	15	1.03 ± 0.07	24.9 ± 2.2
		19	0.90 ± 0.07	8.3 ± 0.9
quercetin−3-glucoside	60	15	0.12 ± 0.01	2.3 ± 0.3
		19	0.91 ± 0.07	4.8 ± 0.5
	90	15	0.50 ± 0.03	12.1 ± 1.0
		19	3.53 ± 0.06	20.7 ± 1.6
catechin	60	15	4.02 ± 0.25	6.3 ± 0.5
		19	4.62 ± 0.33	11.3 ± 1.1
	90	15	5.41 ± 0.40	26.7 ± 2.8
		19	12.32 ± 0.82	29.5 ± 2.7

[a] Expressed in terms of mass of compound per mass of carbon dioxide in the gaseous phase. [b] Data are shown as the means ± standard error of the mean (SEM) for three independent experiments.

Table 1 summarizes the experimental VLE data. This table gives the solubility of the phenolic compound—expressed in terms of mass of compound per mass of carbon dioxide in the gas phase—and the separation factors α of the compounds between the gaseous and liquid phases. These later results are expressed as

$$\alpha = \frac{\left(\frac{wt_{compound}}{wt_{water+ethanol}}\right)_{gas}}{\left(\frac{wt_{compound}}{wt_{water+ethanol}}\right)_{liquid}}$$

The solubility uncertainty, taking in consideration the random uncertainties (statistical, associated to Beer−Lambert's calibration curve and to the average of the experimental measurements) and the systematic uncertainties (uncertainties due to the preparation of standard calibration solutions and to pressure and temperature measurements) were found to be less than 8 %. Each α data point is the average of at least three measurements with a maximum estimated uncertainty of 12 %, listed along with the data in Table 1. Uncertainties reported were calculated from the observed deviation in measurements.

Table 2. Phase Equilibrium Compositions of Water, Ethanol, and CO_2 on the Quaternary Systems CO_2 + Ethanol + Water + Antioxidant Compound[a]

water	p	liquid phase (wt %)			gaseous phase (wt %)		
%	MPa	x_{water}	$x_{ethanol}$	x_{CO_2}	y_{water}	$y_{ethanol}$	y_{CO_2}
60	15	57.9	31.0	11.1	0.6	4.3	95.1
	19	57.8	30.9	11.4	0.8	5.3	93.9
90	15	86.8	7.7	5.5	0.4	1.4	98.2
	19	86.2	7.7	6.2	0.5	2.0	97.4

[a] Calculated from mass balances using the correlation of Duarte et al. of phase equilibrium data for the CO_2 + water + ethanol system.[20]

Table 2 shows the calculated equilibrium compositions of water, ethanol, and CO_2 in the liquid and gaseous phases from mass balances using the correlation of Duarte et al. of phase equilibrium data for the (CO_2 + water + ethanol) system.[20]

In Figure 2, the separation factors are plotted as a function of pressure for all the studied mixtures. Similarly to what was observed for capsaicin in a previous study,[19] for initial hydroalcoholic mixtures richer in water, higher separation factors were obtained. This might be due to the fact that initial mixtures richer in water are in equilibrium with a gaseous phase poorer in ethanol. Additionally, in the case of the mixtures containing quercetin-3-glucoside and catechin, ethanol acted as a cosolvent, enhancing the solubility of these two compounds in the gaseous phase.

The variation of the separation factors with pressure is mainly dependent on the solubility of the ethanol and the compound in the equilibrium phases: it expresses the balance between these two quantities. For the quaternary systems containing *p*-coumaric acid and resveratrol, the decreasing of the separation factor was mainly due to the increase of the solubility of ethanol in CO_2 with pressure. In the case of the mixtures containing quercetin-3-glucoside and catechin, the separation factor increases with pressure due to the increasing of the solubility of both ethanol and polyphenol in the gaseous phase. In fact, in these cases the effect of the solubility of the target molecules prevails.

The equilibrium behavior for the studied quaternary systems is correlated with the chemical structure of the compounds. Similar structures show similar behavior.

Conclusions

In order to examine the feasibility of using $scCO_2$ for the extraction of biophenolic compounds from hydro-alcoholic mixtures, VLE experiments on the quaternary system (phenolic compound + water + ethanol + $scCO_2$) were carried out at 40 °C and pressures of (15 and 19) MPa. In this work, four target molecules were studied. Resveratrol, catechin, *p*-coumaric acid, and quercetin-3-glucoside are among the most important biophenolic present in several edible foods and were used in this work as standard compounds.

The results obtained indicate that the water content effect of the initial hydro-alcoholic mixture in the separation factor is relevant. For mixtures with higher water content, higher separations factors were observed, and a more pronounced effect of pressure is observed. The separation factor decreases with pressure for *p*-coumaric acid and resveratrol, and the opposite trend was observed for quercetin-3-glucoside and catechin. It may be concluded from the experimental results that $scCO_2$ fractionation should be performed from water-rich mixtures and might be optimized varying the operative pressure. These preliminary results encouraged the possibility of extracting phenolic compounds using a continuous counter-current process from hydro-alcoholic mixtures.

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