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Integrated Processes Applied to Obtain Biopotential Extracts from Papaya Seeds

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Abstract

Nowadays, the valorization of food industry by-products and agricultural waste has been the focus of investigation worldwide. In this framework we evaluated two combining high pressure processes, the Supercritical Fluid Extraction (SFE), at 30 MPa and 40 °C with carbon dioxide (CO_2) as solvent, and the Pressurized Liquid Extraction (PLE), at 10 MPa, 80 °C and with ethanol, water and ethanol/water mixture (1:1) as solvents, in order to obtain bioactive compounds from papaya seed extract. The combined methods were applied to selectively recover bioactive extracts. Total phenol content of the resulting extracts was characterized by Folin-Ciocalteu spectrophotometric method. The *in vitro* antioxidant activity was determined by DPPH and β-carotene methods, and the chemical profile was carried out by gas-chromatography-mass spectrometry (GC-MS). The results show better antioxidant performance of the extracts obtained by combined process, compared to the separated methods. The best results for total phenol content (39 $mg_{EAG}.g^{-1}_{extract}$) and DPPH ($EC_{50}$ 199 $\mu g.mL^{-1}$) were found by the combination of SFE and PLE with ethanol/water. The high β-carotene value (92 %) was found by combining SFE and PLE with ethanol. Papaya seeds oil can represent a valuable source of natural compounds, especially benzyl isothiocyanate.

Keywords: Papaya seeds; Process combination; Selective extraction.

1. Introduction

Papaya is a plant very well known and present in tropical and subtropical regions. The fruit is widely consumed for its nutritional characteristics, availability and low cost. The papaya fruit is consumed "*in natura*", and also marketed in the form of pulp, juice, jams and crystallized fruits, with seeds being the main disposal material.

The papaya seed is well known for its medicinal uses and has a broad phytochemical spectrum, especially the BITC (Benzyl-isothiocyanate) compound that is directly linked to the biological activities of the seed [1, 2].

Food and pharmaceutical industries are exploiting new natural sources of bioactive compounds with potential use for food preservation, and application in medicines and cosmetics, which increased studies and application of new technologies for the recovery of these compounds. Among the extraction technologies, methods such as supercritical

fluid extraction (SFE) and pressurized liquid extraction (PLE) are of interest. These are environmentally friendly methods to obtain bioactive compounds due to the use of green solvents such as ethanol, water and CO_2. SFE and PLE are also attractive techniques because of potential reduction of energy consumption and the high quality extracts [3]. However, SFE with CO_2 is normally applied for the recovery of low polarity compounds, whereas PLE with ethanol and/or water allows extraction of compounds of high polarity [4]. Therefore, the objective of this study is to integrate the extraction processes SFE and PLE to improve the recovery of compounds from papaya seed with biological potential.

2. Material and methods

2.1. Papaya seeds and raw material characterization

The papaya seeds were supplied by the company *Kazmierski* from Jaraguá do Sul, Santa Catarina - Brazil. The seeds were received at Laboratory of Thermodynamics and Supercritical Technology from Federal University of Santa Catarina (LATESC/UFSC), water washed, dried in air circulation oven at 45 ºC for 24 h (until moisture of 16.95 ± 0.10%), ground in a knife mill and stored in polyethylene packages at -18 ºC (in domestic freezer) until experiments were carried out.

2.2. Supercritical fluid extraction (SFE)

Mazzutti et al. [5] previously described the experimental unit and the procedure for the high-pressure process. The SFE experiment was carried out with 99.9% pure CO_2 (White Martins, Brazil) in duplicate under the conditions of 30 MPa, 40 °C and solvent flow rate of 0.7 $kg.h^{-1}$, for 4 hours. Approximately 15 grams of sample were placed into the extraction vessel. The selected condition was determined by literature [6]. The extracts were collected in amber glass flasks, weighed and stored under freezing (-18 °C) for further analyses. The extraction yield (X_0) was calculated by the ratio between mass of extract and mass of raw material.

2.3. Pressurized liquid extraction (PLE)

PLE extractions were conducted in an extraction unit described by Mazzutti et al. [5], using ethanol P.A. (Neon, Brazil), distilled water and water/ethanol mixture (1:1) as extraction solvents. The experiments were performed in duplicate of 10 MPa, 80 °C and solvent flow rate of 3 $mL.min^{-1}$, during 67 min. From five to eight grams of sample and glass beads were used to fill the extraction vessel. The extracts were submitted to solvent removal by rotary evaporator (Fitason, Brazil), and the extraction yield was determined.

