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

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17 **Abbreviations**

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

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28

29 **Abstract**

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

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44 **Key words:** Pectin, structure, subcritical water extraction, citric acid extraction, galacturonic acid,
45 *Theobroma cacao*

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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, & Orlie, 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,

77 Rather, Wani, & Gani, 2017; Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014). Nevertheless,
78 the physicochemical properties depends mainly on extraction process used (Chan & Choo, 2013;
79 Rodsamran & Sothornvit, 2019).

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

89 In this context, subcritical water extraction (SWE) allows an efficient and fast extraction of
90 the target compounds from plant matrices by solubilizing both polar and non-polar compounds due
91 to higher concentrations of ions ($[H^+]$ and $[OH^-]$) applying high pressures and temperatures and using
92 the water as solvent. The high temperatures tend to weaken the hydrogen bond, decreasing the
93 dielectric constant value and the water polarity; subsequently, reducing the energy needed for division
94 in solute-matrix interactions and increasing extraction efficiency (Adetunji et al., 2017; Liew, Chin,
95 & Yusof, 2014; Zakaria & Kamal, 2016). Moreover, SWE has demonstrated to be an adequate novel
96 processing technique for commercial pectin extraction from apple pomace and citrus peels (Tanaka,
97 Takamizu, Hoshino, Sasaki, & Goto, 2012; Wang & Lu, 2014); however, the wide variety of
98 technological and biological applications of this polysaccharide has generated an industrial demand
99 of pectin. Consequently, there is a continuous search to find other alternative and non-conventional
100 by-products such as CPH, which could provide competitive yields with interesting properties
101 (Morales-Contreras, Rosas-Flores, Contreras-Esquivel, Wicker, & Morales-Castro, 2018; Marić et

102 al., 2018). To the best of our knowledge, there are no reported data found on the application of SWE
103 for the obtainment of pectin from cocoa by-products.

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

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111 **2. Materials and methods**

112 2.1 Materials and chemicals

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

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119 2.2. Plant material

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

126 2.3. Physico-chemical characterization of samples

127 Previous to phenolic compounds and pectin extractions, a study on the chemical composition
128 was carried out. The content of dry matter was gravimetrically determined as described by the
129 Association of Official Analytical Chemists (AOAC, 2016); ash content was analyzed by calcination
130 at 500-550 °C in a furnace for 2 h; Bradford's method was used to measure the protein content
131 (Bradford, 1976). The phenol-sulfuric acid method (Dubois et al., 1956) was applied to estimate the
132 total carbohydrate content and reducing sugar amount was estimated by DNS (Grajales-Garcia et al.,
133 2012) being the glucose the standard used for both measurements. Soluble dietary fiber, insoluble
134 dietary fiber and total dietary fiber contents were measured applying the 991.43 AOAC methods
135 (AOAC, 2016). The estimation of phenolic content was carried out employing gallic acid as standard
136 and Folin-Ciocalteu's reagent (Fabrowska, Ibanez, Leska, & Herrero, 2016).

137 2.4. Pectin extraction

138 Before pectin production, a certain amount of CPH was subjected to a supercritical fluid
139 extraction in order to recover polyphenols. The optimized conditions applied in the extraction
140 procedure was reported by Valadez-Carmona et al. (2018): 5 g of dried CPH, 300 bar, a flow rate of
141 6 mL/min of CO₂ and 13.7 % ethanol as co-solvent for 150 min. Residues were collected into vials
142 for further pectin extraction.

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

$$145 \quad \text{Yield (\%)} = \frac{\text{weight of dried recovered pectin (g)}}{\text{weight of initial powder(g)}} \times 100 \quad (\text{Equation 1})$$

146 2.4.1 Conventional Extraction (CE)

147 Pectin from CPH was extracted following the method optimized by Vriesmann, Teófilo, &
148 Petkowicz (2012) with some modifications. CPH powder (1:25 w/v solid-to-liquid ratio) were
149 suspended in 4 % (w/v) citric acid solution adjusting the pH to 3.0. Afterwards, the mixture was under
150 continuous stirring at 95 °C for 95 min. Then, the mixture was centrifuged at 3700 g and 4 °C for 15

151 min (Heraeus Multifuge 3SR Plus, Thermo Scientific, Massachusetts, USA). Ethanol (1:2 v/v) was
 152 mixed with supernatant and kept at 4 °C for 16 h in order to precipitate the pectin. A centrifugation
 153 at 3700 g for 30 min at 4 °C was performed to recover the pectin residues. After centrifugation, pectin
 154 residues were washed three times with anhydrous ethanol and pectin samples were lyophilized.
 155 Extractions were performed by triplicate.

