Structural characterization of pectin obtained from cacao pod husk. Comparison of conventional and subcritical water extraction.

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Abreviations

\textbf{Ara:} arabinose; \textbf{CPH:} Cacao pod husk without phenolic extraction; \textbf{CPH-SFE:} Phenolic extraction by Supercritical Fluid; \textbf{CE:} Extracted pectin by conventional method; \textbf{DM:} Degree of methyl esterification; \textbf{ELSD:} Evaporative Light Scattering Detector; \textbf{FT-IR:} Fourier-Transform Infrared Spectroscopy; \textbf{Gal:} Galactose; \textbf{GalA:} Galacturonic acid; \textbf{GC-FID:} Gas Chromatography with Flame ionization detector; \textbf{Glc:} Glucose; \textbf{HPSEC:} High Performance Size Exclusion Chromatography; \textbf{Man:} Mannose; \textbf{Mw:} Molecular weight; \textbf{SEM:} Scanning Electron Microscope; \textbf{SWE:} Extracted pectin by Subcritical Water; \textbf{SFE-CE:} Extracted pectin by conventional method from CPH subjected to supercritical fluid; \textbf{SFE-SWE:} Extracted pectin by subcritical water from CPH subjected to supercritical fluid
Abstract

Pectin was obtained with citric acid and subcritical water extraction from cacao pod husk with or without a previous step consisting of a supercritical fluid extraction of phenols. By subcritical conditions a higher yield (10.9%) was attained in a time 3-fold shorter than that obtained by conventional extraction (~8%) and a greater effectiveness in the recovery of pectin with higher molecular weight (750 kDa) was also found. Regarding pectin structure, galacturonic acid and degree of methyl esterification content were similar (~55 and ~36%, respectively) in both methods. Moreover, pectin recovered by citric acid presented 2-fold higher amount of impurities as compared to subcritical water extraction. Hardly any effects of a previous supercritical treatment were observed in the structure and composition of pectin, indicating the efficiency of the integrated supercritical carbon dioxide and subcritical water extraction as green processes for the obtainment of phenol and pectin from cacao pod husk.

Key words: Pectin, structure, subcritical water extraction, citric acid extraction, galacturonic acid, *Theobroma cacao*
1. Introduction

Cacao pod husks (CPH) are the most abundant by-product of *Theobroma cacao* L., which constitute 52-76% of the cacao fruit in weight. The increasing demand for cocoa beans has led to the accumulation of these undesirable substantial quantities of waste that are usually left to decompose on the cacao plantation, causing a serious environmental problem (Chan & Choo, 2013; Priyangini, Walde, & Ramalingam, 2018). The use of CPH as potential and interesting source of high value compounds such as phenolic compounds (Martinez et al., 2012). As it is widely known, these compounds have a plethora of bioactive activities (de Nazaré do Carmo Brito, Chisté, da Silva Pena, Gloria, & Lopes, 2017; Vu, Scarlett, & Vuong, 2018). In a previous study, Valadez-Carmona, Ortiz-Moreno, Ceballos-Reyes, Mendiola, & Ibanez (2018) succeed in recovering effectively the polyphenols of CPH by supercritical fluid extraction (SFE), which represent environmental-friendly alternatives to conventional methods such as soxhlet, reflux, cold pressing and maceration by organic solvents (Barba, Zhu, Koubaa, Sant’Ana, & Orlien, 2016; Herrero, del Pilar Sanchez-Camargo, Cifuentes, & Ibanez, 2015; Valadez-Carmona et al., 2018). However, no studies have been carried out on the extraction of pectin in the remaining material, in spite of the fact that by-products of cacao have recently emerged as a source of this polysaccharide not only from an economic point of view, but also from an environmental perspective (Priyangini et al., 2018; Vriesmann & Petkowicz, 2017).

Pectin is one of the compounds most important ingredients that are used for its numerous applications in food industry. Gelling and thickening are some pectin functional properties which provide desired mouthfeel and firmness to the foodstuff, being a good substitute of fat and sugar in beverages (Adetunji, Adekunle, Orsat, & Raghavan, 2017; Wang et al., 2016). Moreover, this polysaccharide also presents numerous health benefits in the pharmaceutical industry related with the decreasing of glucose serum levels, the stimulation of the immune response, the reduction of the risk to develop gastrointestinal disorders and their potential antiproliferative effect in colon cancer (Ferreira-Lazarte, Kachrimanidou, Villamiel, Rastall, & Javier Moreno, 2018; Naqash, Masoodi,
Rather, Wani, & Gani, 2017; Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014). Nevertheless, the physicochemical properties depends mainly on extraction process used (Chan & Choo, 2013; Rodsamran & Sothornvit, 2019).

