Valorization of papaya (*Carica papaya* L.) agroindustrial waste through the recovery of phenolic antioxidants by supercritical fluid extraction

Henry I. Castro-Vargas^{1,2} · Wolfram Baumann³ · Sandra R. S. Ferreira⁴ · Fabián Parada-Alfonso¹

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Abstract In this work, supercritical fluid extraction (SFE) for the recovery of phenolic antioxidants from papaya agroindustrial waste (seeds) was explored, making use of neat supercritical CO₂ and CO₂ added with ethanol (CO₂-EtOH). A full factorial design played on in order to evaluate the effect of CO₂ extraction parameters (temperature between 40 and 60 °C, and pressure between 10 and 30 MPa) on yield and total phenols content (TPC), then ethanol was applied as a co-solvent and its effect on the recovery of phenolics was analyzed. The SFE was compared to the conventional extraction using ethanol. The antioxidant activity of all extracts was evaluated, and the phenolic composition in selected extracts was assessed by HPLC-ESI-MS. The highest extraction yields (21.02–26.46%) and TPC (15.34–34.23 mgGAE/g) were found in extracts obtained with CO₂-EtOH and ethanol. Good and selective phenolic recovery was obtained by using CO₂-EtOH, (44.81% of TPC recovered). The CO₂-EtOH extracts showed high radical scavenging activity and higher antioxidant effect against lipid oxidation. Some phenolic acids and flavonoids were observed in the extracts with better antioxidants properties. The results showed that

SFE is a suitable green technology for the phenolic recovery from papaya agroindustrial waste, and also an alternative for its valorization.

Keywords Agroindustrial by-products · Papaya seeds · Green extraction · Phenolic antioxidants · Food preservatives

Introduction

The industrial processing of agricultural food products produces high amounts of waste. On average, it is estimated that 30–50% of processed food is discarded as agroindustrial waste (Dávila et al. 2014; Lin et al. 2013). The industrial consumption of fruits produces large amounts of waste such as pomace, peels, seeds, and kernels. Worldwide, fruit processing generates more than 0.6 billion tons of waste/year, both the wine and beverage industries are the main waste producers (Lin et al. 2013). According to the Food and Agriculture Organization (FAO) of the United Nations, the global production of papaya was 13.1 million tons in 2016. India and Brazil were the major producers with 5.7 and 1.4 million tons, respectively, whereas Colombia produced about 188,305 tons (FAO 2016). Industrial papaya consumption is mainly for the production of papain and the manufacturing of food products. These uses dispose approximately 25%w/w of processed fruit as waste, particularly seeds (Desai and Wagh 1995). Nowadays, the global production of agroindustrial papaya waste is estimated around three million tons/year, 70% are seeds (FAO 2016).

In the last two decades, the consumers' interest in healthy food has increased, meaning that a modern trend to supplement foods with polyphenols, carotenoids, or

✉ Henry I. Castro-Vargas
hicastrov@unal.edu.co

¹ Chemistry Department, Universidad Nacional de Colombia, Carrera 30 No 45-03, Bogotá D.C., Colombia

² Faculty of Engineering, Universidad Libre, Carrera 70 No 53-40, Bogotá D.C., Colombia

³ Chemistry Department, Universidad de Los Andes, Carrera 1 No 18A-12, Bogotá D.C., Colombia

⁴ Chemical and Food Engineering Department, Universidade Federal de Santa Catarina, Florianópolis, SC88.040-900, Brazil

tocopherols obtained from natural sources can be seen. Agroindustrial waste from processing fruits is well known as a source of high-value compounds with wide variety of bioactive properties and promising uses. Seeds and kernels are rich in mono or polyunsaturated fatty acids, whereas pomace, peels, and some seeds are rich in antioxidants (Kao and Chen 2013). Polyphenols, carotenoids or tocopherols from apple and grape pomace (Herrero et al. 2015), cocoa bean hulls (Mazzutti et al. 2018; Valadez-Carmona et al. 2018), mango and banana peels (Martins and Ferreira 2017), and berries waste (Lorenzo et al. 2018) have been studied as preservatives and nutraceutical ingredients in food products.

Papaya agroindustrial waste, particularly papaya seeds, have been reported as a source of antioxidant compounds. A good content of phenolic compounds has been reported in papaya seed extracts and correlated with their antioxidant properties: previous studies reported the free radical scavenging activity of various fractions obtained from papaya seeds; methanol and ethyl acetate fractions showed the better antioxidant properties against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) (Gogna et al. 2015; Faisal et al. 2016; Khor and Wong 2016; Salla et al. 2016). In vitro studies have shown that both methanol and hexane fractions from papaya seeds have high protective effect against induced oxidative stress in cells (Gogna et al. 2015; Salla et al. 2016). Elseways, polyphenols obtained from papaya seeds were added to mackerel (*Rastrelliger kana-gurta*) as preservatives, and as a result, these compounds reduced the lipid and protein oxidation in fish during its storage on ice (Faisal et al. 2016). Different fractions (polar and non-polar) obtained from papaya seeds showed high antioxidant properties against lipid oxidation in vegetable edible oils (Castro-Vargas et al. 2016).

