SUPERCRITICAL CO_2 AND PRESSURIZED SOLVENTS EXTRACTION PROCESSES FOR BIOACTIVE COMPOUNDS FROM MANGO PEEL WASTE (*Mangifera indica L.*).

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Abstract. Processes using supercritical fluid extraction of active compounds represent a great potential for applications in agri- food and pharmaceutical industries. The aim of the present study was to exploit the mango peel waste (*Mangifera indica L.*) for obtain bioactive extracts. Mango skin waste was submitted to extraction process with two sequential steps: supercritical CO_2 extraction ($scCO_2$) followed by pressurized ethanol (PE) from the residue of first stage, extraction operated at 300 bar and 40 °C, conventional ethanol extraction (CE) was done. The extracts obtained were evaluated by spectrophotometric method in terms of Total Carotenoids Content (TCC) (µg carotenoids/g db), Total Phenolic Content (TPC) (mg GAE/g db), Total Flavonoids Content (TFC) (mg CE/g db); and Antioxidant Activity (%AA) by DPPH method. All experiments were performed in duplicate, a ANOVA and Tukey test was performed (p<0.05)(SAS, version 9.1.3). The results demonstrated that TCC value is dependent on the solvent, being high for $ScCO_2$ with carotenoids contents of 5604.60 ± 0.51 µg in contrast to PE, 359.45 ± 0.35 and CE, 704.4 ± 0.9 µg carotenoids/g d.b. TCC results follow the trend $ScCO_2$>CE>PE meanwhile for TPC and %AA trend was CE>PE>$ScCO_2$.The results obtained showed that the combination of extraction methods achieves appreciable results and suggests the potential of this agro-industrial residue for obtaining active compounds.

Keywords: Mango waste, supercritical extraction, pressurized solvents, carotenoids, antioxidant activity.

1. Introduction

In recent years, fruits and vegetables have received considerable attention as sources of biologically active substances, due to their antioxidant properties [1] without leaving behind the properties of the residues that they produce. Large amounts of sub-products and residues generated by different fruits processing industries are traditionally treated as environmental contaminants, but in recent years are being recognized as a source of obtaining valuable components [2], with significant biological properties [3].

Mango (*Mangifera indica L.*) is one of the most important tropical fruits in the world in terms of production and consumer acceptance [4]. With a global production estimated at more than 23.4 million tons per year is commonly called as "the king of fruits" [5] and ranks fifth in the world's most important fruit crop production because of its bright color, characteristic flavor, and high nutritional value [6]. India ranks first among mango producing countries in the world, accounting for 54.2% of total worldwide mango production. Mango production in Colombia is equivalent to 0.63% of world production of the fruit, which places the country in 19th place [7].

During mango processing, the skin is one of the most important waste [2], which constitutes about 15-20% of the fresh fruit weight. At the moment, for some processing industries, the skin is discarded and thus

becomes a source of contamination [8]. Other studies reports use of this waste as a source of pectin, dietary fiber and biogas production [9]. However, within its phytochemical profile, mango skin contains various types of polyphenols, carotenoids and vitamins with different health benefits and properties, mainly for its antioxidant activity [10].

Among the outstanding extraction processes are those employing supercritical carbon dioxide ($scCO_2$) as they take advantage of the ability of some chemicals to act as excellent solvent with temperature and pressure above its critical point. This solvent is non-toxic and safe (GRAS), non-flammable and of low cost [11], highly compressible with surface tension which promote better penetration into vegetable matrix compared with conventional processes [12].

In this study was used mango skin from agro-industrial processing which was submitted to one extraction process with two sequencial steps: the first with supercritical CO_2 ($scCO_2$) and the second, using pressurized ethanol (PE) from the residue of the first extraction.

2. Materials and Methods

2.1. Materials

Mango skin samples (*Mangifera indica L.*) were obtained as a waste from a local processing industry (Valle del Cauca, Colombia), then were lyophilized (Christ, England) for 48 hours, ground (Fritsch pulverisette 14, Germany) and refrigerated (-10 °C) in a refrigerator (model 220 Consul, Brazil). Carbon dioxide, 99.5% w/w (White Martins, Brazil), ethanol 99.5% w/w (Ecibra, Brazil), high purity water Milli-Q (Millipore direct-Q UV, Millipore Corporation , USA), were used as solvents.

2.2. Raw material characterization

Moisture content: it was determined by AOAC gravimetric method [13]. 2 g were used in a capsule sample previously weighed on an analytical balance (model 220A TX, ACCURATE Instruments, Switzerland), and dried in vacuum oven (model Marconi MA-030-12, SP, Brazil) ,It was performed by triplicate.