Conventionally, pectin is extracted employing strong mineral acids (sulfuric, nitric and hydrochloric) at high temperatures for long times (Adetunji et al., 2017; Marić et al., 2018). This process involves pectin degradation, environmental problems by the disposal of hazardous contaminants and the necessity to remove potentially the toxic elements from pectin extracts with special treatments in order to be accepted for consumption (Minjares-Fuentes et al., 2014). Although organic acids (citric and acetic acid) have attracted considerable interest as result of their low hydrolyzing capacity and the minor proton catalyzed depolymerization of polysaccharides, this process is time consuming (Marić et al., 2018; Mzoughi et al., 2018; Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015).

In this context, subcritical water extraction (SWE) allows an efficient and fast extraction of the target compounds from plant matrices by solubilizing both polar and non-polar compounds due to higher concentrations of ions ([H\(^+\)] and [OH\(^-\)]) applying high pressures and temperatures and using the water as solvent. The high temperatures tend to weaken the hydrogen bond, decreasing the dielectric constant value and the water polarity; subsequently, reducing the energy needed for division in solute-matrix interactions and increasing extraction efficiency (Adetunji et al., 2017; Liew, Chin, & Yusof, 2014; Zakaria & Kamal, 2016). Moreover, SWE has demonstrated to be an adequate novel processing technique for commercial pectin extraction from apple pomace and citrus peels (Tanaka, Takamizu, Hoshino, Sasaki, & Goto, 2012; Wang & Lu, 2014); however, the wide variety of technological and biological applications of this polysaccharide has generated an industrial demand of pectin. Consequently, there is a continuous search to find other alternative and non-conventional by-products such as CPH, which could provide competitive yields with interesting properties (Morales-Contreras, Rosas-Flores, Contreras-Esquivel, Wicker, & Morales-Castro, 2018; Marić et...
To the best of our knowledge, there are no reported data found on the application of SWE for the obtainment of pectin from cocoa by-products.

Hence, the purpose of this work was the characterization of pectin from cacao pod husk after an integrated process of extraction based on SFE+SWE. In the residue, after a previous phenolic compounds extraction by SFE (Valadez-Carmona et al., 2018), were carried out the obtainment of pectin using SWE and the structural features (monosaccharide composition, molecular weight (Mw) distribution, functional groups, microstructure) were compared with those of pectin extracted by a conventional process employing citric acid.

2. Materials and methods

2.1 Materials and chemicals

Standard monosaccharides (galactose, rhamnose, glucose, galacturonic acid (GalA), mannose, xylose and arabinose), citric acid monohydrate, hexamethyldisilazane, β-phenylglucoside, Pullulan Standard (0.34–805 kDa), a polymer consists by α(1,6) linked maltotriose units, and trifluoroacetic acid were acquired from Sigma (St. Louis, MO, USA). Ethanol (99.5%) was provided by VWR Chemicals (Barcelona, Spain).

2.2. Plant material

Cacao pods husks (CPH) were from Tapachula in Chiapas, México. Then, a grinding of CPH into a paste was carried out. Whole cacao pods were rinsed with water; the cacao seeds were manually removed from the pods, and the husk was grinded into a paste using a semi-industrial blender (Crypto Peerless K55, Birmingham, England). Then, CPH paste was dried in a drying chamber at 60 °C until a dry matter having a water content of <8% was obtained. The dried CPH was milled using a laboratory mill with grinding tank and sieved to have a particle size ≤ 0.5 mm.

2.3. Physico-chemical characterization of samples
Previous to phenolic compounds and pectin extractions, a study on the chemical composition was carried out. The content of dry matter was gravimetrically determined as described by the Association of Official Analytical Chemists (AOAC, 2016); ash content was analyzed by calcination at 500-550 °C in a furnace for 2 h; Bradford’s method was used to measure the protein content (Bradford, 1976). The phenol-sulfuric acid method (Dubois et al., 1956) was applied to estimate the total carbohydrate content and reducing sugar amount was estimated by DNS (Grajales-Garcia et al., 2012) being the glucose the standard used for both measurements. Soluble dietary fiber, insoluble dietary fiber and total dietary fiber contents were measured applying the 991.43 AOAC methods (AOAC, 2016). The estimation of phenolic content was carried out employing gallic acid as standard and Folin-Ciocalteu’s reagent (Fabrowska, Ibanez, Leska, & Herrero, 2016).

