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SUPERCRITICAL CO_2 EXTRACTION OF RAW PROPOLIS AND ITS DRY ETHANOLIC EXTRACT

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Abstract - Three types of propolis extract were prepared and analyzed with respect to their global extraction yields and with respect to the concentration of the following markers: 3,5-diprenyl-4-hydroxycinnamic acid; 3-prenyl-4-hydroxycinnamic acid; 4-hydroxycinnamic acid and 4-methoxy-3,5,7-trihydroxyflavone. The extract EEP (ethanolic extract of propolis) was obtained by the conventional method from raw propolis using ethanol as solvent. The extracts (SFE) were obtained by supercritical solvent extraction from the raw propolis using supercritical carbon dioxide (sc-CO_2), with and without the addition of ethanol as a co-solvent. The fractionated supercritical extracts (FSCE) were obtained by fractionation (extract and raffinate) of the dry EEP with sc-CO_2. EEP yields of 39.5% were obtained and maximum global extraction yields were 7.3% for SFE with no co-solvent, 51% for SFE with 15% ethanol and 18% for the FSCE extract fraction. The concentrations of the markers in the different extracts differed as a function of the operational parameters, indicating that the addition of co-solvent and the selectivity of sc-CO_2 could be manipulated so as to obtain extracts with the yields and concentrations of interest.
Keywords: Brazilian propolis; Supercritical extraction; Artepillin C; Phenolic compounds.

INTRODUCTION

Propolis is a generic term used to describe a complex mixture of resinous, gummy and balsamic materials obtained from buds, flowers and plant exudates collected by bees; salivary secretions, wax and pollen also made up the final product (MAA/Brasil, 2001). The plant origin of propolis determines its chemical diversity. The chemical composition of bee glue depends on the specificity of the local flora at the site of collection, and thus on the geography and climate of this site. This results in the striking diversity of the chemical composition of propolis, especially of propolis originating from tropical regions (Bankova *et al.*, 2000). Propolis generally contains 50% resin, 30% wax, 5% pollen, 10% aromatic oils and 5% other organic residues (Pietta *et al*., 2002).

Propolis has been used in folk medicine for many years, especially in Europe and Japan, due to the variety of its therapeutic activities (Chen *et al*., 2009) including antibacterial (Kujumgiev *et al*., 1999), antifungal (Valdés *et al*., 1987), antiviral (Amoros *et al*., 1994), anticancer (Matsuno, 1995) (Kimoto *et al*., 2001), immunomodulation (Dimov *et al*., 1992) and antiinflammatory activities (Dobrowolski *et al*., 1991), among others. These activities are associated with the phenolic constituents, especially flavonoids and phenolic acids (Funari and Ferro, 2006).

One important phenolic acid present in Brazilian propolis is 3,5-diprenyl-4- hydroxycinnamic acid (DHCA), also known as Artepillin C (Lee *et al*.,

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2007). One finding indicated that DHCA exhibited inhibitory effects on renal carcinogenesis as a result of its oxy-radical scavenging property (Kimoto *et al*., 2000). Matsuno *et al*. (1997) reported that DHCA was one of the effective anti-tumor compounds found in propolis and Kimoto *et al*. (2001) and Kimoto *et al*. (1998) showed that the DHCA is capable of reducing the mass of the tumor.

Bohlmann and Jakupovic (1979) were the first to identify DHCA, which exists exclusively in Brazilian propolis (Park *et al*., 2004). Midorikawa *et al*. (2001) identified the presence of Artepillin C, 4-hydroxycinnamic acid (*p*-coumaric acid) and 4-methoxy-3,5,7-trihydroxyflavone (kaempferide) in samples of Brazilian propolis. Park *et al*. (2004) identified flavonoids and other phenolic components present in the EEP, such as *p*-coumaric acid, ferulic acid, cinnamic acids, pinobaskin, kaempferol and Artepillin C. Aga *et al*. (1994) suggested that Artepillin C was probably one of the components present in Brazilian propolis with more important antibacterial activity.