Particle diameter: ASAE method was used [14], the sample was conditioned in a set of sieves in the range series of 8-48 Tyler mesh.

2.3. Extraction procedures

They were performed in two stages sequentially in the experimental extraction unit (Extrae Laboratory, Unicamp). The first extraction stage with supercritical CO_2 ($ScCO_2$) and the second stage with pressurized ethanol extraction (PE), In addition to these extractions, an ethanolic conventional extraction (CE) was performed.

First extraction step: was done with supercritical CO_2 (scCO2), the experiments were carried out in the experimental extraction unit Laboratory (Fig. 1).

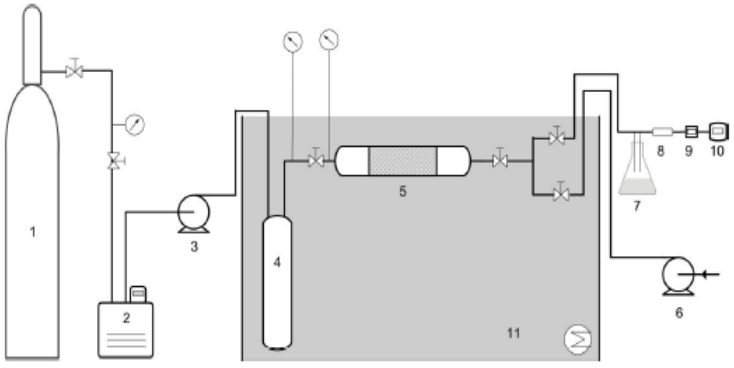

Figure 1. Experimental extraction unit. (Martinez-Correa *et al.* [11]).

Dried and Ground raw material (5 g) were placed within the extracto, operating conditions were adjusted at 300 bar and 40 °C for all experiments. The CO_2 flow corresponded to 1.1 liters/min and the extracts were collected every 30 minutes for 7.5 hours. Ethanol was injected (99.5%, Ecibra) at flow 0.5 liters/min.

Second extraction step: was done with pressurized ethanol (EP) on the residue of $scCO_2$ extraction, it was performed at 300 bar and 40 °C.

Conventional extraction processes (CE): Ethanolic extracts were obtained according to the methodology of Paviani *et al.* [15], where 3 g of raw material and 10 ml of ethanol, under magnetic stirring (Fisatom-752, Brazil) for 24 hours at room temperature (25 °C). It was separated by vacuum filtration (BD pump Dia-8244), the filtrate was stored in a freezer at -10 ° C for 12 hours and filtered to reduce the fat present in the extract. Dried extracts were using a rotary evaporator (Marconi, MA-120, Brazil) and a final evaporation was additionally necessary in a vacuum oven (Model MA-Marconi 030-12, SP, Brazil) at 40 ° C.

2.4. Evaluation of extracts

Chemical characterization (total phenolic content, total flavonoids content, total carotenoid content). To determine the total phenolic content (TPC) was used to extract the Folin-Ciocalteu method according to Singleton *et al.* [16]. Was used a calibration curve using the standard gallic acid (≥ 99%, VETEC, Brazil), at different concentrations and a linear equation was finally obtained TPC = (4.7429Abs + 0.0019), $R^2 = 0.9989$. The TPC final results are presented in mg GAE/g of dry extract (mg GAE/g).

To determine the total flavonoids content (TFC) for the extracts was used the method developed by Zhishen *et al.* [17], was used a calibration curve using the standard catechin (98%, Sigma Aldrich, USA). At different concentrations and finally a linear equation was obtained TFC = (3.2878Abs + 0.0006), $R^2 = 0.9995$. The TFC final results were presented in mg of catechin/g dry matter (mg CE /db).

The total carotenoid content (TCC),was determined spectrophotometrically according to the method of Szydłowska *et al*. [18]. Where, 0,125 grams of each one of the extracts were dissolved in 25 ml of ethanol. The absorbance at 450nm was measured using a spectrophotometer (Lambda 40 UV-VIS, Perkin Elmer, USA) in quartz cuvettes (Q-4 spectrometer) volume of 3.5ml. Subsequently, a calibration curve was carried out with ethanol and betacarotene solutions. The TCC final results were presented in µg of carotenoids/g of dry extract.