2.4. Integrated Process

The integrated process (IP) proposed in this study is composed by SFE followed by PLE (the residue from SFE assay was the raw material for PLE assay). The IP first step (SFE) was performed as described in section 2.2. In sequence, the second step (PLE) was performed using ethanol P.A. (Neon, Brazil), distilled water and water/ethanol (1:1) mixture as extraction solvents at 10 MPa, 80 °C, solvent flow rate of 3 $mL.min^{-1}$, for 67 min. The experiments were performed in duplicate and extracts collected in amber flasks and the solvent removal was performed by rotary evaporator (Fisatom, Brazil).

2.5. Total phenolic content (TPC)

The total phenolic content for each extract samples was determined by the Folin-Ciocalteu method [7]. A stock solution of 2 $mg.mL^{-1}$ of gallic acid in distilled water was

diluted in different concentrations to obtain the standard curve ($R^2 = 0.9965$). The extracts were diluted in ethanol at a concentration of 30 $mg.mL^{-1}$ and a reaction mixture was prepared with 10 μL of extract or solvent, 50 μL of Folin-Ciocateau reagent, 150 μL of 20% sodium carbonate and 800 μL of distilled water. After 2 h incubation at room temperature, the absorbance was measured at 760 nm, using UV-Vis spectrophotometer. The sample was prepared in triplicate and the results expressed in milligrams of gallic acid equivalent per gram of dry matter ($mg_{EAG}.g^{-1}_{extract}$). The chemical profile by gas-chromatography-mass spectrometry (GC-MS) is being investigated.

2.6. Antioxidant activity

The antioxidant activity was determined for the extracts obtained by SFE, PLE and IP. All reagents used in the antioxidant activity analysis were purchased from Sigma–Aldrich (USA).

2.6.1. Free radical scavenging activity (DPPH)

The antioxidant activity by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical method was performed according to the methodology described by Mensor et. al. [8]. The extracts were diluted in ethanol with concentrations of 0 to 500 $\mu g.mL^{-1}$. To the eppendorfs containing the extract dilutions were added 0.3 mM DPPH ethanoic solution, homogenized and incubated in darkness at room temperature. After 30 min the absorbance was measured at 517 nm. The samples were prepared in triplicate and the results expressed in effective concentration (EC_{50}), concentration necessary to reduce by 50% the oxidative activity of the DPPH radical, the EC_{50} values were calculated from the linear regression.

2.6.2. β-carotene bleaching method

Antioxidant activity of extracts by β-carotene/linoleic acid system was carried out according to the method described by Matthäus [9] and Kang et al. [10]. The solution of the β-carotene/linoleic acid system was prepared using 3.4 mg of β-carotene, to which were added 44 μL of linoleic acid, 400 mg of Tween-20 and 5 ml of chloroform. The chloroform was evaporated in a rotary evaporator (Fitason, Brazil) and 100 mL of distilled water were added. Eppendorfs with 1 mL of β-carotene system solution were added 40 μL of ethanoic extract solution (1.667 $mg.mL^{-1}$). And for control the sample was replaced by ethanol. The eppendorfs were placed in water bath at 50 °C and the absorbance was measured at 470 nm at zero incubation times (t:0) and after 2 hours (t:120). The results of antioxidant activity were calculated by Equation 1.

$$AA\% = 1 - \left\{ \left[\frac{(Abs_{t:120} - Abs_{t:0})}{(Abs_{Controlet:120} - Abs_{Controlet:0})} \right] \cdot 100 \right\} \quad (1)$$

3.0. Results and discussion

3.1. Global extraction yield (X_0) and total phenolic content (TPC)

The results of extraction yield and total phenolic content (TPC) of the papaya seeds obtained by SFE, by PLE and by the integrated process (IP) are presented in Table 1.

The yield value obtained for papaya seeds by SFE at condition of 30 MPa and 40 °C was 18.08%, a result suggesting high content of non polar compounds, and higher than yield of other seeds, as reported in literature for butia seed (12.6 %) [11] and chia seed (9.3 %) [12], both obtained at the same conditions of this study. The high yield obtained by SFE

is related to the increase in solvation power with pressure, due to enhancing solvent density.

For PLE, the extraction yield increased with solvent polarity. The highest PLE performance was verified for extraction with ethanol as solvent (23.72%), this condition being statistically different from extractions with water (13.94%) and water/ethanol (12.69%). Morrison and Boyd [13] report that ethanol, although a solvent that solubilizes preferably polar substances, can also extract lipids, giving a higher yield to the extractions. As observed by the high SFE yield, the sample presents a large amount of nonpolar compounds and these were also extracted with the use of ethanol.