2.4. Pectin extraction

Before pectin production, a certain amount of CPH was subjected to a supercritical fluid extraction in order to recover polyphenols. The optimized conditions applied in the extraction procedure was reported by Valadez-Carmona et al. (2018): 5 g of dried CPH, 300 bar, a flow rate of 6 mL/min of CO2 and 13.7 % ethanol as co-solvent for 150 min. Residues were collected into vials for further pectin extraction.

The general scheme of subcritical water and conventional and extractions of pectin from CPH is shown in Figure 1. Yields were calculated according to the formula:

\[
\text{Yield (\%) = } \frac{\text{weight of dried recovered pectin (g)}}{\text{weight of initial powder (g)}} \times 100 \quad \text{(Equation 1)}
\]

2.4.1 Conventional Extraction (CE)

Pectin from CPH was extracted following the method optimized by Vriesmann, Teófilo, & Petkowicz (2012) with some modifications. CPH powder (1:25 w/v solid-to-liquid ratio) were suspended in 4 % (w/v) citric acid solution adjusting the pH to 3.0. Afterwards, the mixture was under continuous stirring at 95 °C for 95 min. Then, the mixture was centrifuged at 3700 g and 4 °C for 15
min (Heraeus Multifuge 3SR Plus, Thermo Scientific, Massachusetts, USA). Ethanol (1:2 v/v) was mixed with supernatant and kept at 4 °C for 16 h in order to precipitate the pectin. A centrifugation at 3700 g for 30 min at 4 °C was performed to recover the pectin residues. After centrifugation, pectin residues were washed three times with anhydrous ethanol and pectin samples were lyophilized. Extractions were performed by triplicate.

Figure 1. Schematic overview of the experimental set-up for pectin extraction in cacao pod husk.

2.4.2 Subcritical Water Extraction (SWE)

To carry out the pectin extractions, an Accelerated Solvent Extraction system ASE 200 from Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit was used. The extraction conditions were selected based on the reports already described in the literature for this methodology. The extraction conditions employed were 121 °C, 103.4 bar and 30 min (Chen, Fu, & Luo, 2015). The extraction cell was heated during 6 min, before each experiment. As well, all
treatments were carried out in 11 mL extraction cells, containing 0.4 g of sample. The extracts obtained were added with ethanol (96%) in a proportion 1:2 (v/v); the mixture was kept for 16 h at 4 °C to precipitate the pectin. Afterwards, the mixture was centrifuged at 1700 g for 30 min at 4 °C. The precipitates were washed with anhydrous ethanol for three times and lyophilised.

2.5. Structural analysis of pectin

2.5.1. Molecular weight (Mw) estimation

The Mw distribution of pectin was estimated following the method described by Muñoz-Almagro, Rico-Rodriguez, Villamiel, & Montilla (2018). Samples (50 µL, 0.1% w/v) previously filtered were separated by HPSEC-ELSD (Agilent Technologies, Boeblingen, Germany) using a TSK-Gel guard column (6.0 mm x 400 mm) and two TSK-Gel columns connected in series, G5000 PWXL (7.8 mm x 300 mm, 10 micron) and G2500 PWXL (7.8 mm x 300 mm, 6 micron) (Tosoh Bioscience, Stuttgart, Germany). The elution of the samples carried out using 0.01 M NH₄Ac as mobile phase at 0.5 mL/min and 30 °C during 50 min. Pullulan standards of different Mw (0.34-805 kDa) were used for the calibration.