Several different methods have been used to extract active compounds from propolis, including extractive solvents such as ethanol (Budock, 1998), water, dilute aqueous ethanol (Shouqin *et al*., 2005). Supercritical carbon dioxide (sc-CO2) has also been applied to propolis, yielding highly valuable products (Catchpole *et al*., 2004; Wang and Yu *et al*., 2004). Raw propolis was extracted by Stahl *et al*. (1988) using supercritical CO_2 at 600 bar and 40°C to extract the wax and leave the insoluble flavonoids behind. Catchpole *et al*. (2004) used sc-CO_2 both as an antisolvent to precipitate high molecular mass components, and also as a solvent to extract the ethanol and the soluble components of the EEP (non-dried). Lee *et al*. (2007) extracted DHCA from Brazilian propolis using sc-CO_2 modified with co-solvent, followed by column chromatography, to obtain very pure DHCA. Chen *et al*. (2009), using a sc-CO_2 extract containing 41.2% (wt) DHCA, successfully suppressed growths of human colo-205 cancer cells, although the total yield of the sc-CO_2 extract was relatively low when compared with the extract obtained with ethyl acetate in a Soxhlet apparatus. Paviani *et al*. (2010) studied the SFE of a dried ethanolic extract from Brazilian propolis, investigating the fractionation of components of interest present in the propolis extract; the results indicated higher selectivity at low solvent density, where there are major differences between phenolic components contents in the extracts and raffinates.

The objective of this work was to investigate the influence of pressure, temperature and the addition of ethanol as co-solvent on the global yield and chemical composition of the supercritical extracts (SFEs) obtained from EEP and from raw propolis. Four main phenolic compounds extracted from propolis were selected for analysis: 3,5-prenyl-4-hydroxycinnamic (DHCA), also known as Artepillin C, 3-prenyl-4-hydroxycinnamic acid (PHCA), 4-hydroxycinnamic acid (*p*-coumaric acid) and 3,5,7-trihydroxyflavone (kaempferide). These compounds were chosen because they characterize the composition of this type of propolis. The sc-CO_2 extracts were compared with the ethanolic extract in terms of yield and the concentration of these compounds.

MATERIAL AND METHODS

Raw Materials

Samples of propolis, native to the State of Minas Gerais, Brazil, classified as group 12 byr Park *et al*. (2002) (Brazil has 12 different groups of propolis, with distinct characteristics), were obtained from Bioessens Ltda. (Cotia, São Paulo, Brazil). The samples showed a dark green color, a characteristic pungent odor and a rigid fragile structure when cold, which became ductile and malleable when heated. The raw materials were packed into plastic bags and stored in a domestic freezer (Consul, model 220, São Paulo, Brazil) at –10°C.

Chemicals

The CO_2 used in the experiments was 99.5% pure and in the liquid phase (White-Martins S.A., Campinas, Brazil), while the ethanol (99.9%) used in the experiments was from Merck (Germany).

DHCA (3,5-Diprenyl-4-hydroxycinnamic acid) and PHCA (3-prenyl-4-hydroxycinnamic acid) were isolated during a previous study by Marcucci *et al*. (2001), 4-hydroxynnamic acid (*p*-coumaric acid) was purchased from Sigma Chemical Co. (USA) with a purity of 98.0% and kaempferide with a minimum purity of 99.0% was obtained from Fluka (USA).

Experimental Procedure

Ethanolic Extract of Propolis (EEP)

Ethanolic extracts of propolis (EEP) were obtained using the methodology of Paviani *et al*. (2010), where 3 g of raw propolis was mixed with 10 mL of ethanol and stirred using a magnet stirrer for 1 day at room temperature. The insoluble portion was then separated by filtration and the filtrates kept in a freezer at –10°C overnight before filtering again to reduce the wax content of the extracts. The solvent

was evaporated off in a vacuum oven at a temperature of 60°C to obtain the dry ethanolic extract of propolis and the yield calculated based on the initial amount of propolis.