Nowadays, the recovery of antioxidants from agroindustrial waste is an attractive option for its exploitation and valorization, for this purpose, different extraction methods are available (Oroian and Escriche 2015). The supercritical fluid extraction (SFE) is considered as an appropriate technique for antioxidants recovery from agroindustrial waste (Herrero et al. 2015; Oroian and Escriche 2015). This method is characterized by to use green solvents as carbon dioxide (CO₂), low thermal degradation of antioxidants compounds, obtaining free solvent extracts, and variable selectivity on the target compounds recovery. Neat CO₂, and CO₂ added with ethanol as co-solvent (CO₂-EtOH) have been used for the recovery of antioxidants from residues generated by the industrial processing of fruits (Herrero et al. 2015; Oroian and Escriche 2015; Banerjee et al. 2017).

The previous background showed that papaya seeds are a promising source of phenolic antioxidants, and that SFE can be an appropriate method for its recovery. The aim of

the present study were to explore the uses of SFE for the recovery of phenolic antioxidants from papaya seeds and to evaluate their antioxidant properties. Neat CO₂ and CO₂-EtOH were used as solvents, and their efficiency for the recovery of phenolic compounds was compared with a conventional extraction method. A three-level full factorial design, together with a response surface analysis (RSA) were used in order to study the effects of the extraction parameters (temperature and pressure) on the extraction yield and total phenol contents (TPC). This work proposes an alternative for the exploitation and valorization of papaya agroindustrial waste as source of antioxidants that could find usefulness in the pharmaceutical and food industries.

Materials and methods

Papaya seed samples

The papaya seeds were supplied by Alimentos SAS S.A. (Bogota Colombia), which were obtained from the agroindustrial processing of papaya. 50 kg of papaya seeds were procured, dried and powdered. The sample with particle size between 0.300 and 0.850 mm (+ 50/– 18 US Standard size sieves) was used in SFE and conventional extraction method. The mean particle diameter was 0.496 ± 0.03 mm, calculation that was made based on the mean size distribution.

Supercritical fluid extraction

Supercritical fluid extraction experiments were performed using neat CO₂ and CO₂-EtOH in the dynamic extraction unit previously described (Zetzel et al. 2003). For extraction, a sample of papaya seeds 10.0 g used, and glass beads were placed inside the extraction column as a fixed bed of particles, and the extraction parameters (temperature, pressure, co-solvent percentage, and solvent flow rate) were adjusted. The extraction time was set up by kinetic assays performed at 50 °C, 20 MPa, and constant CO₂ flow rate of 0.50 ± 0.05 kg/h. All extractions were performed for 180 min and the CO₂ flow above mentioned. The mixture solvent/extract (e.g., CO₂/extract or CO₂-EtOH/extract) was separated to ambient pressure (101.3 kPa) and the extracts were collected in amber flasks. For the CO₂-EtOH extracts, ethanol was removed under vacuum. Finally, the extracts were weighted in an analytical balance (SHIMADZU Model AY220, São Paulo-SP, Brazil), then flushed with a nitrogen stream, sealed, and stored at – 20 °C. The extractions with neat CO₂ were performed using three different levels of temperature and pressure according to the experimental design shown in Table 1

Table 1 Extraction yield, total phenols content, total phenols recoveries and DPPH radical scavenging of the extracts obtained from papaya seeds

Extract	Solvent	Coded variables ^a		Temperature (°C)	Pressure (MPa)	Extraction yield (% w/w d.b.)	TPC ^b (mgGAE/g extract)	TPC recovered ^b (%)	DPPH radical scavenging ^b (IC ₅₀ mg/mL)
		X_1	X_2						
1	CO ₂	− 1.0	− 1.0	40	10	4.59	1.40 ± 0.08 ⁱ	4.09 ± 0.29 ^h	80.60 ± 1.61 ^a
2	CO ₂	− 1.0	0.0	40	20	19.40	1.65 ± 0.07 ^h	4.82 ± 0.25 ^g	79.24 ± 2.84 ^a
3	CO ₂	− 1.0	1.0	40	30	18.80	2.08 ± 0.09 ^g	6.08 ± 0.33 ^f	73.70 ± 1.09 ^b
4	CO ₂	0.0	− 1.0	50	10	1.60	1.56 ± 0.10 ⁱ	4.56 ± 0.36 ^{g,h}	67.50 ± 2.33 ^c
5.1 ^c	CO ₂	0.0	0.0	50	20	20.75	3.78 ± 0.14 ^e	11.04 ± 0.51 ^d	20.95 ± 1.60 ^e
5.2	CO ₂	0.0	0.0	50	20	20.42			
5.3	CO ₂	0.0	0.0	50	20	20.60			
5.4	CO ₂	0.0	0.0	50	20	20.68			
5.5	CO ₂	0.0	0.0	50	20	20.49			
6	CO ₂	0.0	1.0	50	30	19.55	3.20 ± 0.15 ^f	9.35 ± 0.55 ^c	27.77 ± 1.10 ^d
7	CO ₂	1.0	− 1.0	60	10	0.50	0.92 ± 0.04 ^j	2.69 ± 0.11 ⁱ	81.83 ± 0.48 ^a
8	CO ₂	1.0	0.0	60	20	19.63	3.13 ± 0.11 ^f	9.14 ± 0.40 ^e	25.13 ± 0.64 ^d
9	CO ₂	1.0	1.0	60	30	20.35	3.35 ± 0.09 ^f	9.79 ± 0.33 ^e	26.05 ± 0.19 ^d
10	CO ₂ - EtOH 2% _{w/w}	−	−	50	20	21.02 ± 0.05 ^d	7.79 ± 0.38 ^d	22.76 ± 1.38 ^c	14.35 ± 0.45 ^f
11	CO ₂ - EtOH 5% _{w/w}	−	−	50	20	23.75 ± 0.04 ^c	10.42 ± 0.69 ^c	30.44 ± 2.51 ^b	7.02 ± 0.13 ^g
12	CO ₂ - EtOH 8% _{w/w}	−	−	50	20	24.52 ± 0.06 ^b	15.34 ± 0.86 ^b	44.81 ± 3.13 ^a	5.95 ± 0.32 ^h
13	Ethanol	−	−	−	−	26.46 ± 2.66 ^a	34.23 ± 2.75 ^a	−	2.29 ± 0.09 ⁱ