2.5.2. Galacturonic acid and neutral sugars and analysis by GC-FID

Prior to Gas Chromatography with Flame ionization detector (GC-FID), pectin was derivatized after hydrolysis following the method reported by Muñoz-Almagro, Rico-Rodriguez, Wilde, Montilla, & Villamiel (2018b). Sample injection was done in split mode 1:5 and eluted using a DB-5HT column (15 m × 0.32 mm × 0.10 µm, J&W-Agilent, Folson, California, USA). The flow of nitrogen was 1 mL/min. Detector and injector temperatures were kept at 350 and 280 °C, respectively; the temperature gradient started at 150 °C, and raised at 165 °C at 1 °C/min and up to 300 °C at 10 °C/min. Quantitation was carried out using the internal standard method, being the standard, β-phenyl-glucoside (0.05 % w/v).
2.5.3. Degree of methyl esterification (DM)

Lyophilised pectin was analysed by FT-IR in a wide frequency range from 400-4000 cm$^{-1}$ and with 4 cm$^{-1}$ of resolution as reported by Muñoz-Almagro et al. (2018b). The pectin methyl esterification (DM) was calculated as the peak area at 1747 cm$^{-1}$ (COO-R) over the sum of the peak areas of 1632 cm$^{-1}$ (COO$^-$) and 1747 cm$^{-1}$ (COO-R).

2.5.4. Morphological analysis of the cacao extracted pectin

Morphological observations of pectin extracted by the different processes were performed using Scanning Electron Microscopy (SEM). Samples were mounted on aluminium stubs with sticky double-sided conductive metal tape and vacuum-metalized with gold-palladium. Microscopic images were elucidated using a DSM 950 scanning electron microscope (Zeiss Iberia, Madrid, Spain) at 7 kV accelerating voltage, a magnification of 50x and a distance of 10 mm.

2.6. Statistical analysis

All experiments were done by triplicate, the results are show as mean values ± standard deviation (SD). Analyses of variance (P < 0.05, ANOVA) to evaluate the differences were executed using STATGRAPHICS Centurion XVI.I. (Statistical Graphics Corporation, Rockville, MD, USA).

3. Results and discussion

3.1. Chemical composition of initial samples

The chemical properties of CPH and CPH-SFE samples are shown in Table 1. Both samples presented high ash and protein contents (~8%), which were similar to those previously described (Martinez et al., 2012; Vriesmann, Teófilo, & Petkowicz, 2011). The high ash and protein content would suggest that both extracts might be good vegetal sources of minerals and proteins for human nutrition (Vriesmann et al., 2011). Besides, phenolic content of original sample (CPH) (6.2 mg AGE/g dm) was in line to that reported for similar samples (Martinez et al., 2012). Unlike CPH, CPH-SFE phenolic content was lower due to the extraction of phenolic compounds as reported in a previous work (Valadez-Carmona et al., 2018).
Table 1. Proximate composition of cacao by-products before (CPH) or after (CPH-SFE) supercritical extraction (Mean ±SD).

<table>
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<tr>
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<th>CPH</th>
<th>CPH-SFE</th>
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<tr>
<td>Dry matter (dm) (%)</td>
<td>90.5 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94.0 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Protein (g/100 g dm)</td>
<td>8.6 ± 0.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.1 ± 0.48&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Ash content (g/100 g dm)</td>
<td>7.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.9 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Phenolic compounds (mg AGE/ g dm)</td>
<td>6.2 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.1 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Total soluble carbohydrates (g glucose/100 g dm)</td>
<td>58.6 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.9 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Reducing carbohydrates (g glucose/100 g dm)</td>
<td>18.3 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.9 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Total dietary fiber (%)</td>
<td>82.1 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Soluble dietary fiber (%)</td>
<td>4.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Insoluble dietary fiber (%)</td>
<td>77.7 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Ratio SDF/IDF</td>
<td>17.5 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

CPH: initial raw cacao pod husks; CPH-SFE: cacao pod husks after SFE of phenolic compounds. Different letters within each column indicate significant differences according with t-Test (p < 0.05).

In CPH total carbohydrate content was higher than in CPH-SFE; these data were similar to those reported previously in samples of CPH subjected to extraction with different solvents (Martinez et al., 2012; Vriesmann et al., 2011). However, in the case of reducing sugars, although significant differences were found between CPH and CPH-SFE, the mean values were closer than the average values of total carbohydrates, probably ascribed to the fact that phenolic compounds are commonly found as glycoside derivatives which could have been dragged during the SFE (Wollgast & Anklam, 2000). The total dietary fiber content of both samples was around 80% which is a value greater than the published data on cacao pod husks (Martinez et al., 2012; Vriesmann et al., 2011). In both samples, the insoluble dietary fiber fraction was much higher than the soluble dietary fiber (SDF) fraction which can be an indicator of a higher amount of cellulose and hemicellulose in CPH. These results highlight the usefulness of both CPH and CPH-SFE extracts to be incorporated in foods acting as technological ingredients due to the water retention capacity, gelling and stabilizer properties (Martinez et al., 2012).