SFE Apparatus and Experimental Procedure

The experiments were performed using a laboratory scale unit, which basically consists of a CO_2 reservoir, two thermostatic baths, a syringe pump (Isco Inc., Lincoln, Nebraska, USA, Model 500D) and an extractor with an internal volume of approximately 100 mL. About 2 g of EEP or 5 g of raw propolis were loaded into the extraction vessel and 6 mesh glass beads used to fill the empty spaces in the vessel. The CO_2 was pumped into the extraction vessel, which was supported by two 300-mesh wire disks at both ends, at a constant flow rate of 1 $g.min^{-1}$. The amount of sc-CO_2 used in each experiment was about 0.270 kg. A static period of 30 min was used to allow contact between the sample and the supercritical solvent. The global extraction yields (X_o) were calculated as the ratio of the total mass extracted to the initial mass of EEP or raw material (dry basis). The experiments were carried out in triplicate at pressures of 150, 200 and 250 bar, temperatures of 20, 35 and 50°C and with the addition of ethanol as co-solvent in the proportions of 0, 5, 10 and 15% (w/w).

Analytical Method

The chromatographic analyses were carried out using an HPLC equipment (Merck-Hitachi, Darmstadt, Germany), equipped with a pump (Merck-Hitachi, model D-7100) and a diode array detector. Separation was achieved using a Lichrochart column (Merck-Hitachi, Darmstadt, Germany) (RP-18, 125×4mm i.d., 5 μm particle size) using water plus formic acid (95:5, v/v) (solvent A) and methanol (solvent B) as mobile phase. Elution was carried out with a linear gradient and a flow rate of 1 $mL.min^{-1}$. The analysis time was 60 min and detection was at 280 nm. Identification of the substances was based on the UV spectrum (200–400 nm) and the purity peak, using the values of standards acquired commercially and isolated in the laboratory (Marcucci *et al*., 2001). Quantification was done using external standards. The data were analysed by the Chromatography Data Station-DAD Manager software.

One flavonol and three hydroxycinnamic acids that differed with respect to the number of prenyl groups were chosen. These compounds represent approximately 10% in mass of the dry EEP and were chosen for analysis because they represent the main phenolic components of this type of propolis, with Artepillin C being the most important component due to its biological properties.

RESULTS AND DISCUSSION

Extraction Yield of Dry Ethanolic Extract of Propolis (EEP)

Table 1 shows the global yield and the yields of the 4 markers of the dry ethanolic propolis extract (EEP), and also makes a comparison with the ethanolic extracts obtained by Funari *et al*. (2007) and by Biscaia and Ferreira (2009).

The global extraction yield (39.5%) was similar than that obtained by Funari *et al*. (2007) and slightly lower than that obtained by Biscaia and Ferreira (2009). The yields of the selected markers were higher than those obtained in the other studies, except for DHCA, whose value was about 60% of value reported by Funari *et al*. (2007). The yields of *p*-coumaric acid and kaempferide were approximately 60% higher than those obtained by Funari *et al*. (2007) and about 2 times and 3 times higher than the values obtained by Biscaia and Ferreira (2009), respectively. These three samples of propolis were obtained from different places. The sample used in this work was obtained from state of Minas Gerais, the sample of Funari *et al*. (2007) was from "Serra do Japi, state of São Paulo and the sample of Biscaia and Ferreira (2009) was from the state of Paraná. Another important variable that affects the composition, is the season that the sample is collected, as shown in the work of Simões-Ambrosio *et al*. (2010), where the composition of several compounds were analysed, and the concentration of Artepillin C ranged between 1 and 65 mg/g in the course of the year.

Table 1: Global extraction yield of the EEP and extraction yields of the four components analyzed.

	Global yield [a] (%)	DHCA	PHCA	*p*-coumaric acid	Kaempferide
		(mg/g)[b]			
EEP [this work]	39.5 ± 1.2	20.9	5.73	10.94	9.60
Funari *et al*. [30]	38.3 ± 2.0	33.8	Nr	6.30	6.30
Biscaia and Ferreira [31]	46.0 ± 6.0	10.6	1.85	5.14	2.71

[a] Global extraction yield = gram of dry extract per 100 g of raw propolis; nr: not reported;
[b] Extraction yield = mg of solute per gram of raw propolis

FSCE Extracts: Fractionation of Dry Ethanolic Extract of Propolis (EEP) with sc-CO_2

With the objective of obtaining extracts with differentiated chemical compositions, the EEP was fractionated with sc-CO_2 as a function of the temperature and pressure. Fig. 1 shows the extraction curves as a function of temperature and pressure, showing the behavior of the extraction yield as a function of the extraction time under the different operational extraction conditions.

