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1 Impregnation of cinnamaldehyde into cassava starch biocomposite films using supercritical fluid  
2 technology for the development of food active packaging

3

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15 **ABSTRACT**

16 In this work, supercritical solvent impregnation (SSI) has been tested for the incorporation of  
17 natural compounds into biocomposite materials for food packaging. Cinnamaldehyde, with  
18 proved antimicrobial activity against fungi commonly found in bread products, was successfully  
19 impregnated on biocomposite cassava starch based materials using supercritical carbon dioxide as  
20 solvent. Different process experimental conditions were tested (pressure, impregnation time and  
21 depressurization rate) at a fixed temperature (35 °C) in order to study their influence on the  
22 amount of cinnamaldehyde impregnated as well as on the morphology of the films. Results  
23 showed that all conditions permitted to impregnate antimicrobial active amounts superior to those  
24 previously obtained using conventional incorporation methods. Moreover, a significant decrease  
25 of the equilibrium water vapor sorption capacity and water vapor permeability of the films was  
26 observed after SSI processing which is a clear advantage of the process, considering the  
27 envisaged applications.

28

29 **Keywords:** Cassava starch; Antimicrobial agent; Supercritical solvent impregnation; Food  
30 packaging

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33 **1. Introduction**

34 To overcome current increased consumer demands for safer, environmental friendly and higher  
35 quality products, the development of biocomposite films for packaging materials, which can be  
36 used as substitutes for petrochemical based polymers, is an interesting alternative and showed to  
37 be a promising research field (Souza, Ditchfield, & Tadini, 2010).

38 Although many types of biocomposite polymers are being industrially produced (PLA, PHA,  
39 PCL, PEA and others), polymers from agricultural sources, especially polysaccharides, such as  
40 starch or cellulose, are among the most studied.

41 Cassava starch has been extensively used to produce biocomposite films (Kechichian, Ditchfield,  
42 Veiga-Santos, & Tadini, 2010; Mbay, Hoppe, & Thomas, 2012; Müller, Yamashita, & Laurindo,  
43 2008; Paes, Yakimets, & Mitchell, 2008; Souza et al., 2012; Souza, Goto, Mainardi, Coelho, &  
44 Tadini, 2013; Veiga-Santos, Suzuki, Nery, Cereda, & Scamparini, 2008) and also proved to be  
45 an interesting material for several food packaging applications, mainly because of their good  
46 film-forming properties. Films developed from starch are described as isotropic, odorless,  
47 tasteless, colorless, non-toxic and biologically degradable (Flores, Famá, Rojas, Goyanes, &  
48 Gerschenson, 2007).

49 The production of biocomposites is another technological driver that permits to improve the  
50 tensile properties of biopolymer and biodegradable based materials. Also, and as previously  
51 reported, their barrier properties can be further improved by incorporating montmorillonite clay  
52 into the polymer matrix in order to reduce the gas permeability (Priolo, Gamboa, Holder, &  
53 Grunlan, 2010; Souza et al., 2012; Souza et al., 2013). Nanoparticles like mineral clays are  
54 mainly composed of hydrated aluminosilicate with neutral or negative charged layers (Wilhelm,  
55 Sierakowski, Souza, & Wypych, 2003).

56 Packaging is referred as active when it performs some desired role in food preservation other than  
57 providing an inert barrier to external conditions. It permits to change the condition of the

58 packaged food to extend its shelf life or to improve safety or sensory properties, while  
59 maintaining its quality (Souza et al., 2010). Biocomposite films carrying natural additives, such  
60 as antimicrobial agents, could be considered an emergent tendency of functional food packaging.  
61 The antibacterial and antifungal activities of essential oils are well known and present clear  
62 advantages because of their greater activity when compared with the effects of the individual  
63 active compounds, probably due to the synergistic effects (Bakkali, Averbeck, Averbeck, &  
64 Idaomar, 2008; Du Plooy, Regnier, & Combrinck, 2009).

