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ETHANOLIC AND HYDROALCOHOLIC EXTRACTS OF *PITANGA* LEAVES (*Eugenia uniflora* L.) AND THEIR FRACTIONATION BY SUPERCRITICAL TECHNOLOGY

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Abstract - Hydroalcoholic extracts prepared by conventional methods (in stirred vessel at 25 °C and 1 bar) and, hydroalcoholic and supercritical carbon dioxide ($scCO_2$) extracts prepared in fixed bed extractors (60 ºC and 400 bar) were obtained from *pitanga* leaves (*Eugenia uniflora* L.) in order to recover phenolic compounds. Hydroalcoholic and ethanolic extracts obtained in fixed bed were further fractionated in four separators by gradual pressure reduction, using $scCO_2$ as an antisolvent. All extracts and fractions were characterized in terms of extraction yield and the presence of total phenolics and flavonoids. Solvents were found to significantly influence the extraction yields and composition. Yields increased as a function of polarity, whereas the hydroalcoholic extractions displayed the highest yields and the highest amounts of extracted phenols and flavonoids.
Keywords: *Eugenia uniflora* L.; Fractionation; Supercritical extraction; Phenolic compounds.

INTRODUCTION

During the last decades, there has been a growing interest in the bioavailability and biological effects of the phenolic compounds present in many kinds of plants (Cartea et al., 2011; Vieitez et al., 2018). *Eugenia uniflora* L., known as *pitanga*, presents high phenolic contents and its leaves have been used in infusions and decoctions in folk medicine to treat inflammations, rheumatic pain and fever (Rattman et al., 2012). *Pitanga* has also been found to be hypoglycemic, diuretic and to avoid stomach disorders (Amorim et al., 2009), hypertension (Consolini et al., 1999), antifungal (Ferreira et al., 2013), trypanocidal and leishmaniasis activity (Santos et al., 2012; Santos et al., 2013).

Supercritical carbon dioxide ($scCO_2$) is widely used as an extraction solvent, since it is inert, non-flammable, non-toxic and low cost. Due to its non-polar nature, it extracts mainly non-polar solutes, but can also be extended to polar solutes by using ethanol, water or its mixtures as $scCO_2$ co-solvents. The use of co-solvents may be advantageous since increases specificity and reduces solvent consumption (Del Valle et al., 2005; Wijngaard et al., 2012).

Martinez-Correa et al. (2011) studied the combined process of supercritical extraction followed by conventional extraction (with ethanol or aqueous in a stirred vessel at 25 °C and 1 bar) of *pitanga* leaves. Later, Garmus et al. (2014) studied the sequential extraction process using $scCO_2$ in the first step, followed by ethanol and water in a second and third steps. They

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found significant differences in the global extraction yields and in the extract composition. Ethanolic and aqueous extracts produced in fixed bed (Garmus et al., 2014) and by conventional methods (Martinez-Correa et al., 2011), after supercritical extraction, had more total phenolic compounds, indicating that previous $scCO_2$ extraction pre-concentrates phenolic compounds for the following extraction steps.

Another option for obtaining differentiated extracts is to use $scCO_2$ as an antisolvent, a technique known as SAF (Supercritical Antisolvent Fractionation). SAF is based on the continuous contact between $scCO_2$ and the polar liquid extract (ethanolic, aqueous or hydroalcoholic) in pressurized separation chambers. This contact reduces the polarity of the solution, allowing the precipitation of the most polar components in the liquid mixture. If many separators in series with different temperature and pressure conditions are employed, it is possible to achieve selective extract fractionation. This process causes the precipitation of the most polar and heavier compounds preferably in the first separators, whereas the other components as well as another part of the solvent mixture (less polar components and ethanol) remain diluted in $scCO_2$ and are recovered by pressure reduction (Catchpole et al., 2004; Gonzalez-Coloma et al., 2012; Sánchez-Camargo et al., 2016; Visentínet al., 2011).

The ethanolic, hydroalcoholic and supercritical extracts of *pitanga* leaves were compared in terms of the extraction yields and the phenolics contents. Further fractionation of the ethanolic and hydroalcoholic extracts using $scCO_2$ as an antisolvent was also evaluated. All extracts/fractions were analyzed in terms of total phenols and total flavonoids and fractionation products had their phenolic compounds characterized by mass spectrometry.

MATERIALS AND METHODS

Characterization of the raw material

The samples of *pitanga* leaves (*E. uniflora* L.) were collected in the experimental cultivation field of the Chemical, Biological and Agricultural Pluridisciplinary Research Center, State University of Campinas experimental field (22°45′00″ south and 47°10′21″ west; Paulinia, Brazil), dried in an air circulation oven (Marconi, model MA-030/12, SP, Brazil) at 40 ºC for 24 h and then ground in a knife mill (Marconi, MA 340 model, Brazil). The corresponding voucher specimen (1816) was deposited at the CPQBA-Herbarium. Raw material properties were quantified in terms of total volatiles and moisture, mean particle diameter, real and apparent density, and bed porosity. The total content of volatiles and moisture (VU; %) was determined by the AOAC 934.04 (1997) gravimetric method, at 105 °C under vacuum at 525 mmHg, weighing every 2 h until the variation was less than or equal to 3 mg. Wet basis moisture (U; %) contents were measured according to the Karl-Fisher method, using an automatic titrator (Model 701 KF Titrino, Metrohm) and an oven (832 KF Thermoprep). The mean particle diameter was estimated by the ASAE method (ASAE, 1997), employing a vibratory sieve system (Model 1868, Bertel, SP, Brazil), from the means of the materials retained on the sieve meshes 24, 32,48, 60, 100 and 270. Real density (ρ_r) was determined by helium gas pycnometry (Micromeritics Accu Pyr II 1340). For the calculation of bed apparent density (ρ_a), the vegetal matrix mass required to fill a volume of 10 cm^3 was used. The porosity of the bed (ε) was calculated from the relationship between the real density of the sample and the apparent density of the bed according to Rahman et al. (1996).