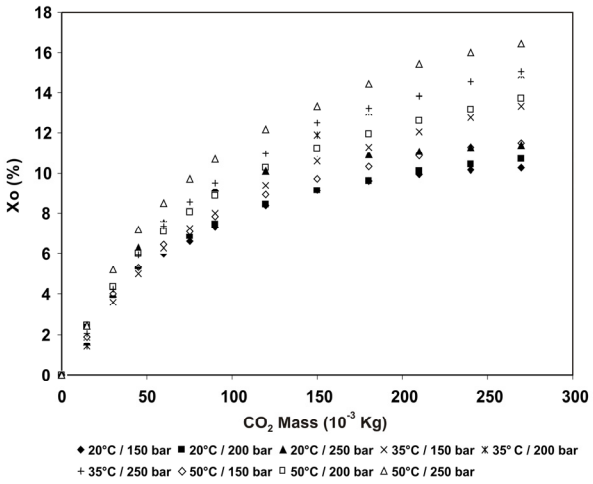


Figure 1: Global yield curves for FSCE obtained for the sc-CO_2 extraction of the dry ethanolic extract of propolis (EEP).

The extraction curves seen in Fig. 1 indicate that, in general, the yield increased with increasing pressure and temperature, and that the mean values (triplicates) of the global yields presented a maximum of 18% under the highest conditions of temperature and pressure studied (50ºC/250 bar) and a minimum of 11.90% under the mildest conditions of 20ºC/150 bar. The effect of temperature and pressure on the extraction yield could be associated with the increase in solubility of the solutes in sc-CO_2 due to the increase in density of the CO_2 with the increase in pressure at constant temperature, and to the increase in vapor pressure of the solutes with increase in temperature. The effects of temperature and pressure on the chemical composition of the extract are associated with alterations in selectivity of the sc-CO_2 with respect to the different solutes, which depends on alterations in the physical properties of the solvent, such as polarity and density. It can be seen that an increase in pressure at a temperature of 20ºC did not influence the extraction yields, which remained practically the same at 12%, considering that the density of the supercritical solvent at 250 bar is only 6% higher than at 150 bar. At a temperature of 50ºC there was a greater variation in extraction yield with pressure, since in this case the density of the CO_2 at 250 bar is 19% higher than at 150 bar. The maximum yield obtained at 50ºC was 18% at 250 bar, as against 12.3% at 150 bar. With respect to the effect of temperature, varying the temperature from 20 to 50ºC at a constant pressure of 250 bar resulted in an increase in global yield from 12.4% at 20ºC to 18% at 50ºC. In this case, the increase in temperature resulted in an increase in vapor pressure of the solute, which predominated over the negative effect of a reduction in density from 963 to 834 kg/m^3. The data shown in Table 2 also indicate that, at constant temperature, an increase in pressure caused an increase in global yield due to the increase in solvent density.

Table 2: Global yield and chemical profile of FSCE obtained by fractionation of EEP with sc-CO_2.

	CO_2 density[a]	Global yield X_o [b]	DHCA	PHCA	*p*-coumaric acid	Kaempferide
	(Kg/m^3)	(% w/w)	(mg/g)[c]			
20°C/150 bar	903.6	11.9 ± 2.1	47.7	4.34	4.86	3.69
20°C/200 bar	951.4	12.2 ± 1.3	44.7	3.72	3.73	2.59
20°C/250 bar	963.3	12.4 ± 1.4	47.9	4.62	4.94	1.76
35°C/150 bar	815.0	13.35 ± 0.02	45.6	4.85	3.35	2.88
35°C/200 bar	866.1	14.9 ± 0.1	45.1	3.58	3.58	2.74
35°C/250 bar	901.7	15.4 ± 0.4	51.1	5.12	5.84	3.80
50°C/150 bar	699.2	12.3 ± 1.1	41.0	4.60	3.37	4.76
50°C/200 bar	784.6	14.8 ± 1.0	48.8	4.38	4.50	4.09
50°C/250 bar	834.7	18.0 ± 2.2	52.4	7.43	5.73	9.34
EEP	-	100	52.8	14.5	27.7	24.3