Antioxidant activity (%AA). Antioxidant activity (%AA) was measured by the DPPH method described by Mensor *et al.* [19]. From an extract solution of 1 mg/ml concentration, dilutions of the extracts at concentrations of 5, 10, 25, 50, 125 and 250 µg/ml in volumetric flasks of 10 ml. A solution was prepared of 0.3 mM of reagent DPPH (2.2 Diphenyl-1-picrylhydrazyl) (Sigma-Aldrich Chemie, Germany) with 3mg of reagent and 25 ml of ethanol. In test tubes were added 5 ml of each solution of various concentrations, was added 2 ml of the solution of 0.3 mM of reagent DPPH and after 30 minutes the absorbance was determined at 518 nm visually observing color changes indicating the reaction of the reactive antioxidant compound with the reagent. The reference solution used as blank was prepared with 5 ml of each extract solution at various concentrations and 2 ml of ethanol and the negative control was prepared in a proportion of 2 ml of 0.3 mM solution of DPPH reagent and 5 ml of ethanol. For these two solutions, the absorbance was measured at 518 nm. The spectrophotometer was calibrated with ethanol.

To calculate the antioxidant activity (% AA) the equation (1) was used:

$$\%AA = 100 - \left[\frac{\left(Abs_{\,sample} - Abs_{\,blank}\right)x \;\; 100)}{Absnegcontrol}\right] \qquad (1)$$

The extract concentration responsible for a 50% decrease in the initial activity of DPPH (EC_{50} µg/ml) was calculated by regression analysis [20].

2.5. Statistical Analysis

Was performed a variance analysis ANOVA ($p<0,05$) and Tukey test (SAS, version 9.1.3) for experiments by duplicate and analysis by triplicate.

3. Results and Discussion

Regarding the procedures for characterizing the vegetable matrix, a moisture content was 8.62 ± 0.52% and average particle diameter of 0.2944mm.

In Table 1, are represent the overall yields of the extractions studied and the respective concentrations and quantified yields in mango skin (*Mangifera indica L.*).

Table 1. Yields and concentrations of quantified compounds in the vegetal matrix

Process	Global Yield (%)	Phenols (TPC)		Flavonoids (TFC)		Carotenoids (TCC)	
		Concent. (mg GAE/g)	Yield (mg/g)	Concent. (mg CE./g)	Yield (mg/g mp)	Concent. (mg carot./g)	Yield (mg/g)
$ScCO_2$	1.7 ± 0.3[a]	15.8 ± 0.4[a]	0.3 ± 0.05	8.0 ± 0.12[a]	0.1 ± 0.02	5.6 ± 0.5[a]	0.1 ± 0.02
PE	37.0 ± 3.1[b]	23.5 ± 0.4[b]	8.7 ± 0.7	4.1 ± 0.06[b]	1.5 ± 0.1	0.4 ± 0.4[b]	0.1 ± 0.01
CE	10.4 ± 0.5[c]	41.6 ± 0.2[c]	4.3 ± 0.2	10.5 ± 0.02[c]	1.1 ± 0.05	0.7 ± 0.9[c]	0.1 ± 0.003

The different letters represent the significant differences for the different treatments in the same column, Tukey test ($p < 0.05$). Supercritical extracts ($ScCO_2$), pressurized ethanolic extracts (PE), conventional extracts (CE).

Was observed in the second extraction stage (PE), had the highest overall extraction yield, this behavior is due to the use of high pressures in organic solvents for extraction processes promotes mass transfer of solute to the solvent, improving extraction yield as suggested by Mustafa *et al.* [21]. However, it is important to note that polar nature of the solvent also has a positive influence on the compounds that are present in the matrix indicating a greater presence of polar substances in mango peel extracts, this influence in the global extraction yields was reported by Martinez-Correa *et al.* [11]. CE extraction yield was higher than $scCO_2$, this behavior was reported in several studies [22,23], which also obtained global yields for the conventional processes higher than supercritical extraction.

3.1. EVALUATION OF EXTRACTS

Chemical composition: the Figure 2a shows the contents of phenols, flavonoids and total carotenoids for the first stage; supercritical extracts ($scCO_2$) and second stage; with pressurized ethanol extracts (PE). Figure 2b shows the content of phenols, total flavonoids and carotenoids in conventional ethanolic extracts (CE).

Total Phenolic content: In this study, TPC had significant differences for the first and second extraction stage. The conventional ethanol extraction (CE) presented the largest phenol content (41.6 ± 0.2 mg GAE/g db), results are below those obtained by Abdul Aziz *et al.* [6], and close reported values by Ajila *et al*. [24] the for polyphenols content in mango peel extracts. These behavior could be attributed of the samples, which a possible oxidation of compounds by enzymatic reactions. According to Palafox-Carlos *et al.* [25], the physiological processes of ripening in mango directly affects the polyphenol content.