Chemicals

Ethanol 99.5% (Synth, Brazil) was used for the processes of conventional hydroalcoholic extraction, fractionation, sequential and hydroalcoholic extractions in fixed bed. Ultrapure water Milli-Q (Millipore direct-Q3 UV, Millipore Corporation, USA) was used to prepare the solvents used in the hydroalcoholic extractions and extract analysis. CO_2 (99.5%, White Martins Gases Industriais, Brazil) was used as solvent for the sequential extractions. For the chromatographic analyses, acetonitrile of HPLC grade purchased from Merck S.A. (Rio de Janeiro, Brazil) was used, and deionized water was obtained from a Milli-Q (Millipore, Billerica, MA) purification unit.

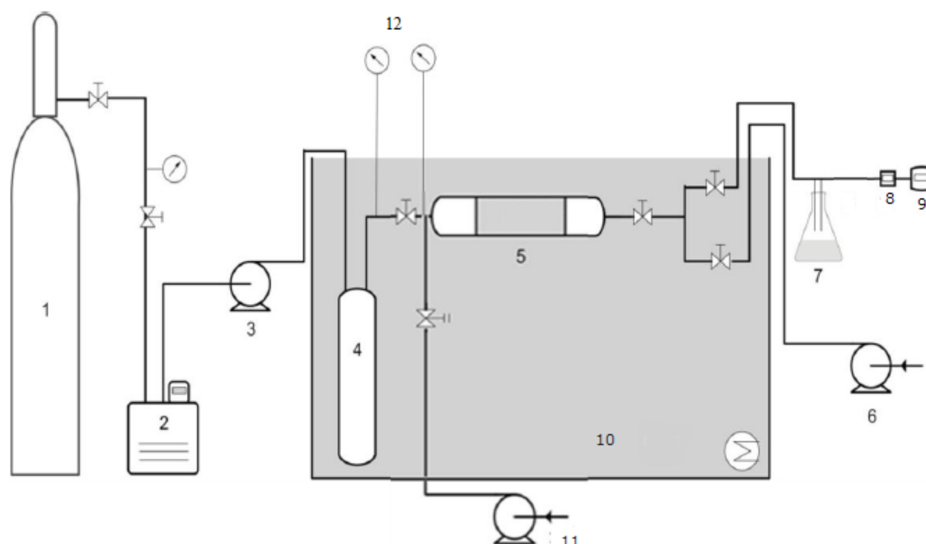
Experimental system for extraction in fixed bed

Experiments were performed in an experimental unit (EXTRAE Laboratory, UNICAMP, Brazil) according to the scheme presented in Figure 1.

The unit basically consists of a CO_2 cylinder (1), a refrigerated bath (Cole-Parmer, Polystat 12101-31, EUA) (2), a high pressure pump (Eldex Laboratories, PN 1018 AA-100-S, EUA) (3), a buffer tank (stainless steel AISI 316 de 500 mL, Suprilab, Brazil) (4), an extractor (stainless steel AISI 316 de 50 mL, Suprilab, Brazil) (5), an extract collector (7), a gas flow meter (Cole-Parmer, 32908-69, EUA) (8), a volume totalizer (Lao-G1, Brazil) (9), a thermostatic bath (Sulab, Brazil) (10), two Bourdon manometers (Record, Brazil) (one located in the buffer tank and the other in the extractor entrance), a peristaltic pump (Cole-Parmer, Masterflex 77200-62, EUA) (6) and a pump (Eldex Laboratories, PN 1018 AA-100-S, EUA) (11) used for hydroalcoholic extraction.

Supercritical extraction

The extractor (5) was manually filled with approximately 7.0 g of dry and milled leaves and glass



(1) CO_2 cylinder, (2) refrigerated bath, (3) high pressure pump, (4) buffer tank, (5) extractor, (6) and (11) peristaltic pump, (7) collection flask, (8) gas flow meter, (9) volume totalizer, (10) thermostatic bath , (12) Bourdon manometer

Figure 1. Schematic diagram of the experimental fixed bed extraction unit.

beads (6 mesh) to fill in the empty spaces and to avoid preferential routes by the solvent. The operational conditions were adjusted to 400 bar and 60 ºC for all the assays, based on previous work by Garmus et al. (2014). When the established conditions were achieved, a half-hour static period for the system's stabilization was adopted and then the extraction was initiated with a CO_2 flow rate of 1.5 L/min (ρCO_2 = 1.65 g/L) through the bed. The extracts were collected in the collector (7). The CO_2 in the gaseous state that leaves the collector (7) was led to a flow meter (8) and volume totalizer (9) to measure the volume of CO_2 used.