^aTemperature (X_1); Pressure (X_2)^bTPC, TPC recovered and DPPH radical scavenging columns shows the mean ± standard deviation (n = 3)^cCentral point was replicate five times (extract 5.1–5.5). TPC, TPC recovered and DPPH radical scavenging values for central point correspond to average (n = 5)

Means in columns followed by the same letter are not statistical different

(extract 1 to 9), and explained in further section. On the other hand, the extractions with CO₂-EtOH were performed at 50 °C, 20 MPa, and three levels of co-solvent percentage (2, 5 and 8%w/w), temperature and pressure were established according to extraction yield and total phenol content results observed for the neat CO₂ experiments. All extractions were performed by triplicate; the extraction yields were expressed as weight-to-weight percentage on dry basis (% w/w d.b.).

Experimental design

For the SFE with neat CO₂, three-level full factorial design coupled to response surface analysis (RSA) were used to evaluate the effect of temperature (X_1) and pressure (X_2) on the response variables on extraction yield (Y_1) and TPC (Y_2). The temperature and pressure levels and their coded

variables are shown in Table 1 (extracts 1–9). The experimental design consisted of 9 experimental runs, and was performed in randomized in order to minimize the effects of unexpected variability on the responses. Central point was replicated five times (extract number 5.1–5.5). The second order models were obtained [according to Eq. (1)] to aim at explaining the Y_n response variables as a function of the extraction parameters:

$$Y_n = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (1)$$

where β_0 is the intercept, β_1 and β_2 are linear regression coefficients, β_{11} and β_{22} are quadratic regression coefficients, and β_{12} is the interaction coefficient. The response surface plots were generated to show the effect of the extraction parameters on the response variables. The fit of the model was evaluated by analysis of variance (ANOVA).

Conventional extraction

The conventional extraction with ethanol by Soxhlet method was developed as an exhaustive extraction process of phenolic antioxidants. The extractions were performed under reduced pressure in order to avoid the thermal degradation of target compounds. The pressure of the extraction system was adjusted to reduce the boiling point of the ethanol at 35 °C. The papaya seed samples (10.0 g) were extracted for 8 h. After the extraction, the ethanol was removed under vacuum. The extractions were performed in triplicate and the extraction yield was expressed as % w/w d.b.

Total phenols content

The Total Phenols Content (TPC) in all extracts was determined by the Folin-Ciocalteu method with the slight modifications proposed by Castro-Vargas et al. (2010). The dry extracts were resuspended in a mixture of ethyl acetate:ethanol (1:1) at final concentration range from 10 to 20 mg/mL. The 100 µL of each extract solution was mixed with 750 µL of the Folin-Ciocalteu reagent (10%w/w); after 5 min, 750 µL of sodium carbonate solution (6%w/w) were added. Finally, the mixture was stirred and left to react in the dark and room temperature for 90 min. Subsequently, the absorbance at 765 nm (Thermo Scientific Evolution 600 UV/Vis) was measured. The TPC was expressed as mg of gallic acid equivalents per g of dry extract (mgGAE/g extract), calculated using a standard curve of gallic acid (concentrations between 0 and 100 µg/mL). All measurements were carried out in triplicate.

Antioxidant activity

The antioxidant properties of papaya seed extracts were evaluated using two methods: the *in vitro* free radical-scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the antioxidant activity against the lipid oxidation in vegetable edible oil (VEO).

DPPH scavenging activity

The DPPH[•] scavenging activity was determined by following the procedure that has been previously described by Castro-Vargas et al. (2010), where 1 mL of DPPH[•] solution 0.1 M in ethanol, with initial absorbance measured at 517 nm (A_0), was added to 50 µL of each extract (dissolved in a mixture of ethyl acetate:ethanol 1:1), and the final absorbance at 517 nm (A_f) was measured after 60 min. The scavenging percentage was determined as % = $(A_0 - A_f)/A_0$. The extract concentration (mg/mL) necessary to decrease the initial DPPH[•] concentration by

50% (IC_{50}) was determined by interpolation in the standard curve. Gallic acid was used as reference phenolic antioxidant in order to compare its DPPH[•] scavenging activity versus the papaya seed extracts. All measurements were performed by triplicate.