Asghar et al. [14] evaluated the extraction by two-week maceration of papaya seeds and obtained yield values for water extraction of 12.84 % and ethanol 12.36 %. Muhamad et al. [15] evaluated the extraction of the papaya seed by orbital shaker and obtained yield values for solvent hexane of 0.12 %, for water of 0.04 % and for ethanol 0.02 %. The conditions evaluated by the authors are different from those used in the study, but it is noteworthy that in both studies the yields increased with the polarity of the solvents.

For the integrated process (1st step SFE and 2nd step PLE) the highest yield was obtained for the test that used water as extraction solvent (18.55 %), a result that is statistically the same as that obtained for the water/ethanol solvent (17.13 %). Comparing IP results with single PLE extraction of papaya seeds in the same condition, we observe higher yield performance from the IP, because the SFE removed the lipids, favoring the greater interaction of the solvent with the more polarity compounds. The integration of the processes, SFE followed by PLE, allows to obtain two products with different characteristics and good yield.

Table 1. Extraction yield and total phenol content of papaya seed extracts obtained by different extraction techniques.

Extraction method	P (MPa)/T (°C) and/or solvent	SPI[(3)]	Extraction yield (%)	Total phenol content (mg GAE g^{-1})
SFE Papaya seeds	30/40[(1)]	-	$18.08^{b} \pm 0.32$	$2.11^{d} \pm 0.11$
PLE Papaya seeds	10/80/water	9.0	$13.94^{cd} \pm 0.96$	$38.48^{a} \pm 2.75$
	10/80/water/ethanol	7.2	$12.69^{d} \pm 1.24$	$38.33^{a} \pm 1.78$
	10/80/ethanol	5.2	$23.72^{a} \pm 1.12$	$12.48^{c} \pm 0.77$
IP Residue SFE	10/80/water[(2)]	9.0	$18.55^{b} \pm 0.09$	$40.81^{a} \pm 1.16$
	10/80/water/ethanol[(2)]	7.2	$17.13^{bc} \pm 1.06$	$38.63^{a} \pm 1.53$
	10/80/ethanol[(2)]	5.2	$4.94^{e} \pm 0.11$	$29.38^{b} \pm 0.86$

IP: [(1)]1st step SFE and [(2)] 2nd step PLE; Different superscript letters mean groups statistically different ($p < 0.05$) in each column. [(3)] SPI: solvent polarity index [16].

Analyzing the total phenolic content (TPC) from the extracts obtained by PLE, the highest values were reached using water and the water/ethanol mixture as solvents. Moreover, the lowest TPC result was observed for the extract obtained by SFE at 30MPa and 40°C (2.11 $mg_{EAG}.g^{-1}_{extract}$), this behavior is justified by the low affinity of the CO_2 with the phenolic compounds, mostly polar substances, and therefore with more affinity to polar solvents.

The TPC values reported for Muhamad et al. [15] for papaya extract obtained with water by low pressure extraction was 5.57 ± 0.10 $mgEAG.g^{-1}_{extract}$. The difference observed

between the literature data is related to the conditions of the raw material and to the preparation of the samples.
For the IP extraction method, the content of phenolic compounds ranged from 29.38 to 40.81 $mg_{EAG}.g^{-1}_{extract}$, with the highest value observed for the treatment using water as solvent. The SFE removed mostly low polar compounds, which allowed the PLE to a better interaction between the solvent and phenolic compounds. It is important to observe that the integrated process (SFE and subsequent extraction with PLE) favored the increase of TPC recovery. This demonstrates that the extraction with SFE besides removing the oily fraction present in the sample can be used as a pretreatment for the PLE step.
In general, SFE with CO_2 removes compounds with nonpolar characteristics and PLE using polar solvents (ethanol and water) recovers more polar compounds. Therefore, the sample treatment with SFE before PLE provides a gain in total yield and in TPC. Highlighting the importance of a sequential process for raw material fractionation.