Hydroalcoholic extraction

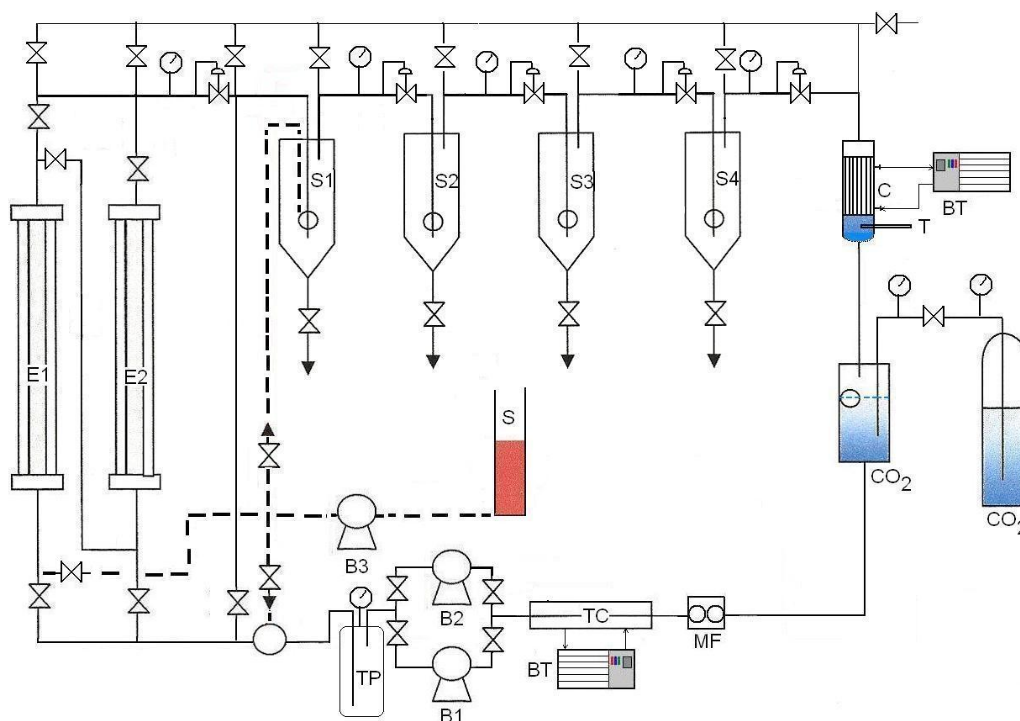
The extraction in a single step was performed in a fixed bed (Figure 1) with ethanol plus water in the 70:30 v/v ratio (ρ = 0.865 g/cm³) at 400 bar and 60 ºC, where approximately 7.0 g of leaves were placed in extractor and a solution flow rate of 0.5 mL/min (flow rate measured in ambient conditions, 25 ºC and atmospheric pressure) for 3 h. The extraction temperature and pressure were based on the conditions used by Garmus et al. (2014). The extraction was performed in triplicate.

For comparison, a conventional hydroalcoholic extraction of *pitanga* leaves in a stirred vessel was also performed according to the methodology proposed by Piantino et al. (2008) with some modifications: 3.0 g of dry sample were mixed with 20 mL of the solvent (ethanol:water, 70:30 v/v) and then the mixture was stirred at ambient temperature (25 ºC) for 24 h. Later, the mixture was vacuum filtrate, saving the filtered. To the solid residue, 10 mL of ethanol were added, centrifuged at 3000 rpm for 5 min (centrifuge - BR44, Jouan, France), and re-filtered with help from the vacuum pump (MA-057-13, Marconi, Brazil). This last filtrate was mixed to the previous one, constituting the ethanolic extract.

Experimental system for extraction and fractionation

Figure 2 shows details of the experimental unit used for extractions with fractionation. The unit had two extractors (E1 and E2), offering the possibility of being operated in two different diameters and in various lengths and being operated in series or parallel, four separators (S1 and S4) with pressure controlled by back pressure, CO_2 pumping system (B2 and B1), solvent (S) pumping system (B3), condenser (C) for recycling CO_2, mass flow meter (MF) of the Coriolis type, and a thermostable bath (BT) for controlling CO_2 temperature for pumping and condensation control. Temperatures of the extractors and separators were controlled by an electric heating system with electric resistances. Paula et al. (2017) have described the system in detail.

For the extraction process, 30.0 g of the sample was placed in the first extractor (E1). First, the pressurization with CO_2 was done in the S1, S2 and S3 separators at 300, 200, and 100 bar, respectively. The S4 separator remained at atmospheric pressure. The extractor was then pressurized with the 70:30 ethanolic or hydroalcoholic solution (v:v) until the extraction conditions of 400 bar and 60 ºC were achieved. The ethanol flow rate was then regulated to 1.89 mL/min



E1 and E2: extractors; S1, S2, S3 and S4: separators; B1, B2 and B3: pumps; TC: heat exchanger;
BT: thermostatic bath; MF: gas flow meter; TP: buffer tank; S: solvent, C: condenser

Figure 2. Scheme of the experimental extraction and fractionation unit.