It was determined that in the first extraction, ($scCO_2$), nonpolar polyphenolic fraction was extracted but remained within the matrix compounds that were not exhausted during this extraction which were extracted in the second stage (PE), these compounds correspond to polar character and representing a better solubility with the solvent. Thus the $scCO_2$ removes the non-polar substances, the polar ones are more accessible to be extracted by PE as a sequential extraction process. These results were similar to those reported by Vatai *et al.* [26] and Santos *et al.* [27].

Total Flavonoids: TFC also presented significant differences, e conventional ethanolic extraction (CE) presented the higher content flavonoids (10.5 ± 0.02 mg CE/g db), this value is within the range reported by Abdul *et al*. [6] for mango peel extraction. Therefore it is considered that $scCO_2$ demonstrated its effectiveness for extracting low polarity fraction of flavonoids, and the second stage (PE) extracted a minor polar fraction (4.1 ± 0.06 mg CE/g db) .a behavior comparable with the results presented by Toledo-Guillen *et al*. [28].

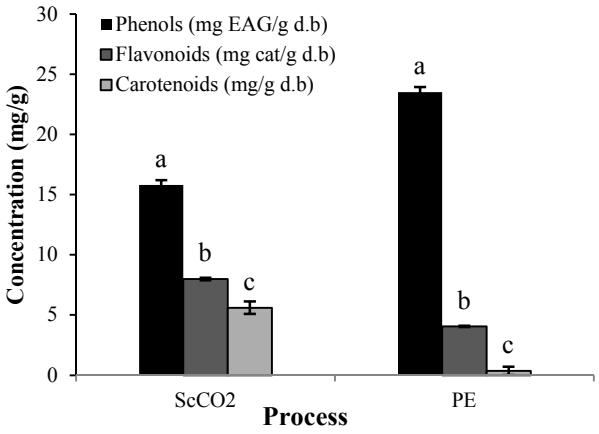

Figure 2a. Phenols content, total flavonoids and carotenoids. Error bars represent standard deviation for triplicate treatments and the statistical differences representation. Tukey test ($p<0,05$). Supercritical extraction with CO_2 ($scCO_2$), pressurized ethanol extraction (PE).

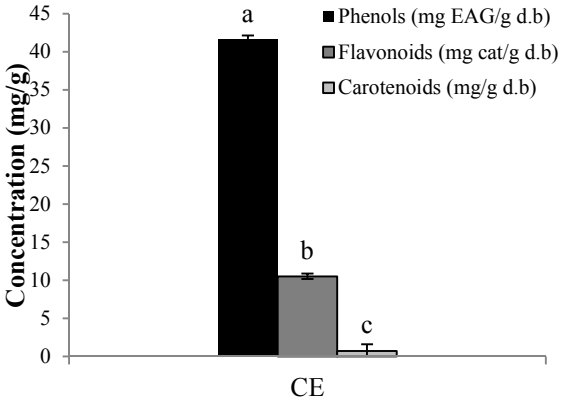

Figure 2b. Phenolic content, total flavonoids and carotenoids in conventional ethanolic extraction(CE). Error bars represent the standard deviation for triplicate treatments and statistical differences representation. Tukey test ($p<0,05$).

Total Carotenoids:, TCC also presented significant differences, $scCO_2$ extraction stage allows to produce highest carotenoid content (5604.6 ± 0.5 µmg carotenoids/g db). These confirm affinity of the solvent with nonpolar compounds such as carotenoids, which structurally possess unsaturated hydrocarbon chains with cyclohexene rings at their ends and according to Sun *et al.* [29] and the absence of strong polar fraction in the molecular structure explains its high solubility of this compounds in $scCO_2$. This high solvating power and selectivity behavior is reported by Filho *et al.* [30] and Vági *et al.* [3], in extraction of carotenoids in pitanga and tomato pomace , respectively.

The CE process, represents an alternative procedure for recovering carotenoids (704.4 ± 0.9 µg of carotenoid/g db,) this result is higher than the values reported by by Ajila *et al.* [31], the solvent nature and ripening of sample could be explain these results. Carotenoids content is high in peel with advance physiological ripening compared with the peel with partial ripening [8, 32],.

Antioxidant activity: The antioxidant activity (%AA) was measured by the DPPH method described by Mensor *et al*. [19]. In this method, we evaluated the capability of possible antioxidant to neutralize a possible radical. The compound 1,1-diphenyl-2-pricrylhidrazyl (DPPH) is a stable radical having an intense violet color that absorbs radiation at 518 nm, so that its concentration can be determined by spectrophotometric methods.