3.2. Antioxidant activity

The antioxidant activity of papaya seed extracts was evaluated by β-carotene/linoleic acid system and by DPPH radical method, presented in terms of EC_{50} (effective concentration) which expresses the minimum concentration required to inhibit 50% free radicals. Consequently, lowest EC_{50} values indicate highest free radical scavenging activity. Table 2 shows antioxidant activity values obtained by the different extraction methods. The synthetic compound BHT was used as standard for comparison purpose.
The EC_{50} results ranged from 199.7 to >1000 $\mu g.mL^{-1}$. The best results were obtained by PLE and IP with the use of water/ethanol as solvent, with values of 201.01 and 199.7 $\mu g.mL^{-1}$, respectively. These EC_{50} values were close to the one verified for the standard antioxidant BHT (261 $\mu g.mL^{-1}$), demonstrating a strong antioxidant potential of these extracts. The water/ethanol mixture provided the extraction of compounds with high antioxidant activity compared to other solvents. According to Bobinaité et al. [17] the hydro-alcoholic mixtures are rather few selective and scanned a wide range of polarities regarding the compounds to be extracted.
The extracts obtained by SFE and PLE with ethanol showed EC_{50} values higher than 1000 $\mu g.mL^{-1}$. In this case, it can be assumed that under the analyzed conditions the extracts did not present detectable activity by DPPH radical method. An important factor to be observed for the antioxidant activity by the DPPH method is that the extracts obtained by IP presented EC_{50} values better than the extract obtained by SFE and PLE in separate processes. In this way, the combination of the processes favored the extraction of compounds with higher antioxidant activity by the DPPH radical method.
A similar behavior to the DPPH method was observed for the β-carotene/linoleic acid system method. Where the extracts obtained by IP presented higher values of AA when compared with the SFE and PLE techniques.
The IP method provided an increment in antioxidant potential of the extract, which can be observed when comparing the antioxidant activity from the extract obtained by PLE with ethanol with the extract obtained by IP with ethanol, 36.44 % and 92.30 %, respectively. This behavior indicates that the treatment of the raw material with the SFE (first step of the IP) provided good fractionation of the extract. In the 1st step of the IP, the SFE method removed mostly nonpolar compounds, with affinity to CO_2, and in the 2nd step (PLE) the compounds with higher polarity and with antioxidant activity were removed. It can once again highlight the efficiency of the combination of processes for the greater extraction of compounds with biological activities.

Barroso et al. [6] and Corrêa [18] found benzyl isothiocyanate as the main constituent of papaya seed. This compound is related to the biological activities found in this study. According to Clarke [19] benzyl isothiocyanate (BITC) is a bioactive compound derived from the hydrolyzate of myrosinase, present in papaya seeds.

Table 2. Antioxidant activity by DPPH and β-carotene bleaching for papaya seed extracts obtained by different extraction techniques.

Extraction method	P (MPa)/T (°C) and/or solvent	Sample	DPPH EC_{50} (μg mL^{-1})	β-carotene bleaching AA (%)
SFE	30/40[(1)]	Papaya seeds	$>1000^{e}$	$24.29^{e} \pm 2.32$
PLE	10/80/water	Papaya seeds	$768.61^{c} \pm 10.51$	$70.25^{bc} \pm 0.49$
	10/80/water/ethanol	Papaya seeds	$201.01^{a} \pm 7.40$	$71.29^{bc} \pm 1.18$
	10/80/ethanol	Papaya seeds	$>1000^{e}$	$36.44^{d} \pm 2.54$
IP	10/80/water[(2)]	Residue SFE	$1000^{d} \pm 48.10$	$74.22^{b} \pm 4.28$
	10/80/water/ethanol[(2)]	Residue SFE	$199.7^{a} \pm 3.19$	$62.51^{c} \pm 1.88$
	10/80/ethanol[(2)]	Residue SFE	$529.92^{b} \pm 3.29$	$92.30^{a} \pm 6.67$
	BHT		261 ± 12	

IP: [(1)] 1st step SFE and [(2)] 2nd step PLE; Different superscript letters mean groups statistically different ($p < 0.05$) in each column.

4 Conclusion

The integrated process (1st step SFE and 2nd step PLE) provided extracts with better extraction yield and biological activities when compared to the individually applied SFE and PLE techniques. Thus, the integration of processes can be an alternative to improve the performance of the SFE and PLE techniques, in addition to providing a better use of the raw material which are considered an agroindustrial residues without definite applicability. Highest phenolic content and antioxidant activity were achieved when papaya seeds was previously defatted by SFE (30 MPa/40 °C) and then submitted to PLE (10 MPa/70°C/water/ethanol), a combination mode.

Acknowledgements

The authors wish to thank CNPq (projects number 454025/2014) and Capes, Brazilian funding agencies, for the financial support and post-doctoral fellowships which sponsored this work.

5 References

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