and CO_2 flow rate to 0.8 kg/h to ensure a CO_2:solvent ratio (ethanol or hydroalcoholic solution) of 90:10 w/w. The extraction process lasted for 2 h and, at the end of the process, the separators were depressurized and the 4 fractions (E1 to E4 for the ethanolic fractionation and H1 to H4 for the hydroalcoholic fractionation) collected in flasks for further analysis. Figure 3 shows

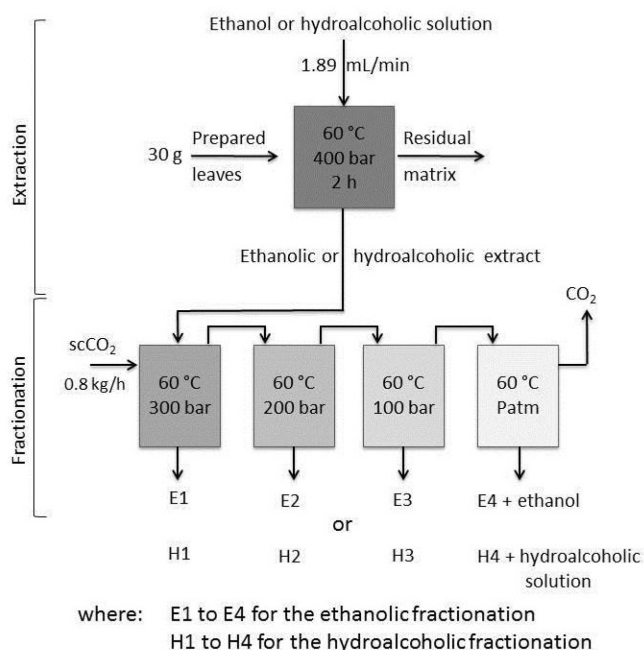


Figure 3. Flowchart of the ethanolic or hydroalcoholic extraction process in one step and fractionation

an illustrative scheme for the process of extraction and fractionation.

Determination of total phenolics and flavonoids content

The determination of total phenolics was performed using Folin-Ciocalteu reagent, according to the procedure proposed by Singleton et al. (1999), and expressed as gallic acid equivalent (mg GAE/g extract). The absorbance was determined at 750 nm using a UV spectrophotometer (Orion AquaMate 8000, Thermo Scientific, USA). The total flavonoids were measured by the method reported by Zhishen et al. (1999) and the results were expressed as catechin equivalent (mg CE/g extract). The absorbance of the solution was determined at 510 nm.

UPLC-ESI-MS/MS

To characterize by MS the composition of extracts, a UPLC-ESI-MS/MS analysis was performed. First, each extract was solubilized in water:methanol (1:1; v/v) solution to a concentration of 1 µL/mL. Then, the analyte solution was separated by HPLC (Agilent 1290 Series Liquid Chromatography equipment, Agilent Technologies, USA) using a C18 Kinetex 2.6 µm, 3.0 mm i.d., 100 mm column (Phenomenex, California, US) and a mobile phase consisting of acetonitrile (phase B) and water (phase A). The gradient method was as follows: 0-28 min, 3-97% B; 28-30 min, 97-3% B; 30-33 min, 3% B. The flow rate was 0.5 mL/

min at 40 °C, and the injected volume was 2 μL. Mass spectra were collected using a QTOF instrument (Q-TOF 6550) and ESI ionization (Dual AJS-ESI) using the following conditions: drying gas at 290 °C, drying gas flow 11 L/min, nebulizer at 20 psi; sheath gas at 350 °C; sheath gas flow 12 L/min, VCap 3000; fragmentor 110 V, OCT 1 RF Vpp 750 V, different collision energy using N_2 and a *m/z* 50 to 1600 range. Automatic MS/MS experiments were carried out using three different collision energies, that is: 20 eV, 30 eV and 40 eV. Integration and data elaboration were performed using MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

RESULTS AND DISCUSSION

Raw material characterization

Table 1 shows the properties that characterize the processed *pitanga* leaves.

Note in Table 1 a 1.9% difference between the water content determined by the Karl Fisher method (7.30%) and that from gravimetry (9.15%). Such a difference is normal since the Karl Fischer method is selective to water whereas the gravimetric method is unable to distinguish the volatile compounds that are evaporated (Isengard, 2001), counting the volatiles as moisture.

The leaves of *Eugenia uniflora* were collected at the same local as the samples used by Martinez-Correa et al. (2011) and Garmus et al. (2014), however in different years. The moisture (7%) found in this sample is among the values found by Garmus et al. (2014) (6%) and Martinez-Correa et al. (2011) (10%).

Table 1. Results of the properties of loose particles and bed of the processed *pitanga* leaves.

Property	*Pitanga* (*E. uniflora L.*)
Volatile and moisture content (VU%)	9.15 ± 0.07%
Moisture (U)	7.30 ± 0.04%
Mean particle diameter (dp)	0.307 mm
Real density (ρ_r)	1.530 ± 0.004 g/cm³
Bed apparent density (ρ_a)	0.421 ± 0.003 g/cm³
Porosity (ε)	0.725 ± 0.002

Hydroalcoholic extraction

Figure 4 shows the kinetics of the three hydroalcoholic extraction curves.