Antioxidant activity in vegetable edible oil

The antioxidant properties of papaya seed extracts against the lipid oxidation in vegetable edible oil (VEO) were evaluated and compared with the synthetic antioxidant tert-butylhydroquinone (TBHQ). The VEO sample (without antioxidants) was a mixture of different kinds of edible oil, whose general composition was 70% of unsaturated fatty acids triglycerides (40% oleic, 55% linoleic, and 5% of others), and 30% of stearic acid triglycerides. Previously, we studied the VEO oxidation process and its variables, and an experimental procedure for antioxidant activity (AA) measures was establishing (Castro-Vargas et al. 2016). The AA experiments were performed following two steps: (1) accelerated oxidation of VEO samples added with ferrous ion as pro-oxidant, and each extract (dry) or TBHQ (liquid) as antioxidants at a final concentration of 300 mg/kg. One VEO sample without antioxidants was used as control. All VEO samples were kept in oxidation for 15 days at 60 °C. (2) The antioxidant properties of extracts and TBHQ were tested by measuring some lipid oxidation products; lipid hydroperoxides (LHP), thiobarbituric acid reactive substances (TBARS), and hexanal. The LHPs were measured by conjugated dienes method; the TBARS were analyzed using their absorbance at 532 nm, whereas the hexanal was measured by headspace-solid phase microextraction-gas chromatography. The oxidation products were quantified after 15 days of oxidation and the results were expressed as the percentage of inhibition of lipid oxidation products formation according to Eq. (2):

$$\text{Inhibition (\%)} = \left[\frac{[\text{LOP}]_C - [\text{LOP}]_A}{[\text{LOP}]_C} \right] \times 100 \quad (2)$$

where $[\text{LOP}]_C$ and $[\text{LOP}]_A$ are the lipid oxidation products concentration in control and added with antioxidants samples, respectively.

Analysis of phenolic compounds

The extracts with the best qualities (extraction yield, TPC, and AA) were selected and analyzed by HPLC-ESI-MS in order to identify some phenolic compounds. Analyses were performed using an Agilent Technologies 1260 chromatograph (Palo Alto, CA, USA) equipped with a binary pump (G1312B), a degasser, an autosampler, and a thermostatted column compartment. Each extract was

dissolved in acetonitrile at 20 mg/mL and the injected sample was 1 μ L. Chromatographic separation was performed on a Bischoff ProntoSIL 300-5-C-18 column (150 mm $\times$ 4 mm $\times$ 5 μ m) kept at 30 °C. The mobile phase was composed of 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.3 mL/min. The gradient program was as follows: 0% B (0 min), 10% B (20 min), 50% B (28 min), 70% B (30 min), 90% B (50 min), 0% B (55 min), finally the initial conditions were maintained for 10 min. The choice of the mobile phase gradient was based on not reported experiments. The chromatographic system was coupled to a Quadrupole-Time-of-Flight MS detector (Agilent Technologies 6520 Q-TOF), equipped with an electrospray ionization (ESI) source operating at the negative ionization mode. The MS and MS/MS analysis settings were as follows: ESI capillary voltage, 3.5 kV; nitrogen as drying and nebulizing gas at flow rate 5 L/min, temperature 300 °C, and pressure 30 psi; collimator voltage 175 V; and octopole voltage 750 V. The spectra were acquired over a mass to charge ratio (m/z) ranging from 50 to 1000. For the MS/MS analysis, the selected ions were fragmented using nitrogen as collision gas at 350 °C and 20 eV. All results were analyzed using the MassHunter Workstation Agilent Technologies software. The phenolic compounds were identified using their mass spectra information, the molecular formula, and bibliographic data.

Statistical analysis

The statistical differences of extraction yield, TPC, LHP, hexanal, and TBARS were determined by means of ANOVA. All the statistical analyses were developed using the software Statgraphics Centurion XVII with a confidence level of 95%.

Results and discussion

Recovery of phenolic antioxidants from papaya seeds

In this work, the effects of pressure and temperature on the recovery of phenolic compounds with neat CO₂ were studied by RSA. The suitable extraction pressure and temperature were selected; after that, ethanol was used as a co-solvent, and the effects on the recovery of target compounds was evaluated. Table 1 presents the extraction yields obtained from papaya seeds using SFE with CO₂ and CO₂-EtOH, and exhaustive extraction with ethanol. The yields show the efficiency of the extraction methods and solvents for the recovery of extractable solutes. From Table 1, it can be seen that higher yield was obtained when using ethanol (26.46%), followed by the extracts number

11 and 12 obtained with CO₂-EtOH (23.75 and 22.52%, respectively), and the extract number 5 (20.59%) obtained with neat CO₂. The high extraction yield observed in the ethanolic extract suggests that the extraction process was exhaustive. Khor and Wong (2016) previously reported on phenolics extraction from papaya seeds using exhaustive maceration with ethanol, obtaining lower extraction yield (5.2%). On the other hand, the SFE with neat CO₂ at 20 and 30 MPa allowed to obtain low polarity extracts with yields between 18.80 and 20.59%. Previously, reports showed that papaya seeds are a source of low polarity extracts; da Silva and Jorge (2017) reported oil content in papaya seeds above 20%, while Li et al. (2015) recovered oil with yields between 22 and 26% by ultrasound assisted extraction. In these studies, a high content of polyunsaturated fatty acids in oils (particularly linoleic acid) were observed, however, phenolic compounds were not reported. In disagreement with the above, Kothari and Seshadri (2010) observed high content of phenolic compounds (mostly flavonoids) in papaya oil obtained using hexane. This background suggests that papaya seed extracts obtained by SFE with neat CO₂ may contain polyunsaturated fatty acids and some phenolic compounds, which is in agreement with the TPC results observed in the present work and explained in further text.