[a] Angus *et al*. (1976); [b] Global yield = mg of FSCE extracted per 100 gram of EEP,
[c] Concentration = mg of solute per gram of FSCE

With respect to the concentration of Artepillin C in the fractionated extracts (Table 2), the concentration did not vary substantially with temperature and pressure, and was slightly lower than that found in the starting EEP. The concentrations of the other components in the supercritical extracts were also lower than their concentrations in the EEP. Greater variations in the concentrations occurred at 50ºC, where greater variations in the extraction yield and density of the sc-CO_2 also occurred with an increase in pressure. Fig. 2 shows the variation in concentration of Artepillin C in the fraction extracted from the FSCE extracts.

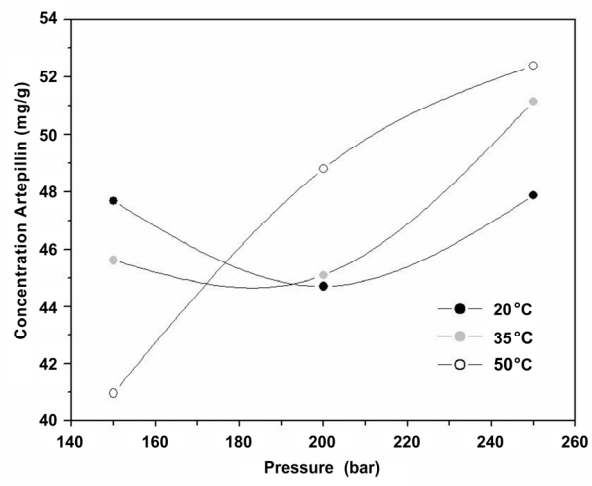


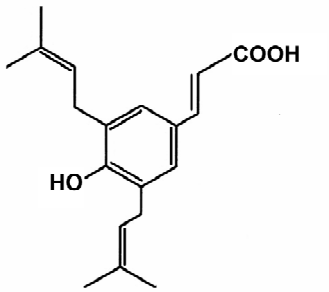
Figure 2: Artepillin C concentration in supercritical extracts of the EEP.

At higher pressures, the concentration of Artepillin C increased with an increase in temperature, but at lower pressures the inverse occurred, the concentration decreasing with an increase in temperature at constant pressure. This type of behavior can be explained based on the effect of the temperature and pressure on the variation in solvent density and by the effect of temperature on the vapor pressure of the solute. The isotherms crossed between 150 and 200 bar. In this region, the effect of temperature on the increase in vapor pressure compensated the effect of temperature on the decrease in solvent density. Due to the existence of this point, the lowest and highest concentrations occurred on the same isotherm of 50ºC.

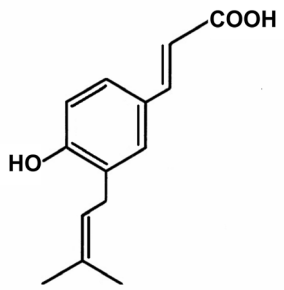
It can also be seen that, at higher pressures, the differences between the concentrations in the extracts and those in the original EEP were smaller. At lower pressures, it can be seen that CO_2 was more selective for fractionation, since the differences between the concentrations were greater, but, on the other hand, the yields were very small. Fractionation depends on the degree of polarity of the molecules, CO_2 preferably extracting non-polar molecules. However, with an increase in pressure and consequent increase in density and increase in polarity of the carbon dioxide, its capacity to extract more polar components increases. It can be seen that, for the majority of the substances investigated, there was an increase in concentration with increase in pressure, due to the increase in the density of carbon dioxide and the increase in its polarity.