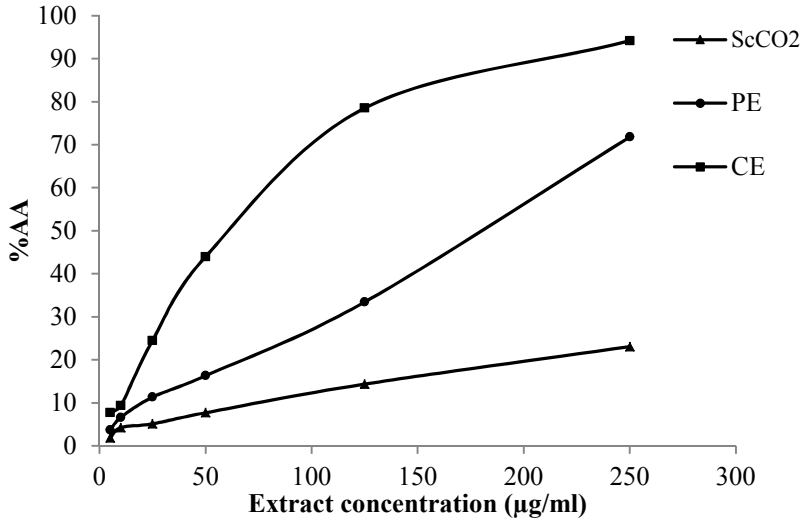

Figure 3. Capture activity of the DPPH radical. $scCO_2$ (▲),PE (●),CE (■).

Figure 3, shows that analyzed extracts showed an increasing activity with extract concentration in the range of 0-250 µg / ml, and the maximum %AA obtained for each extracts showed significant differences. At concentration 250 µg/ml PE and CE extracts showed %AA higher than $scCO_2$ (Fig. 4).

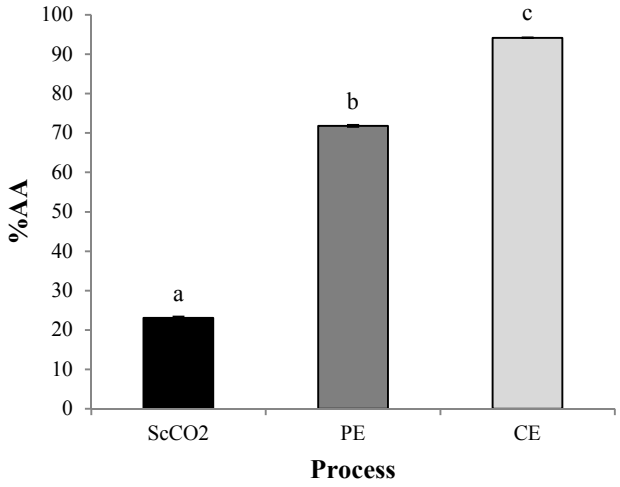

Figure 4. Antioxidant activity of the extracts at 250 µg / ml. Error bars represent standard deviation for triplicate treatments and statistical differences representation. Tukey test ($p<0.05$)

The highest antioxidant activity was obtained for CE extracts, this result is agree with those reported by Faller *et al*. [33]. The lowest radical scavenging capacity was obtained for $scCO_2$ extract, and was observed generalized behavior with low polarity solvents [34, 35].

Effective concentration (EC_{50}): %AA concentration resulting was ploted against the extract to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. A smaller EC_{50} describes a higher antioxidant potential of compounds [36]. Table 2 are reported the EC_{50} values, value for $scCO_2$ extract was not possible to calculate, because of the higher value of %AA does not exceed the 50% required to perform this calculation.

Table 2. CE_{50} values for extracts EP and EC.

PE		**EC**	
CE_{50} (μg/ml)	R^2	CE_{50} (μg/ml)	R^2
174.52	0.995	96.69	0.89

It can be seen that EC extract could be considered as moderately active and PE extract as slightly active, according to Reynertson *et al.* [37]. This behavior may be related to phenols content of in the extracts because of the activity and content of these compounds can influence antioxidant activity. This vegetable matrix submitted to pressurized ethanol extraction (PE) as the second stage allowed to obtain extracts with antioxidant activity higher than those obtained in the first stage ($scCO_2$)

4. Conclusions

The combination of extraction methods achieves appreciable results in terms of phenolic content in pressurized ethanol. Flavonoids and total carotenoids in Mango mesocarp (*Mangifera indica L.*) are low polarity and were preferentially extracted with supercritical carbon dioxide. Mango peel waste represents a valuable source of natural antioxidants and represents potentiality for processes of extraction of value-added compounds with important biological activity.

Acknowledgements

To Laboratory ExTraE (Universidade Estadual de Campinas-Unicamp –Brazil).

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