3.2. Pectin extraction yields
As shown in Table 2, SWE was more efficient for pectin extraction than conventional method, reporting significantly higher yields in a shorter time (30 vs 95 min). These differences might be related to the high reactivity of the ions generated as consequence of water dissociation under subcritical conditions. Physicochemical property of subcritical water including dielectric and solubility are significantly altered with the elevated temperature resulting in a number of physical advantages such as high diffusion, low viscosity, increased vapour pressures, and higher mass transfer rate. Thus, SWE provides reduction of energy for division in solute-matrix interactions increasing the extraction efficiency (Adetunji et al., 2017; Liew et al., 2014). Similar results were also obtained by Ueno, Tanaka, Hosino, Sasaki, & Goto (2008), who recovered more pectin from the flavedo of *Citrus junos* with SWE compared to conventional hydrochloric acid extraction.

On the other hand, yields of pectin from CPH obtained by the conventional process were lower than those found by Vriesmann et al. (2012) who reported values of 10.1% in the recovery of cacao pectin under the same conditions (95°C, pH 3, 95 min). These dissimilarities could be due to the different origin of the CPH and environmental growth conditions (Chan & Choo, 2013). On the contrary, these values were higher than those described by Chan & Choo (2013) who recovered a 7% of pectin from CPH applying the same temperature and extracting agent at pH value slightly lower (2.5) for longer duration (180 min). This reduction could be explained by the breakdown of pectin molecules as a result of the increment of time (Abid et al., 2016).

Moreover, no significant differences were detected in the pectin yields obtained before and after phenolic compounds extraction, indicating the harmlessness of supercritical CO2 step in the recovery of this polysaccharide. This fact can be associated to the high molecular weight of pectin that prevents them from being extracted by CO2 (Femenia, Garcia-Marin, Simal, Rossello, & Blasco, 2001).

Table 2. Molecular parameters and sugar composition of pectin extracted from cacao pod husk using two different extraction methods (Mean ±SD)
### Monosaccharides composition

<table>
<thead>
<tr>
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<th>CPH CE</th>
<th>SWE</th>
<th>SFE-CE</th>
<th>SFE-SWE</th>
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<tr>
<td>Yield (%)</td>
<td>8.3 ± 0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.4 ± 0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
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#### 3.3. Pectin structural characterization

#### 3.3.1. Molecular weight estimation

As illustrated in Figure 2a and 2b, hardly any variations between CPH pectin subjected or not to SFE were detected, demonstrating the absence of interference of this technique in the recovery of this polysaccharide. This result is in agreement with Femenia et al. (2001) who reported the difficulty to modify fragments of high Mw by CO₂. However, very different profiles were observed in pectin samples obtained by using the two tested processes, CE and SWE (Figure 2c).
Figure 2. Chromatographic HPSEC profiles for pectins extracted from CPH: a) conventionally with and without previous SFE; b) by subcritical water with and without previous SFE c) comparison between conventional and SWE without previous SFE treatment.
Five peaks eluted in the samples extracted with citric acid while four peaks were present in the chromatograms of pectin obtained using SWE. These qualitative differences resulted in quantitative changes in Mw, as depicted in Figure 3. SWE highlighted for being a technique more selective than CE for the extraction of pectin from CPH since showed a considerable fragment (>35%, 750 kDa) corresponding to pectin (peak 2’). Moreover, it only contained a 10% of molecules of Mw upper than 805 kDa (peak 1’) could correspond to the hemicelluloses and celluloses which is glued to the pectin. Therefore, a low presence of this peak could indicate a successful extraction of pectin with high purity. On the contrary, CE demonstrated not to be as effective as SWE due to the abundant presence (≥50%) of molecules of Mw upper as 805 kDa (peaks 1+2+3), indicating that the conditions used in the conventional method were not strong enough to recover pectin from cell wall of the plant without hydrolyzing it. Regardless of extraction method, in all treatments occurred a hydrolysis caused by acid species (citric acid and H₃O⁺, peaks 4’ and 5) that produced a considerable fragment (peak 3’ and 4) corresponding to modified pectin of diverse Mw (125.2 and 61.2 kDa) (Cocero et al., 2018; Vriesmann et al., 2012).