65 Cinnamon bark essential oil, mainly constituted by cinnamaldehyde, showed a strong  
66 antimicrobial activity against *Pseudomonas putida* strain isolated from meat (Oussalah, Caillet,  
67 Saucier, & Lacroix, 2006). Scanning electron microscopic observations revealed that the  
68 antimicrobial activity of cinnamaldehyde is bacteriocidal since bacterial cells suffered severe  
69 damages in their surface structure (Kim, Park, & Park, 2004). Nielsen and Rios (2000)  
70 demonstrated the high activity of essential oil against the most important spoilage fungi of bread,  
71 whereas Oussalah, Caillet, Saucier and Lacroix (2007) showed that the essential oil of  
72 *Cinnamomun cassia* was the most active against pathogenic bacteria.

73 The incorporation of essential oils into viscous biopolymer solutions (that are normally used to  
74 prepare biocomposite and active films by the conventional solvent casting method) has some  
75 drawbacks mainly due to the oil hydrophobic character that makes difficult to obtain a  
76 homogeneous material (Kechichian et al., 2010; Mayachiew & Devahastin, 2010; Mayachiew,  
77 Devashastin, Mackey, & Niranjana, 2010; Seydim & Sarikus, 2006; Souza et al., 2013). In these  
78 cases supercritical solvent impregnation (SSI) method can represent an efficient and  
79 advantageous alternative. This technique has been largely used in the development of materials  
80 for applications in the biomedical field mostly to prepare improved sustained drug delivery  
81 devices (Braga et al., 2008; Costa et al., 2010a; Costa et al., 2010b; Dias et al., 2011; Natu, Gil, &  
82 Sousa, 2008) and scaffolds with different porosities for tissue engineering applications  
83 (Reverchon & Cardea, 2012). Applications of this new technique in antimicrobial packaging are

84 quite scarce (Bierhalz, Silva, Sousa, Braga, & Kieckbusch, 2013; Sonkaew, Sane, & Suppakul,  
85 2012).

86 Among the main advantages of the SSI technique it can be referred that: *i*) it permits the  
87 impregnation of a large number of different natural and synthetic based polymers if they swell  
88 when in contact with scCO<sub>2</sub>; *ii*) it is particularly advantageous to impregnate hydrophobic  
89 molecules, as is the case of essential oils; *iii*) solute loading and depth of impregnation can be  
90 tuned by changing process conditions; *iv*) it originates final products that are free from organic  
91 solvent residues since scCO<sub>2</sub> is released as a gas after depressurization; and finally *v*) the  
92 technique permits to work at relatively mild conditions in an oxygen free environment which is  
93 often desirable when the objective is to impregnate natural based compounds with biological  
94 activity.

95 In this way, the main goal of this work was the development and characterization of active starch  
96 based films to be used for food packaging purposes. Films were impregnated with  
97 cinnamaldehyde, an antimicrobial agent that inhibit the proliferation of *Penicillium commune* and  
98 *Eurotium amstelodami*, fungi commonly found in bread products, using supercritical carbon  
99 dioxide (scCO<sub>2</sub>) as solvent at different process conditions. The antimicrobial agent was also  
100 incorporated in film matrix by the conventional solvent casting method for comparison. The  
101 antimicrobial quantification and other important properties for food packaging applications such  
102 as the equilibrium water sorption capacity and water vapor permeability of scCO<sub>2</sub> processed and  
103 non-processed films were also evaluated.

104

## 105 **2. Materials and Methods**

### 106 *2.1 Materials*

107 Native cassava starch, Amilogill® 1500, kindly supplied by Cargill Agrícola, Brazil (amylose:  
108 19.7 g/100 g; amylopectin: 80.3 g/100 g; maximum moisture: 14.0 g/100 g) was used as the film  
109 forming component to provide a continuous biocomposite film matrix. Glycerol (Synth, Brazil)

110 was added as plasticizer and natural Na-montmorillonite clay (commercial product Argel T,  
111 Bentonit União, Brazil, used as received) was used as filler. Pure cinnamaldehyde (Synth,  
112 Portugal) was used as antimicrobial agent. Distilled water and ethanol (95 %, Synth, Brazil) were  
113 used as solvents for the filmogenic solutions. Carbon dioxide (99.998 %, White Martins,  
114 Portugal) was employed as solvent in the SSI experiments. Silica gel (Sigma Aldrich, Brazil),  
115 potassium sulfate (99 %, Sigma Aldrich, Portugal) and sodium chloride (99.5 %, Sigma Aldrich,  
116 Brazil) were used to create the controlled relative humidity (RH) conditions.