156

157 Figure 1. Schematic overview of the experimental set-up for pectin extraction in cacao pod husk.
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159 2.4.2 Subcritical Water Extraction (SWE)

160 To carry out the pectin extractions, an Accelerated Solvent Extraction system ASE 200 from
 161 Dionex Corporation (Sunnyvale, CA, USA) equipped with a solvent controller unit was used. The
 162 extraction conditions were selected based on the reports already described in the literature for this
 163 methodology. The extraction conditions employed were 121 °C, 103.4 bar and 30 min (Chen, Fu, &
 164 Luo, 2015). The extraction cell was heated during 6 min, before each experiment. As well, all

165 treatments were carried out in 11 mL extraction cells, containing 0.4 g of sample. The extracts
166 obtained were added with ethanol (96%) in a proportion 1:2 (v/v); the mixture was kept for 16 h at 4
167 °C to precipitate the pectin. Afterwards, the mixture was centrifuged at 1700 g for 30 min at 4 °C.
168 The precipitates were washed with anhydrous ethanol for three times and lyophilised.

169 2.5. Structural analysis of pectin

170 2.5.1. Molecular weight (Mw) estimation

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

179 2.5.2. Galacturonic acid and neutral sugars and analysis by GC-FID

180 Prior to Gas Chromatography with Flame ionization detector (GC-FID), pectin was
181 derivatized after hydrolysis following the method reported by Muñoz-Almagro, Rico-Rodriguez,
182 Wilde, Montilla, & Villamiel (2018b). Sample injection was done in split mode 1:5 and eluted using
183 a DB-5HT column (15 m × 0.32 mm × 0.10 µm, J&W-Agilent, Folson, California, USA). The flow
184 of nitrogen was 1 mL/min. Detector and injector temperatures were kept at 350 and 280 °C,
185 respectively; the temperature gradient started at 150 °C, and raised at 165 °C at 1 °C/min and up to
186 300 °C at 10 °C/min. Quantitation was carried out using the internal standard method, being the
187 standard, β-phenyl-glucoside (0.05 % w/v).

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190 2.5.3. Degree of methyl esterification (DM)

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

195 2.5.4. Morphological analysis of the cacao extracted pectin

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

201 2.6. Statistical analysis

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

205 3. Results and discussion

206 3.1. Chemical composition of initial samples

207 The chemical properties of CPH and CPH-SFE samples are shown in Table 1. Both samples
208 presented high ash and protein contents ($\sim 8\%$), which were similar to those previously described
209 (Martinez et al., 2012; Vriesmann, Teófilo, & Petkowicz, 2011). The high ash and protein content
210 would suggest that both extracts might be good vegetal sources of minerals and proteins for human
211 nutrition (Vriesmann et al., 2011). Besides, phenolic content of original sample (CPH) (6.2 mg AGE/g
212 dm) was in line to that reported for similar samples (Martinez et al., 2012). Unlike CPH, CPH-SFE
213 phenolic content was lower due to the extraction of phenolic compounds as reported in a previous
214 work (Valadez-Carmona et al., 2018).

215 Table 1. Proximate composition of cacao by-products before (CPH) or after (CPH-SFE) supercritical
216 extraction (Mean $\pm$ SD).

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

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

219

220 In CPH total carbohydrate content was higher than in CPH-SFE; these data were similar to
221 those reported previously in samples of CPH subjected to extraction with different solvents (Martinez
222 et al., 2012; Vriesmann et al., 2011). However, in the case of reducing sugars, although significant
223 differences were found between CPH and CPH-SFE, the mean values were closer than the average
224 values of total carbohydrates, probably ascribed to the fact that phenolic compounds are commonly
225 found as glycoside derivatives which could have been dragged during the SFE (Wollgast & Anklam,
226 2000). The total dietary fiber content of both samples was around 80% which is a value greater than
227 the published data on cacao pod husks (Martinez et al., 2012; Vriesmann et al., 2011). In both
228 samples, the insoluble dietary fiber fraction was much higher than the soluble dietary fiber (SDF)
229 fraction which can be an indicator of a higher amount of cellulose and hemicellulose in CPH. These
230 results highlight the usefulness of both CPH and CPH-SFE extracts to be incorporated in foods acting
231 as technological ingredients due to the water retention capacity, gelling and stabilizer properties
232 (Martinez et al., 2012).