3.3.2. Neutral sugars and galacturonic acid

Figure 4 depicts the chromatographic profile obtained by GC-FID of the monosaccharides of SWE after hydrolysis with 2 N TFA. As can be seen, the monomers found were xylose, arabinose, galactose, rhamnose and GalA derived from pectin, whereas mannose and glucose came probably from cellulose and hemicellulose which is bound to pectin (Muñoz-Almagro et al., 2018). The quantitative analysis of these carbohydrates is shown in Table 2. Regarding the monomeric composition, the most abundant monosaccharide was GalA, reaching values significantly higher in SWE than in CE (59 vs ~50%). These values are close to those reported by (Chan & Choo, 2013; Vriesmann et al., 2012) in a range of 59 to 65% for the extraction of pectin from cacao husks under similar conventional conditions.
Figure 3. Estimation of Mw (kDa) and main fragments formed (%) before and after polyphenols recovery by SFE of cacao pectin extraction (a) conventional (b) subcritical water.

Note: values with different small case superscript letters (a-c) in the same line within each pectin indicate significant differences as estimated by Tukey’s test (P < 0.05); CPH-SFE: cacao pod husks whose phenolic compounds were extracted by supercritical fluid extraction; CE: extracted pectin by conventional method; SFE-CE: extracted pectin by conventional method from CPH submitted by supercritical fluid; SWE: extracted pectin by subcritical water; SFE-SWE: extracted pectin by subcritical water from CPH submitted by supercritical fluid.
Figure 4. Chromatographic profile obtained by GC-FID of TMS oximes of monosaccharides after hydrolysis with 2 N TFA of extracted pectin from CPH by subcritical water. 1: Xylose; 2: Xylose + Arabinose; 3 and 4: Rhamnose; 5: Galactose; 6: Mannose; 7: Glucose; 8: Galactose + Mannose + Glucose; 9 and 10: Galacturonic acid; 11: Internal standard.

In general terms, as shown in Table 2, rhamnose and galactose were the most abundant neutral sugars in all pectins followed by xylose and arabinose; hardly any differences were detected in the content found after SWE, being GalA the component of pectin most positively affected by SWE. This distribution of carbohydrates as well as the corresponding ratios (Xyl+Ara+Gal)/Rha and GalA/Rha, suggested that the structure consists mainly of homogalacturonan backbone and a considerable part of rhamnogalacturonan with limited arabinogalactan and/or arabinan side chains (Amorim, Vriesmann, Petkowicz, Martinez, & Noleto, 2016; Vriesmann & Petkowicz, 2017).

Besides, it is noticeable that pectins extracted conventionally with or without a previous SFE step, contained 2-fold greater amount of glucose and mannose than the ones achieved using SWE process, thus demonstrating that SWE was more suitable for the recovery of pectin with small amounts of compounds derived from other sources.
Regarding the effect of a previous SFE step for phenolic compounds extraction, the most striking feature was the significant increase in the level of GalA and galactose in both pectin extractions (conventional and subcritical water). According to Femenia et al. (2001) the application of SC-CO₂ gives rise to the breaking of hydrogen bonds between hemicelluloses and cellulose contributing to increase the porosity of the wall and, therefore, improving its exhibition for the subsequent pectin extraction by CE and SWE. These results could indicate that the treatment with SC-CO₂ allows the recovery of pectin without damaging monosaccharides with a significant role in the anti-cancer activity as galactose (Maxwell, Belshaw, Waldron, & Morris, 2012).

3.3.3. Degree of methyl esterification (DM)

The FT-IR spectra were used to elucidate if the different extraction methods used to obtain pectin affect the functional groups and their bonding configurations. In general, slightly changes were observed in the spectral profiles of pectin extracted before and after SFE (Figures 5a and 5b). The wide and marked absorption band at 3400 cm⁻¹ which correspond to O-H stretching vibration of hydroxyl groups was sharper in SFE-SW pectin in comparison to its control (Figures 5a and 5b), probably as consequence of the extraction of the phenolic compounds by SFE technique (Wang et al., 2014). The same behavior was observed in CE and SWE (Figure 5c) indicating the breakage of bonds –O-H. This result could be attributed to the high dissociation constant of the citric acid in comparison to the water (14 vs 3.1) (Pereira et al., 2016). The absorption band at 2938 cm⁻¹ due to C-H (-CH, -CH₂, -CH₃) exhibited the same intensity in all pectin samples, indicating that CE did not affect these functional groups. Furthermore, as it can be observed, in the pectin extracted conventionally, it is noticeable that the intensity of the band near 1740 cm⁻¹ related to C=O stretching vibration of methylsterified carboxyl groups decreased, whereas the absorption at about 1620 cm⁻¹ corresponding to carboxylate ion stretching increased after supercritical treatment (Figure 5a).