Piantino *et al*. (2008) studied the extraction of phenolic compounds from *Baccharis dracunculifolia* leaves by extraction with supercritical carbon dioxide, ethanol and methanol, with a view to obtaining the same compounds studied in the present work. They chose this vegetable because it is the main source (Park *et al*., 2004) used by the bees to make the propolis of group 12 in the States of São Paulo and Minas Gerais, and thus, like the propolis, contains large amounts of these phenolic compounds. In this case the global yields in the alcoholic extracts were higher than those in the supercritical extracts, although, for the supercritical extraction at 60ºC and pressure of 400 bar, the concentration and extraction yield of three of the four markers were much higher in the supercritical extract, only that of *p*-coumaric acid being lower. The difference in the concentrations and extraction yields of these four compounds could be explained based on their chemical structures, as shown in Fig. 3. Piantino *et al*. (2008), comparing the chemical structure of hydroxycinnamic acid and of its prenylated derivatives (DHCA and PHCA), showed that the addition of a prenyl group to the molecule increased the yield in the supercritical extraction. The extraction of Artepillin C (2 prenyl groups) with supercritical carbon dioxide was much better than the extraction of *p*-coumaric acid (0 prenyl groups) and better than that of PHCA (1 prenyl group). This indicates that the addition of prenyl groups decreases the polarity of the molecule, favoring supercritical extraction (Piantino *et al*., 2008).

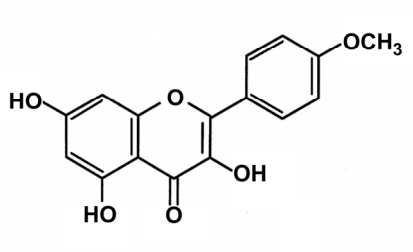
With a view to enriching the propolis extracts with Artepillin C, the results of the fractionation of the EEP suggest that increasing the pressure and temperature above those used in the present work could provide extract fractions richer in Artepillin C than the original EEP and improve the fractionation yield of the extract. They also suggest the possibility of adding small amounts of ethanol as a co-solvent to the CO_2 to alter its polarity and fractionate the extract with a greater yield, promoting a difference in the concentration of the components of interest.



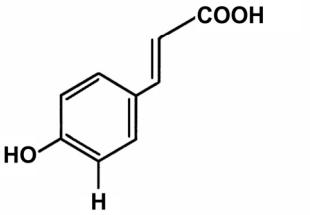
Artepilin C (DHCA)



3-prenyl-4-hydroxycinnamic acid (PHCA)



Kaempferide



p-coumaric acid

Figure 3: Chemical structures of the selected phenolic compounds.

Supercritical Fluid Extraction of Raw Propolis

Table 3 compares the results obtained for the supercritical extraction of raw propolis with and without the addition of a co-solvent.

The global yields obtained from the raw propolis without the addition of ethanol as co-solvent were much lower than those obtained with the addition of ethanol. According to Catchpole *et al*. (2004), propolis is not very soluble in supercritical CO_2, but much more soluble in the mixture of CO_2 + ethanol. As can be seen in Table 3, the results show that the supercritical extractions using ethanol as co-solvent produced extracts with much higher global yields than those extracted without the co-solvent. In addition, the extractions made with the addition of 15% ethanol produced extracts with global yields equal to or greater than the EEP reference extract (extraction with pure ethanol at atmospheric pressure).

Table 3: Comparison between the global extraction yields, X_o (% w/w), of the ethanolic and sc-CO_2 extractions of raw propolis and the concentration of some markers in the respective extracts.

T	P	Co-solvent	Global Yield	DHCA	PHCA	*p*-coumaric acid	Kaempferide
(°C)	(bar)	(%)	(X_o%)[a]	(mg/g)[b]			
20	150	0	3.39	20.4	1.35	1.89	24.9
20	250	0	4.03	24.6	2.09	2.87	29.5
50	150	0	3.50	-	0.17	0.13	1.15
50	250	0	7.30	57.8	1.88	1.82	0.00
20	150	5	18.5	29.6	9.33	16.3	0.88
20	250	5	20.1	29.0	8.79	15.4	0.00
50	150	5	20.3	12.3	0.97	1.85	0.97
50	250	5	27.8	19.4	2.52	4.33	0.71
35	200*	10	37.5 ± 3.1	52.0	10.8	23.7	44.4
20	150	15	39.0	2.27	1.25	0.72	0.00
20	250	15	40.8	2.54	1.66	1.68	0.00
50	150	15	42.1	36.6	15.3	27.1	11.9
50	250	15	51.3	12.8	2.86	4.23	0.00
EEP		39.5 ± 1.2		52.8	14.5	27.7	24.3