The hydroalcoholic extraction in fixed bed (60 ºC, 400 bar) was performed in triplicate, of which two repetitions (run 1 and 2) were performed with the measurement of 11 points with distinct time gaps in a total of 3 h, with a twelfth point corresponding to the system's depressurization. However, the last repetition was done with a single point also after 3 h. The global yields were 29.7%; 29.3% and 26.3%, respectively, leading to an average hydroalcoholic extraction yield of 28 ± 2%.

Note in Figure 4 that, at the end of the second hour of extraction (solvent mass 51.96 g), the yield

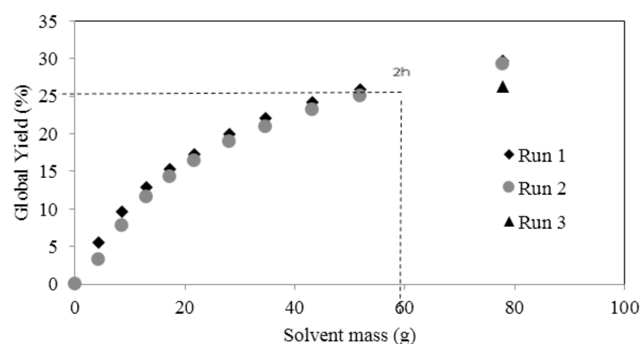


Figure 4. Extraction yields at 3 h (400 bar and 60 ºC), using an ethanol:water solution of 70:30 v/v as solvent with a flow of 0.5 mL/min.

corresponded to approximately 86.35% of the yield obtained at the end of the first run's extraction (3 h extraction), indicating that, from this point forward, the rate of mass transfer is controlled primarily by the diffusion phenomena inside the solid particles. Such kinetic behavior was also observed by Garmus et al. (2014). Considering the extraction process on an industrial scale, the extraction can therefore be interrupted at 2 h, due to the low extraction rates characteristic of the diffusional mass transfer.

Global extraction yields

Table 2 summarizes the extraction yields as well as phenol and flavonoid concentrations for the *pitanga* leaves extracts, including data from previous works by Martinez-Correa et al. (2011) and Garmus et al. (2014).

Note in Table 2 major differences for the extraction yields using hydroalcoholic solvents (ethanol + water), $scCO_2$ or ethanol. The hydroalcoholic extracts and the first fraction of hydroalcoholic and ethanolic extracts showed the highest contents of phenolic compounds, ranging from 230 to 260 (mg GAE/g extract), whereas supercritical fractionational resulted in low yields and phenolic concentrations. Thus, the use of antisolvent in the fractionation process is therefore beneficial.

The hydroalcoholic extraction in fixed bed (HFB) displayed the highest global yield (28 ± 2%), which is an intermediate value when compared to those of 20% and 30% for the extractions in fixed bed obtained by Garmus et al. (2014) using ethanol and water at 400 bar and 60 ºC. The conventional procedure in stirred vessel using hydroalcoholic solvent (HC) displayed the second highest global yields (25 ± 1%), which is also intermediate as compared to those of 18% and 27% obtained by Garmus et al. (2014). The yield found for fixed bed, which is superior to that for the conventional method, indicates a positive influence of pressure on the extraction yield, as was discussed by Garmus et al. (2014). The lowest yields of 4.6% were obtained by the CO_2 supercritical extraction, which is similar to those of 5% and 3.5% obtained (Garmus et

Table 2. Global extraction yields and phenol and flavonoid concentrations for the *pitanga* leaves extracts.

Extraction	Extract*	Yield (%)	Total phenols		Total flavonoids	
			Concentration (mg GAE/g extract)	Yield (mg GAE/ g leaves)	Concentration (mg CE/g extract)	Yield (mg CE/g leaves)
Conventional						
This work	HC	25 ± 1	255 ± 40	63 ± 11	40 ± 2	10.0 ± 0.2
[1]**	EC	18 ± 2	151 ± 1	28 ± 3	61 ± 2	11 ± 2
	AC	27 ± 1	109 ± 1	30 ± 1	20 ± 2	6 ± 1
Fixed Bed						
This work	HFB	28 ± 2	265 ± 22	70 ± 6	30 ± 2	7.8 ± 0.6
[1]**	EFB	20 ± 1	164 ± 1	32 ± 2	40 ± 2	8 ± 1
[1]**	AFB	33.3 ± 0.4	152 ± 1	51 ± 1	15 ± 1	5.0 ± 0.4
[1]**	SC	5 ± 1	32.7 ±	1.8 ±	153 ± 4	8 ± 2
[2]***	SC	3.5 ± 0.1	51 ± 7	1.7 ± 0.3	63 ± 1	2.2 ± 0.1
This work	SC	4.6	42 ± 15	2.0 ± 0.7	74 ± 5	3.6 ± 0.2
EFB (Ethanolic) $scCO_2$ Fractionation	E1	8.7 ± 0.6	264 ± 32	23 ± 2.8	41 ± 3	3.6 ± 0.2
	E2	0.6 ± 0.1	63 ± 6.7	0.35 ± 0.05	51 ± 6	0.28 ± 0.03
	E3	1.6 ± 0.6	25 ± 0.7	0.41 ± 0.01	60 ± 6	0.99 ± 0.09
	E4	1.9 ± 1.6	176 ± 7.2	3.4 ± 0.1	71 ± 17	1.4 ± 0.3
	Σ	13 ± 3	$\overline{M}$ = 212	27.3	$\overline{M}$ = 48.4	6.2 ± 0.7
HFB (Hydroalcoholic) $scCO_2$ Fractionation	H1	30.1	231.1	69.6	33.7	10.1
	H2	0.27	26.1	0.07	59.6	0.16
	H3	1.36	46.9	0.63	71.7	0.98
	H4	1.44	16.5	0.24	32.4	0.47
	Σ	33.2	212	70.5	35.3	11.7