233 3.2. Pectin extraction yields

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

244 On the other hand, yields of pectin from CPH obtained by the conventional process were
245 lower than those found by Vriesmann et al. (2012) who reported values of 10.1% in the recovery of
246 cacao pectin under the same conditions (95°C, pH 3, 95 min). These dissimilarities could be due to
247 the different origin of the CPH and environmental growth conditions (Chan & Choo, 2013). On the
248 contrary, these values were higher than those described by Chan & Choo (2013) who recovered a 7
249 % of pectin from CPH applying the same temperature and extracting agent at pH value slightly lower
250 (2.5) for longer duration (180 min). This reduction could be explained by the breakdown of pectin
251 molecules as a result of the increment of time (Abid et al., 2016).

252 Moreover, no significant differences were detected in the pectin yields obtained before and
253 after phenolic compounds extraction, indicating the harmlessness of supercritical CO₂ step in the
254 recovery of this polysaccharide. This fact can be associated to the high molecular weight of pectin
255 that prevents them from being extracted by CO₂ (Femenia, Garcia-Marin, Simal, Rossello, & Blasco,
256 2001).

257 Table 2. Molecular parameters and sugar composition of pectin extracted from cacao pod husk using
258 two different extraction methods (Mean $\pm$ SD)

259

	CPH		CPH-SFE	
	CE	SWE	SFE-CE	SFE-SWE
Yield (%)	8.3 ± 0.62 ^a	10.9 ± 0.10 ^b	7.4 ± 0.26 ^a	10.9 ± 0.17 ^b
Monosaccharides composition (%)				
GalA	53.7 ± 2.1 ^a	59.5 ± 1.4 ^b	48.9 ± 2.8 ^a	58.8 ± 1.8 ^b
Xyl	1.5 ± 0.1 ^a	1.7 ± 0.0 ^b	1.6 ± 0.2 ^{ab}	1.8 ± 0.1 ^b
Ara	2.5 ± 0.2 ^a	3.0 ± 0.2 ^b	2.2 ± 0.2 ^a	2.5 ± 0.1 ^a
Rha	15.3 ± 0.7 ^b	14.5 ± 1.2 ^{ab}	16.6 ± 0.5 ^c	14.0 ± 0.3 ^a
Gal	18.3 ± 1.8 ^{ab}	16.6 ± 1.3 ^a	21.5 ± 0.8 ^c	19.2 ± 1.9 ^b
Man	2.5 ± 0.2 ^b	1.2 ± 0.1 ^a	2.6 ± 0.1 ^b	1.0 ± 0.1 ^a
Glc	6.2 ± 0.4 ^c	3.5 ± 0.2 ^b	6.6 ± 0.3 ^c	2.7 ± 0.2 ^a
GalA/Rha	3.5 ± 0.2 ^b	4.1 ± 0.4 ^c	2.9 ± 0.3 ^a	4.2 ± 0.1 ^c
Xyl+Ara+Gal/Rha	1.5 ± 0.1 ^b	1.6 ± 0.1 ^c	1.6 ± 0.0 ^a	1.7 ± 0.2 ^c
DM	37.5 ± 0.3 ^a	39.8 ± 0.9 ^b	36.8 ± 0.3 ^a	42.2 ± 2.7 ^b

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 ($P < 0.05$); Xyl: xylose; Ara: arabinose; Rha: rhamnose; Gal: Galactose; Man: mannose; Glc: glucose; GalA: galacturonic acid. CPH: initial raw cacao pod husks; 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. DM: degree of methylesterification.

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.