Similar but not as prominent behavior was found also in the pectin extracted by subcritical procedures. According to Femenia et al. (2001), the increase of the number of carboxylate ions in the pectins extracted from CPH without phenolic compounds, could be due to the breakage of ionic bonds
as consequence of the application of the high pressures in SC-CO₂ (Chaharbaghi, Khodaiyan, & Hosseini, 2017; Wang & Lu, 2014).

Moreover, the reduction of the peaks 1146 and 1045 cm⁻¹ of CE in comparison with SWE (Figure 5c), corresponding to the stretching vibrations C–OH side groups and the C–O–C glycosidic bond vibration, could be due to the minor presence of glycosidic linkages between sugar units (Zhang et al., 2018). This fact could corroborate the degradation of pectin obtained by conventional methods as it has been mentioned previously.

On the other hand, some bands were maintained regardless of the type of the extraction method, such as at 1250 cm⁻¹ (-CH₃CO stretching), 957 cm⁻¹ (C-O bending), 912 cm⁻¹ (rocking mode of -CH₃), and 820 cm⁻¹, (-CCH and -COH bending at the C-6 position) (Wang & Lu, 2014).

In the samples analyzed, these qualitative changes resulted in quantitative changes in the DM, as illustrated in Table 2. The four samples analyzed were low-methoxyl pectins with values of DM in the range 36.8-42.2%, in line with those reported previously in the literature for pectin derived from CPH (Chen et al., 2015; Vriesmann et al., 2012). No significant differences (p>0.05) were detected in DM between pectins subjected or not to SFE. This could indicate that the previous recovery of phenolic compounds with SFE caused only slight structural modifications in this polysaccharide, with respect to this parameter. However, DM of CE was significantly lower in comparison to SW (~37 vs ~40%), probably due to the effect of the high acid citric concentration (4% w/v) and long contact time during the pectin extraction (95 min) which could accelerated the de-esterification (Chan & Choo, 2013; Cocero et al., 2018). Unlike CE, SWE allows the effective extraction of pectin without using low pH avoiding any degradation and modification in their structural and technological features (Cocero et al., 2018; Liew et al., 2014).
Figure 5. FT-IR spectra of pectin extracted from CPH: a) conventionally with and without previous SFE; b) by subcritical water with and without previous SFE; c) comparison between conventional and subcritical water extraction without previous SFE treatment.
3.3.4. Morphological analysis of the cacao extracted pectins

Figure 6. SEM images of freeze dried pectins extracted by conventional procedure and subcritical water without (a) CE and c) SWE) or with supercritical fluid (b) SFE-CE and d) SFE-SWE) at 50x magnification.

SEM microstructure of pectins extracted by conventional and subcritical water extraction without (Figure 6 a and c) and with (Figure 6 b and d) a previous treatment by SC-CO₂ is shown in Figure 6. As it is illustrated, no changes were appreciated between the samples subjected to the supercritical treatments and their corresponding counterparts (a) and b) for CE and c) and d) for SWE). The pectin obtained with citric acid presented a smooth surface with some long cracks in their surface. Nonetheless, pectins obtained by subcritical procedures, exhibited a different structure. This
heterogeneous architecture consisted of several irregular and rough surfaces which are compact and flaky in shape, which can be due to the higher content of neutral sugar sides in their structure.

4. Conclusions

In this study the use of an environmental friendly technique such as subcritical water let us to obtain pectin as high added value compound from cacao pod husk by-products. The most striking features were: higher pectin yield, higher galacturonic acid content and higher degree of methyl esterification using SWE as compared to the conventional extraction with citric acid. In addition, as reflected by the estimation of molecular mass and monomeric composition, subcritical water pectin presented less interference compounds derived from other polysaccharides of the cell wall. Considering that a previous extraction of phenolic compounds by supercritical fluid extraction with CO$_2$ did not give rise to any structural detriment in pectin, a combination of integrated green process employing SFE, as a first step for the sequential recovery of phenolics, and SWE as a second step for pectin recovery from CPH, constitutes an environmentally green approach and efficient waste management proposal based on biorefinery that can be generalized to other by-products.

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