* Hydroalcoholic in fixed bed (HFB), conventional hydroalcoholic in stirred vessel at 25 °C and 1 bar (HC), supercritical (SC), conventional ethanolic (EC), conventional aqueous (AC), ethanolic in fixed bed (EFB), aqueous in fixed bed (AFB); (E1) to (E4) for the ethanolic fractionation; (H1) to (H4) for the hydroalcoholic fractionation; [1]**(Garmus et al., 2014); [2]*** (Martinez-Correa et al., 2011).

al., 2014; Martinez-Correa et al., 2011) when applying the same method.

Phenols and Flavonoids Analysis

Table 2 shows the yield data (mg/g leaves) and concentration (mg/g extract) of the phenols and flavonoids.

Total Phenols

By analyzing the phenols contents in *pitanga* leaves, a great difference of the hydroalcoholic extracts in relation to the supercritical ones were observed. Among the hydroalcoholic extracts (HFB and HC), there was not a great divergence in results, but the highest values obtained for the fixed bed procedure in relation to the conventional one can be related to the positive influence of pressure. The phenolic yields of 70 and 63 mg GAE/g leaves of the hydroalcoholic extracts were superior to the best yields of 51 mg and 28 mg GAE/g leaves obtained (Garmus et al., 2014; Martinez-Correa et al., 2011) through the aqueous procedure in fixed bed and the conventional ethanolic one, respectively. This result indicates the possibility of a stronger affinity of phenols with the solvent of intermediate polarity, extracting compounds that would not be extracted using pure water or ethanol.

For the supercritical extraction, the phenolic yield of 2.02 ± 0.72 mg GAE/g leaves was close to those of 1.8 and 1.7 mg GAE/g leaves obtained by Garmus et al. (2014) and Martinez-Correa et al. (2011), respectively. The substantial effect of solvent polarity for best extraction of phenolic compounds is therefore clear, with polar solvents reducing the extract content. Table 2 also shows the superiority of hydroalcoholic extracts in terms of phenolic contents.

Total Flavonoids

Unlike the phenols' content, it was not possible to observe superiority of the flavonoids in the hydroalcoholic extracts in relation to the supercritical ones. Among the extracts obtained using the hydroalcoholic solvent, a different relation was also observed than the one for the phenols' content, since, in this case, the highest flavonoids content was shown in the conventional extract. This suggests that, possibly, the extraction at atmospheric pressure is more favorable for the separation of these compounds than high pressure (400 bar).

The yields for the hydroalcoholic extraction in fixed bed (7.8 ± 0.6 mg CE/g leaves) is within the interval found by Garmus et al. (2014), (8 ± 1 and 5.0 ± 0.4 mg CE/g leaves) for the extractions in fixed bed using, respectively, ethanol and water. It is therefore possible to suggest that, for flavonoids, medium polarity of the solvent leads to intermediate yields. For conventional extraction with hydroalcoholic solvent, the yield (10.0 ± 0.2 mg CE/g leaves) was higher than that found by Martinez-Correa et al. (2011) (3.37 ± 0.07 mg CE/g leaves) using only alcohol as solvent. This similarity suggests that highly polar solvents, particularly aqueous solutions, favor flavonoid extraction.

Table 2 shows that, since flavonoids are sub-class phenolic compounds, the content of flavonoids was inferior to that of phenolics, whereas total flavonoid contents vary much less than that of phenols, with the highest content being observed for the supercritical extract. But, as previously discussed, the lower global yields of both supercritical steps contributed, again, to a low yield of flavonoids.

Fractioning

Table 2 and Figure 5 summarize the fractionation results with $scCO_2$ at 60 ºC and pressures of 300, 200, 100 bar and atmospheric pressure of ethanolic and hydroalcoholic 70:30 (v/v) extractions at 400 bar and 60 ºC, as well as the selective precipitation of the target compounds according to the gradual pressure reduction. At 60 ºC, the mixture of CO_2 and ethanol creates a single phase above approximately 110 bar (Lim et al., 1994), indicating that all ethanol flows into the second separator. Below this pressure, therefore, the mixture forms two phases and CO_2 is separated from ethanol.