Table 1 shows the TPC observed in papaya seed extracts, these results are present as mg of gallic acid equivalents per g of extract (mgGAE/g extract). In addition, the efficiency of the SFE to phenolic recovery is presented as recovered %TPC. These values were obtained by comparison between the TPC values for supercritical extracts and the TPC value for ethanolic extract. The highest TPC was observed in the ethanolic extract (34.23 ± 2.75 mgGAE/g extract), followed by CO₂-EtOH extracts (from 7.79 ± 0.38 to 15.34 ± 0.86 mgGAE/g extract), and neat CO₂ extract number 5 (3.78 ± 0.14 mgGAE/g extract). The high TPC observed in ethanolic extract is due to the ethanol polarity, which allows extracting medium to high polarity compounds such as polyphenols. Additionally, conventional extraction was performed in this work under low temperature in order to avoid the thermal degradation of the target compound. Phenolic extraction from papaya seeds with polar solvents by conventional extraction methods has been previously reported: the aqueous extracts showed TPC 0.98 mgGAE/mg extract (Obob et al. 2013), methanolic extracts presented 118 mgGAE/g extract (Khor and Wong 2016), and ethanolic extracts 307 mgGAE/g extract (Kothari and Seshadri 2010). The TPC observed in the ethanolic extract obtained in the present work was lower than the one reported by Kothari and Seshadri (2010); difference that can be caused by the variations in the concentration of phenolic compounds in papaya seeds. Several reports have

shown that the concentration and distribution of secondary metabolites in fruits (e.g., polyphenols) can be affected by parameters associated with their crop, post-harvest handling, and industrial processing (Patthamakanokporn et al. 2008).

The TPC recoveries between 2.69 and 11.04% were obtained by using neat CO₂. The lower recovery was obtained at 60 °C and 10 MPa, and the higher recovery was observed at 50 °C and 20 MPa. The low recoveries observed are mainly due to the non-polar character of CO₂, although some simple phenolic compounds and phenolic acids may be extracted using neat CO₂, its solubility is rarely high (Bitencourt et al. 2016, 2018). Despite the low TPC recoveries observed, these were higher compared with the development of similar studies with agroindustrial wastes, such as passion fruit by-products (%TPC recovered between 4.9 and 10%) (Oliveira et al. 2016) and cocoa bean hulls (%TPC recovered between 3.5 and 7.8%) (Mazzutti et al. 2018).

In the SFE with neat CO₂, RSA was used to evaluate the effect of temperature (X_1) and pressure (X_2) on response variables such as extraction yield (Y_1) and TPC (Y_2). Table 2 presents the ANOVA results used to evaluate the fit of the second order model and the effect of extraction parameters. For the experimental region explored, the experimental data was fitted to the second order model. The Eq. (3) presents the resulting model equation for extraction yield with R^2 (99.61%), adj- R^2 (99.27%), standard error (0.691), and lack of fit ($p = 0.873$). The R^2 and the lack of fit indicate that the regression model explains the process behavior in a satisfactory manner. On the other

hand, the parameters with the highest effect on the extraction yield were the linear and quadratic coefficients of pressure (both with $p < 0.0001$), followed by the interaction between temperature and pressure ($p = 0.0058$), and the linear coefficient of temperature ($p = 0.0249$). Figure 1 shows the response surface graph of the second order model for extraction yield, this plot permitted to visualize the effect of the extraction parameters on the response variable. The response surface graph shows that the extraction yield improved with the increase of the extraction pressure between 10 and 20 MPa; for example, at 60 °C the yield increased 41 times (from 0.50 to 20.50%) when the pressure was changed from 10 to 20 MPa. This behavior is mainly due to the increment of the supercritical CO₂ density and therefore its solvent power. High pressure favors the solutes extraction from the vegetable matrix, therefore the global yield can be enhanced. On the other hand, the negative effect of temperature on the extraction yield was observed at low and middle pressure level; for example, at 10 MPa the increase of temperature between 40 and 60 °C reduced the global yield from 4.59 to 0.50%. This result could be explained by the decrease of CO₂ density with increased temperature.

$$Y_1 = 20.363 - 0.360X_1 + 8.693X_2 + 1.447X_1X_2 - 0.3X_1^2 - 9.240X_2^2 \quad (3)$$

For the phenolic extraction using neat CO₂, TPC data was fitted to the second order model and Eq. (4) was obtained. The R^2 and the lack of fit values showed that the regression model satisfactorily explains the effect of pressure and temperature on phenolic compound extraction (Table 2). The RSA indicates that the extraction parameter with the higher effect was the linear coefficient of pressure, followed by the quadratic coefficients of pressure and temperature. The response surface graph of the second order model for TPC is presented in Fig. 2. The positive effect of pressure was observed at all temperatures, the pressure increased improving the phenolic compounds extraction from papaya seeds, this effect was more notorious when the pressure was enhanced from 10 to 20 MPa; above 20 MPa, the change in TPC is little and the surface showed a plateau zone. The pressure effect that was observed may be due to the little enhance in the dielectric constant of CO₂ generated with the increase of pressure, however, highest pressures are necessary to obtain more appreciable changes (Mukhopadhyay 2000). Noteworthy, Pérez et al. (2015) observed a similar effect in the extraction of grape seed oil using neat supercritical CO₂; at a constant temperature, the increase of extraction pressure from 10 to 30 MPa enhanced the TPC in the extracts from 3 to 16 mgGAE/g.