282
283 Five peaks eluted in the samples extracted with citric acid while four peaks were present in
284 the chromatograms of pectin obtained using SWE. These qualitative differences resulted in
285 quantitative changes in Mw, as depicted in Figure 3. SWE highlighted for being a technique more
286 selective than CE for the extraction of pectin from CPH since showed a considerable fragment (>35%,
287 750 kDa) corresponding to pectin (peak 2'). Moreover, it only contained a 10% of molecules of Mw
288 upper than 805 kDa (peak 1') could correspond to the hemicelluloses and celluloses which is glued
289 to the pectin. Therefore, a low presence of this peak could indicate a successful extraction of pectin
290 with high purity. On the contrary, CE demonstrated not to be as effective as SWE due to the abundant
291 presence ($\geq 50\%$) of molecules of Mw upper as 805 kDa (peaks 1+2+3), indicating that the conditions
292 used in the conventional method were not strong enough to recover pectin from cell wall of the plant
293 without hydrolyzing it. Regardless of extraction method, in all treatments occurred a hydrolysis
294 caused by acid species (citric acid and H_3O^+ , peaks 4' and 5) that produced a considerable fragment
295 (peak 3' and 4) corresponding to modified pectin of diverse Mw (125.2 and 61.2 kDa) (Cocero et al.,
296 2018; Vriesmann et al., 2012).

297 3.3.2. Neutral sugars and galacturonic acid

298 Figure 4 depicts the chromatographic profile obtained by GC-FID of the monosaccharides of
299 SWE after hydrolysis with 2 N TFA. As can be seen, the monomers found were xylose, arabinose,
300 galactose, rhamnose and GalA derived from pectin, whereas mannose and glucose came probably
301 from cellulose and hemicellulose which is bound to pectin (Muñoz-Almagro et al., 2018). The
302 quantitative analysis of these carbohydrates is shown in Table 2. Regarding the monomeric
303 composition, the most abundant monosaccharide was GalA, reaching values significantly higher in
304 SWE than in CE (59 vs ~50%). These values are close to those reported by (Chan & Choo, 2013;
305 Vriesmann et al., 2012) in a range of 59 to 65% for the extraction of pectin from cacao husks under
306 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 oxymes of monosaccharides after hydrolysis with 2 N TFA of extracted pectin from CPH by subcritical water. 1: Xylose 1; 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, & Noletto, 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.

333 Regarding the effect of a previous SFE step for phenolic compounds extraction, the most
334 striking feature was the significant increase in the level of GalA and galactose in both pectin
335 extractions (conventional and subcritical water). According to Femenia et al. (2001) the application
336 of SC-CO₂ gives rise to the breaking of hydrogen bonds between hemicelluloses and cellulose
337 contributing to increase the porosity of the wall and, therefore, improving its exhibition for the
338 subsequent pectin extraction by CE and SWE. These results could indicate that the treatment with
339 SC-CO₂ allows the recovery of pectin without damaging monosaccharides with a significant role in
340 the anti-cancer activity as galactose (Maxwell, Belshaw, Waldron, & Morris, 2012).

341 3.3.3. Degree of methyl esterification (DM)

342 The FT-IR spectra were used to elucidate if the different extraction methods used to obtain
343 pectin affect the functional groups and their bonding configurations. In general, slightly changes were
344 observed in the spectral profiles of pectin extracted before and after SFE (Figures 5a and 5b). The
345 wide and marked absorption band at 3400 cm⁻¹ which correspond to O-H stretching vibration of
346 hydroxyl groups was sharper in SFE-SW pectin in comparison to its control (Figures 5a and 5b),
347 probably as consequence of the extraction of the phenolic compounds by SFE technique (Wang et
348 al., 2014). The same behavior was observed in CE and SWE (Figure 5c) indicating the breakage of
349 bonds –O-H. This result could be attributed to the high dissociation constant of the citric acid in
350 comparison to the water (14 vs 3.1) (Pereira et al., 2016). The absorption band at 2938 cm⁻¹ due to C-
351 H (-CH, -CH₂, -CH₃) exhibited the same intensity in all pectin samples, indicating that CE did not
352 affect these functional groups. Furthermore, as it can be observed, in the pectin extracted
353 conventionally, it is noticeable that the intensity of the band near 1740 cm⁻¹ related to C=O stretching
354 vibration of methylesterified carboxyl groups decreased, whereas the absorption at about 1620 cm⁻¹
355 corresponding to carboxylate ion stretching increased after supercritical treatment (Figure 5a).
356 Similar but not as prominent behavior was found also in the pectin extracted by subcritical
357 procedures. According to Femenia et al. (2001), the increase of the number of carboxylate ions in the
358 pectins extracted from CPH without phenolic compounds, could be due to the breakage of ionic bonds

359 as consequence of the application of the high pressures in SC-CO₂ (Chaharbaghi, Khodaiyan, &
360 Hosseini, 2017; Wang & Lu, 2014).

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

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

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

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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.

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

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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.

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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 CO₂ 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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