The majority of the ethanolic extract (8.7 of a total 13%) was precipitated in the first separator, when the

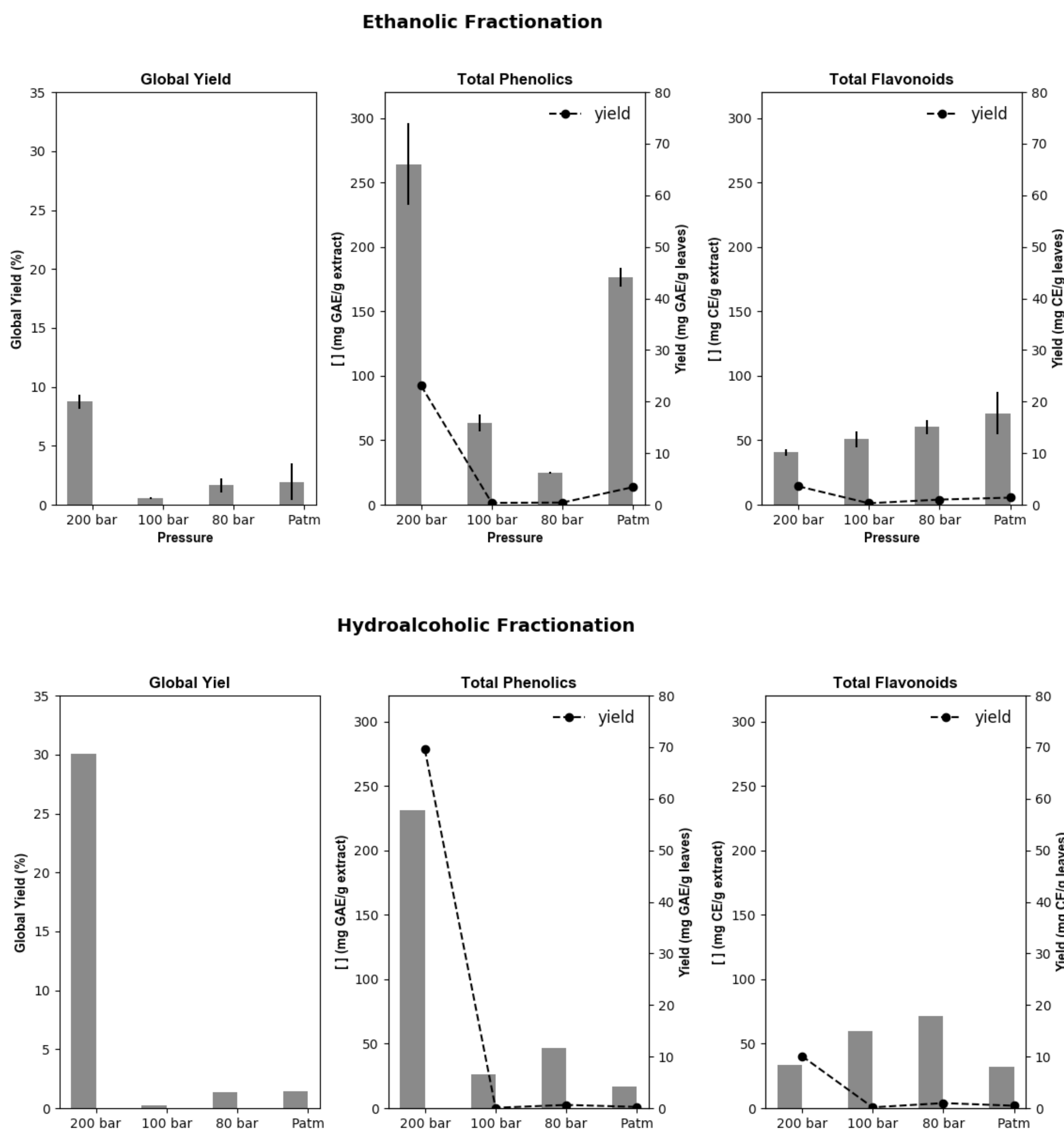


Figure 5. Representation of concentration (mg/g extract) and yield (%) obtained in the analysis of phenol and flavonoid fractions.

CO_2 was mixed with the ethanolic extract, thus pointing to the drastic reduction of the solvent polarity. When the pressure is reduced from 300 bar (first separator) to 200 bar (second separator), a low yield of precipitate is observed with low phenolic concentrations. In the third and fourth separators, there is a growing increase in precipitates, but with lower phenolics concentrations than those found in the first separator. An important result in these fractionation conditions is that the flavonoids concentrations increased from one fractionation phase to the other. The tuning of the operational conditions (temperature and pressure) in the separators can therefore be used for separating ethanol from the extract as well as for separating total phenolics from flavonoids and, consequently, to obtain differentiated extracts in the composition of target bioactive compounds.

The proposed fractionation process was inefficient for the hydroalcoholic extract since about 91% of all extract obtained was precipitated in the first separator. The water present in the extract decreases extract solubility in supercritical CO_2, creating two phases in separator 1, and precipitating most of the desired compounds. A similar behavior was observed in the fractionation of the hydroalcoholic extract of *A. chica*, in which 98% of the extract was precipitated in separator 1 (Paula et al., 2018).

UPLC-ESI-MS/MS

All the extracts obtained from the fractionation were analyzed by UPLC-ESI- MS/MS and 12 compounds could be tentatively assigned (Table 3), which was made based on their exact mass and MS/MS fragmentation patterns as compared to reported data.

Among the 12 compounds identified, one is an organic acid (quinic acid); four are phenolic acids (chlorogenic acid, gallic acid, 3-O-p-coumaroylquinic acid and 5-O-galloylquinic acid); six are flavonoids (myricetin-O-rhamnoside, myricetin-O-hexoside, quercetin-O-deoxyhexoside, quercetin glucosyl rhamnoside, myricetin-O-pentose and morin) and one is a phenolic acid derivative (uralenneoside). Table 4 shows the extracts in which each of the identified compounds were detected.