Table 2 Analysis of variance of regression coefficients and statistical indicators of second order model for extraction yield (Y_1) and total phenols content (Y_2)

	Y_1		Y_2	
	Coefficient	p value	Coefficient	p value
X_1^a	− 0.360	0.0249*	0.378	0.0636
X_2^a	8.693	< 0.0001**	0.791	0.0032*
$X_1 X_2$	1.447	0.0058**	0.437	0.0757
X_1^2	− 0.3	0.5052	− 0.916	0.0105*
X_2^2	− 9.240	< 0.0001**	− 0.926	0.0100*
Lack of fit	$(p = 0.873)$		$(p = 0.778)$	
R^2	99.61%		92.41%	
Adj- R^2	99.27%		86.09%	
Standard error ^b	0.691		0.408	

^a Temperature (X_1); pressure (X_2)

^b Standard error relative to Y_1 and Y_2 values

**highly significant ($p < 0.01$)

*significant ($0.01 < p < 0.05$)

Fig. 1 Response surface plot for the effect of SFE parameters on extraction yield from papaya seeds. Temperature and pressure coded levels (− 1, 0, + 1) are described in the Table 1

Fig. 2 Response surface plot for the effect of SFE parameters on total phenols content in papaya seeds extracts. Temperature and pressure coded levels (− 1, 0, + 1) are described in the Table 1

$$Y_2 = 3.6221 + 0.378X_1 + 0.791X_2 + 0.437X_1X_2 - 0.916X_1^2 - 0.926X_2^2 \quad (4)$$

Table 1 shows that by the SFE the higher extraction yield and TPC recovered values were obtained with neat CO₂ at 50 °C and 20 MPa. Moreover, Figs 1 and 2 showed their maximum response values at pressures above 20 MPa, and temperatures between 50 and 60 °C. Considering these results, 50 °C and 20 MPa were the selected extraction conditions for the SFE experiments with CO₂ added with ethanol as co-solvent. It is worth mentioning that these conditions correspond to middle temperature and pressure levels, which from the industrial-scale view can be suitable to reduce both energetic and technical

requirements. Ethanol was employed as co-solvent in order to improve the phenolic compounds extraction from papaya seeds. Furthermore, ethanol is a GRAS solvent, which allows its safe usage in food, and pharmaceutical and cosmetic products. Three levels of ethanol were used (2, 5, and 8%), and its effect on extraction yields and TPC recoveries was observed (Table 1). The addition of 5 and 8% of co-solvent improved the extraction yields compared to the neat CO₂; an increase from 20.59% (extract number 5) to 23.75 and 24.52%, (extracts number 11 and 12, respectively), was observed. As expected, the TPC recoveries were also enhanced; the ethanol addition to CO₂ augmented the TPC recoveries between 2 and 4 times compared to the neat CO₂. These results could be explained

considering that ethanol can increase the polarity of the supercritical solvent, improving the solubilization and extraction of medium polarity solutes (Manna et al. 2015). Additionally, the solute-matrix bindings could be broken due to the fact that the hydroxyl group from ethanol forms a hydrogen bond with the matrix, so the solutes that are released from matrix increase. The improvement of the extraction yields and TPC recoveries on function of the ethanol percentage increase is due to the increase of CO₂ solvation power, however, this behavior is affected by the behavior of the mixture phase under the extraction conditions (Valadez-Carmona et al. 2018).

In this study, 44.81% of phenolic compounds were recovered using CO₂-EtOH at 50 °C, 20 MPa and 8% of co-solvent (extract number 12). Compared with other kinds of waste generated in the industrial processing of fruits, which have been explored as sources of phenolic compounds using SFE with CO₂-EtOH, extract number 12 showed a TPC (15.34 mgGAE/g extract) that is higher than in the extracts obtained from grape skins (3 mgGAE/g, at 40 °C, 20 MPa, and 5% EtOH) (Manna et al. 2015), blackberry bagasse (12.73 mgGAE/g at 60 °C, 15 MPa, and 10% EtOH) (Pasquel et al. 2014), cacao pod husk (13.63 mgGAE/g at 50 °C, 30 MPa, and 15% EtOH) (Valadez-Carmona et al. 2018), and passion fruit seeds cake (15 mgGAE/g at 40 °C, 30 MPa, and 5% EtOH) (Oliveira et al. 2016). It is worth mentioning that grape and blackberry waste are recognized as good sources of phenolic compounds.