[a]Global yield = gram of dry extract per 100 g of raw propolis; [b]Concentration = mg of solute per gram of dry extract;
*Central point conditions (temperature, pressure and percent co-solvent), trial carried out in triplicate

Biscaia and Ferreira (2009) obtained extraction yields of between 1.5 and 12% for the supercritical extraction of raw propolis obtained in the State of Paraná (Brazil), using pressures between 100 and 250 bar and temperatures between 30 and 50ºC, without the use of a co-solvent. The addition of 2 to 7% ethanol as co-solvent in sc-CO_2 at 150 bar and 40ºC increased the yield from 8.6% without co-solvent to 14.6% with the addition of 2% ethanol and to 24.2% with 7% ethanol.

In the extracts obtained with the addition of 5% or 15% co-solvent (Table 3), an increase in pressure from 150 to 250 bar with a fixed temperature of 20ºC promoted an insignificant increase in extraction yield, producing extracts with similar concentrations of the markers. However, at 50ºC, in all cases, an increase in pressure from 150 to 250 bar promoted a considerable increase in extraction yield and altered the concentrations of the marker compounds. This difference in behavior of the systems at 20ºC and at 50ºC can be attributed to differences in the values of solvent density, a behavior similar to that observed in the fractionation of the EEP with sc-CO_2 shown in Table 2.

In general, the results (Table 3) showed differentiated behavior with variations in the yield and concentrations as a function of temperature, pressure and proportion of ethanol. At 20ºC, a maximum yield of 40% was obtained using 15% ethanol, which is approximately equal to the yield in EEP, but the concentrations were much lower than those found in the EEP. Using an intermediate condition (central point) of 35ºC and 200 bar and 10% ethanol, an extract similar to the ethanolic extract EEP was obtained in terms of both yield and concentrations of the markers. At 50ºC with 0 or 5% ethanol, the yields were smaller than those of the EEP and an increase in pressure increased the extraction yield and the concentrations of the markers, with the exception of the concentration of kaempferide. However, with 15% ethanol, the extraction yields were higher than the yield of EEP and an increase in pressure produced the inverse behavior with respect to the compositions, decreasing the concentrations, possibly due to an increase in the co-extraction of undesirable substances.

According to Negri *et al*. (1998), CO_2 shows non-polar characteristics and the supercritical extraction of raw propolis preferably extracts non-polar substances, represented by waxes such as hydrocarbons and monesters, present in large amounts in the raw propolis, as can be observed from the low extraction yields.

CONCLUSIONS

The global yields obtained in the supercritical extraction of raw propolis without the use of a co-solvent were of the order of 3 to 7%, much lower than the yield of 39.5% obtained by conventional ethanolic extraction. However the addition of ethanol as a co-solvent significantly increased the global extraction yield. The addition of 5% ethanol as co-solvent increased the global yield to 18 to 28% for the different conditions of temperature and pressure, and the addition of 10% ethanol under the conditions of 200 bar and 35ºC, produced an extract with yield and chemical profile very similar to those of the conventional ethanolic extract. The use of 15% ethanol produced extracts with highly differentiated chemical profiles and extraction yields equal or greater to those of EEP, obtaining a yield of 51%.

Fractionation of the EEP with sc-CO_2 produced extract fractions with yields on the order of 11 to 18% and small difference with respect to Artepillin C composition between the extracts and the original EEP, although the chemical profile of the 4 markers was well differentiated from the chemical profile of EEP. The effect of the selectivity of sc-CO_2 was apparent in the variation of the chemical profile of the extracts as a function of temperature and pressure, indicating that an increase in temperature and pressure above the values used in the present study could produce extracts with greater yields and higher concentrations of the markers, principally of Artepillin C.

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