With the exception of morin, all identified compounds were present in the initial ethanolic and hydroalcoholic extracts. Considering the composition of the fractions obtained in the different separators, it is observed that the fractionation of the hydroalcoholic extract resulted in extracts with greater composition differences. For this extract, the operating conditions employed in the first separator (H1) were sufficient to completely precipitate several phenolics, that is, myricetin-O-hexoside, quercetin glucosyl rhamnoside, quercetin glucosyl rhamnoside and 3-O-p-coumaroylquinic acid. In general, these same phenolics were also completely separated in fractionation of the ethanolic extract along the separators.

The flavonoids identified in the extracts (myricetin-O-rhamnoside, myricetin-O-hexoside, quercetin-O-deoxyhexoside, quercetin glucosyl rhamnoside, myricetin-O-pentose and morin) presented a more pronounced fractionation for the hydroalcoholic extract (Figure 5 and Table 4) since in the last separator

Table 4. Detection of compounds in the different extracts by UPLC-ESI-MS/MS.

Compound	E1	E2	E3	E4	H1	H2	H3	H4
Myricetin-O-rhamnoside	X	X	X	X	X	X	X	nd
Chlorogenic acid	X	X	X	X	X	X	X	nd
Gallic acid	X	X	X	X	X	X	X	nd
Quinic acid	X	X	X	X	X	X	X	X
Myricetin-O-hexoside	X	X	nd	nd	X	nd	nd	nd
Quercetin-O-deoxyhexoside	X	X	X	X	X	X	X	X
Quercetin glucosyl rhamnoside	X	X	nd	nd	X	nd	nd	nd
Myricetin-O-pentose	X	X	nd	nd	X	X	X	nd
Morin	X	X	X	X	nd	nd	nd	nd
Uralenneoside	X	X	X	X	X	X	X	X
3-O-p-Coumaroylquinic acid	X	X	X	X	X	nd	nd	nd
5-O-Galloylquinic acid	X	X	X	X	X	X	X	nd

X = detected; nd = not detected; E1 to E4 for the ethanolic fractionation; H1 to H4 for the hydroalcoholic fractionation.

Table 3. Identification of compounds by UPLC-ESI -MS/MS.

Compound	*m/z* experimental	*m/z* calculated	Molecular formula	Fragments	Error (ppm)	Reference
Myricetin-O-rhamnoside	463.0873	463.0877	$C_{21}H_{20}O_{12}$	316, 179	-0.77	a,b
Chlorogenic acid	353.0885	353.0890	$C_{16}H_{18}O_9$	191	-1.42	c
Gallic acid	169.0138	169.0137	$C_7H_6O_5$	125.0250; 69.0353	0.59	d
Quinic acid	191.0558	191.0555	$C_7H_{12}O_6$	85.0306	1.57	e
Myricetin-O-hexoside	479.0834	479.0826	$C_{21}H_{20}O_{13}$	316.0236	1.73	e, f, g, h
Quercetin-O-deoxyhexoside	447.0937	447.0927	$C_{21}H_{20}O_{11}$	300.0274; 151.0036	2.15	b, f, g
Quercetin glucosyl rhamnoside	609.1465	609.1455	$C_{27}H_{30}O_{16}$	446.0172; 300.0271	1.61	c
Myricetin-O-pentoside	449.0736	449.0742	$C_{20}H_{18}O_{12}$	316.0245; 271.0259; 179.0002	-1.34	b
Morin	301.0348	301.0348	$C_{15}H_{10}O_7$	125.0276; 151.0043	-0.10	f
Uralenneoside	285.0612	285.0610	$C_{12}H_{14}O_8$	152.0111; 108.0211	0.70	i
3-O-p-Coumaroylquinic acid	337.0921	337.0923	$C_{16}H_{18}O_8$	191.0566	-0.73	j
5-O-Galloylquinic acid	343.0669	343.0665	$C_{14}H_{16}O_{10}$	169.00141; 191.0556; 125.0243	1.09	l

[a](Negri and Tabach, 2013), [b](Simirgiotis et al., 2013), [c](Pascoal et al., 2015), [d](Munekata et al., 2016), [e](Saldanha et al., 2013), [f](Sun et al., 2007), [g](Díaz-De-Cerio et al., 2015), [h](Fracassetti et al., 2013), [i](Díaz-de-Cerio et al., 2016), [j](Gouveia and Castilho, 2010), [l](Wyrepkowski et al., 2014).

(ambient pressure condition) only the flavonoid quercetin-O-deoxyhexoside was detected, with a decrease in concentration of total flavonoids.

Although quantification of the compounds was not performed, the substantial qualitative variations in composition of these phenolic compounds herein described provided information for the understanding of the applied fractionation process of *pitanga* leaves.

CONCLUDING REMARKS

Increasing solvent polarity significantly increases global extraction yields for *pitanga* leaves. Hydroalcoholic extraction, applied for the first time for such leaves resulted in intermediate yields when compared to the data in the literature for aqueous and ethanolic solutions. For total phenols, best extraction yields were found for hydroalcoholic extraction. Hydroalcoholic extraction therefore provides better extraction of phenolics when compared to the literature and the supercritical method. The use of $scCO_2$ in the fractioning phase of the ethanolic extraction showed that it is possible to separate ethanol from the extract while obtaining differentiated extracts in terms of phenol and flavonoid compositions.

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