Antioxidant activity

DPPH scavenging activity

Table 1 presents the results of the DPPH[•] scavenging expressed as IC₅₀; the low IC₅₀ values mean high AA against DPPH[•]. The higher DPPH[•] scavenging activity was exhibited by ethanolic extract (IC₅₀ = 2.29 mg/mL), followed by the extracts obtained by using SFE with CO₂-EtOH (IC₅₀ between 5.95 and 14.35 mg/mL). Gallic acid was used as reference phenolic antioxidant in order to compare its DPPH[•] scavenging activity versus the papaya seed extracts, this compound showed IC₅₀ = 6.09 ± 0.25 mg/mL. Extracts number 12 and 13 showed higher AA compared to the gallic acid, furthermore, the ANOVA results indicate that extract number 11 and gallic acid have similar AA. Table 1 shows that the addition of ethanol as a co-solvent in SFE improved the DPPH[•] scavenging activity of papaya seed extracts; this result is due to the increase of phenolic compound concentration in extracts. A Pearson correlation analysis between TPC and DPPH[•] scavenging activity indicates a high positive correlation ($R^2 = 0.9785$), thus, extracts with

high TPC have large DPPH[•] scavenging activity. The results observed suggest that papaya seeds are a source of phenolic antioxidants with free radical-scavenging activity. Previous reports showed that papaya seed extracts have high DPPH[•] scavenging activity. Khor and Wong (2016) reported IC₅₀ = 0.078 mg/mL for methanol extract, whereas Zunjar et al. (2015) observed IC₅₀ = 3.11 mg/mL for aqueous extract.

Antioxidant activity in vegetable edible oil

Papaya seed extracts and TBHQ as reference antioxidant compound were added to VEO, and their antioxidant properties against lipid oxidation were evaluated. The antioxidant effectiveness was evaluated as %inhibition of HPL, TBARS, and hexanal formation [calculated according Eq. (2)]. Figure 3 shows the AA results, all extracts and TBHQ showed antioxidant effect against VEO oxidation (although the extracts number 1 and 4 did not show inhibitory effect on the hexanal formation). In general, it can be observed that the LHP formation was inhibited between 67.9% and 93.0% (extracts number 13 and 11, respectively), while the production of TBARS was delayed between 41.5% and 90.9% (extracts number 1 and 11, respectively), and the hexanal formation was reduced between 1.6% and 58.7% (extracts number 7 and 11, respectively). The higher AA was observed for the extract number 11, obtained using CO₂-EtOH at 50 °C, 20 MPa, and 5% of co-solvent. Other extracts with high AA were number 10 and 12, obtained with CO₂-EtOH, and number 5 and 9, recovered with neat CO₂. Table 1 shows that the mentioned extracts also presented high TPC values (particularly those obtained by using CO₂-EtOH), these results suggest that the phenolic compounds in extracts are related with their antioxidant properties. The Pearson correlation analysis between TPC and %inhibition of HPL, TBARS, and hexanal indicate a good positive correlation between TPC and TBARS inhibition ($R^2 = 0.9201$), and TPC and hexanal inhibition ($R^2 = 0.9314$), however, a poor correlation between TPC and LHP inhibition was observed ($R^2 = 0.5119$).

The SFE with neat CO₂ allowed to obtain extracts with good AA in VEO (e.g., extracts number 5, 6, 8, and 9), moreover, the addition of ethanol as a co-solvent improved the antioxidant properties of extracts. Figure 3 shows that the extracts number 10, 11, and 12 (obtained with CO₂-EtOH) presented high effectiveness to inhibit the TBARS and hexanal formation compared to extract number 5 (obtained using neat CO₂). These results are due to the enhancement in the concentration of phenolic compounds in papaya seed extracts generated by the use of ethanol as a co-solvent (discussed in previous section).

Fig. 3 Antioxidant activity in vegetable edible oil of papaya seeds extracts and TBHQ presented as inhibition percentage of lipid hydroperoxides (LHP), thiobarbituric acid reactive substances

(TBARS) and hexanal formation. For each lipid oxidation product, different letter means statistical differences

Lipid oxidation in foods is a complex process, which can be developed by different ways and reaction mechanisms. The oxidation of polyunsaturated fatty acids can generate a wide range of products: hydroperoxides, aldehydes, ketones, esters, ethers, short-chain acids, alcohols, lactones, furans, and aliphatic and aromatic hydrocarbons (Kazutoshi and Takayuki 2004). Hydroperoxides are the initial lipid oxidation products, while some TBARS and the hexanal are considered as intermediate and final products (Guillén-Sans and Guzmán-Chozas 1998; Kazutoshi and Takayuki 2004). A suitable antioxidant for food products should reduce/inhibit the formation of oxidation products in its initial, intermediate, and final stages in order to avoid/minimize food spoilage and its hazardous effects on consumers. We have observed that papaya seed extracts obtained by SFE, particularly CO₂-EtOH extracts, inhibits the VEO oxidation in its different stages; extract number 11 showed high efficacy to reduce the initial LHP, and secondary-final TBARS and hexanal. Additionally, Fig. 3 shows that various papaya seed extracts (number 5, 6, 8, 9, 10, 11, and 12) exhibited higher AA compared to the antioxidant reference TBHQ. Noteworthy, TBHQ is widely used by the edible oil industries in Colombia and other American countries due to its high antioxidant effectiveness and low price (between USD\$20 and USD\$50 per kg). However, this compound has been very questioned respect to its thermal instability and possible carcinogenic effects

(Eskandani et al. 2014). Considering the facts mentioned above, papaya seeds could be considered a promising source of phenolic antioxidants (via SFE) with possible use in edible oils as preservatives, in addition, these extracts may be explored to replace TBHQ in food products.

Analysis of phenolic compounds

The results presented in the previous sections showed that papaya seeds could represent a valuable source of phenolic extracts, which have good free radical scavenging activity and high protective effect against lipid oxidation in edible oils. Two extracts were selected and analyzed by HPLC-ESI-MS/MS; the ethanolic extract was selected due to its high values of extraction yield, TPC, and DPPH[•] scavenging activity; on the other hand, CO₂-EtOH extract number 11 was selected considering its good yield, TPC values, DPPH[•] scavenging activity, and high AA in VEO. Table 3 shows the chromatographic and mass spectra information of phenolic compounds tentatively identified in selected papaya seed extracts. Five phenolic acids, three flavonoids, and two flavonoid glycosides were identified in the ethanolic extract, whereas five phenolic acids were observed in CO₂-EtOH extract. The phenolic compounds detected in the present work have been previously observed in different parts of papaya fruit; kaempferol-3-O-glycoside, caffeic acid, ferulic acid, p-hydroxybenzoic acid,

Table 3 Tentatively identified phenolic compounds in papaya seeds extracts by HPLC-ESI-MS/MS

Rt (min)	Molecular formula	[M-H] [−] (<i>m/z</i>)	MS ² (<i>m/z</i>)	Tentative identification
10.5	C ₁₆ H ₁₈ O ₉	353	191	Chlorogenic acid ^{a,b}
12.9	C ₉ H ₈ O ₄	179	135	Caffeic acid ^{a,b}
17.6	C ₁₀ H ₁₀ O ₄	193	134, 117	Ferulic acid ^{a,b}
20.7	C ₇ H ₆ O ₃	137	93	p-Hydroxybenzoic acid ^{a,b}
25.3	C ₉ H ₈ O ₃	163	119, 153	p-Coumaric acid ^{a,b}
33.5	C ₁₅ H ₁₀ O ₈	317	137	Myricetin ^a
36.8	C ₁₅ H ₁₀ O ₇	301	271, 151	Quercetin ^a
43.5	C ₁₅ H ₁₀ O ₆	285	175, 153	Kaempferol ^a
48.1	C ₂₁ H ₂₀ O ₁₁	447	285	Kaempferol-3-O-glycoside ^a
49.7	C ₂₁ H ₂₀ O ₁₂	463	301	Quercetin 3-O-glycoside ^a

^a Compounds identified in ethanolic extract^b Compounds identified in CO₂-EtOH extract

and p-coumaric acid were identified in the seeds (Kadiri et al. 2017); caffeic acid, ferulic acid, and p-coumaric acid were observed in the peel (Nieto Calvache et al. 2016); while all the compounds cited in Table 3 were detected in the pulp (Zunjar et al. 2015). Kadiri et al. (2017) study reported concentrations of these substances between 0.07 and 0.38 mg/g dry extract, ferulic acid was observed as the most abundant compound with concentrations between 0.26 and 0.38 mg/g. The phenolic compounds observed in papaya seed extracts possess notable antioxidant properties. Some compounds as ferulic acid and quercetin have been approved in certain countries as food additives to prevent lipid oxidation and as color ingredients (Srinivasan et al. 2007).

Ethanol by conventional extraction method allowed to recover more polarity phenolic compounds compared to the SFE with CO₂-EtOH, for example, some flavonoids and their glycosides were only observed in the ethanolic extract. As expected, the use of CO₂-EtOH as solvent extracted medium polarity compounds such as simple phenolic acids. Previous studies showed that ferulic acid and caffeic acid are soluble in CO₂-EtOH, in addition, their solubility improves when the co-solvent amount is increased (Bittencourt et al. 2016, 2018). The phenolic composition observed in the ethanolic extract can be related with its high TPC and DPPH[•] scavenging activity; polyhydroxy phenolics have been reported as compounds with high reduction–oxidation potentials and free radical scavenging properties (Oroian and Escriche 2015). On the other hand, CO₂-EtOH selectivity on the phenolic acids may be related with the high AA in the VEO observed for extract number 11.

Conclusion

The SFE using neat CO₂ and CO₂-EtOH was explored for the recovery of phenolic antioxidants from papaya agroindustrial wastes and compared with a conventional extraction method with ethanol. The ethanolic extract showed the higher extraction yield and TPC values. In SFE with neat CO₂ the extraction pressure showed the higher effect on yield and TPC, and as expected, the addition of ethanol as co-solvent improved the extraction yield and TPC, allowing recover up to 44.81% of phenolic compounds. Ethanolic and CO₂-EtOH extracts exhibited good DPPH[•] scavenging activity, while CO₂-EtOH extracts showed the higher AA in VEO. On the other hand, the phenolic composition observed in the extracts analyzed indicates that CO₂-EtOH is a selective solvent to extract phenolic acids. In general, the present study showed that papaya seeds are a promising source of phenolic extracts with interesting antioxidant properties. The SFE using CO₂-EtOH is an attractive green extraction technique for the recovery of phenolic antioxidants (particularly phenolic acids) from this residue. Papaya seeds and its extracts can be of interest to food, pharmaceutical, and cosmetic industries as source of high value ingredients. This is an alternative for the most efficient use and potential valorization of papaya agroindustrial waste.

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