117

## 118 *2.2 Film preparation*

119 Firstly, Biocomposite Active Films (BAF) were produced by the solvent casting method: the  
120 filmogenic solution was prepared by mixing 0.8 g of cinnamaldehyde with 1.0 g of glycerol at 38  
121  $\pm 2$  °C, using a magnetic stirrer. This mixture was further homogenized with a solution previously  
122 prepared according to Souza et al. (2012), with 0.1 g of clay nanoparticles, 5.0 g of cassava starch  
123 and 95 g of distilled water. After cooling, this solution was diluted with 14.25 g of ethanol and  
124 poured onto cylindrical plates and dried at  $(35 \pm 2)$  °C for (18-24) h, in an oven with forced air  
125 circulation (Nova Ética, series N480, Brazil). The maximum employed amount of antimicrobial  
126 agent was defined in previous work (0.080 g/100g of filmogenic solution) and its incorporation  
127 was only possible using sucrose ester of fatty acids, an emulsifier specific for oil/water emulsions  
128 in order to avoid a phase separation, corresponding to 0.080 for emulsifier content/essential oil  
129 content proportion (Souza et al., 2013).

130 For SSI experiments, Biocomposite Films (BF) were also prepared, according to same procedure  
131 using 1.5 g of glycerol, by solvent casting method, without antimicrobial agent. The BF were  
132 than impregnated with cinnamaldehyde using the SSI technique to produce BAF.

133

## 134 *2.3 Supercritical solvent impregnation (SSI) experiments*

135 The supercritical carbon dioxide (scCO<sub>2</sub>) high pressure equipment used in this work, described in  
136 detail by Sousa, Gil, Leite, Duarte, & Duarte (2006), is comprised of a compressed CO<sub>2</sub> liquid  
137 pump, a sealed high pressure stainless steel vessel with sapphire windows (with an internal  
138 volume of ~10 cm<sup>3</sup>), a temperature-controlled bath ( $\pm 0.1$  °C), a pressure transducer and a  
139 magnetic stirring plate placed under the cell (working at 300 rpm). Briefly, an experimental SSI  
140 assay, at batch mode, consists in introducing liquefied CO<sub>2</sub> into the thermostated high pressure  
141 cell until the desired operational pressure is achieved. The cell was previously loaded with  
142 squared film samples to be impregnated (6 samples of 1.2 cm  $\times$  1.2 cm) fixed by stainless steel  
143 supports and with 0.20 mL of cinnamaldehyde. The employed amount of antimicrobial agent was  
144 calculated taking into account the solubility of the antimicrobial agent at the operational  
145 conditions (Baseri, Haghghi-Asl, & Lotfollahi, 2010), the volume of the cell and the weight of  
146 the films (approximately 50 mg for each sample). A magnetic stirrer was also placed into the cell  
147 to promote the solubility of cinnamaldehyde in the scCO<sub>2</sub> phase. The operational conditions  
148 tested were (150 and 250) bar at 35 °C and for two impregnation times (3 and 15) h and at two  
149 depressurization rates (1 and 10) bar·min<sup>-1</sup> (Table 1). These working conditions were previously  
150 defined in the sample films using only scCO<sub>2</sub>.

151 Before impregnation tests, all samples were stored at a controlled relative humidity (RH) of 75  
152 %. After the pre-established impregnation time (the period in which the antimicrobial agent and  
153 the films are in direct contact under pressure), the system was then depressurized and the  
154 impregnated films were stored at -4 °C to avoid cinnamaldehyde sublimation until further  
155 analyses. In order to confirm their stability when subjected to different process conditions  
156 (different pressures and depressurization rates), samples of BF were processed with scCO<sub>2</sub>  
157 without using antimicrobial agent.

158

159 *2.4 Antimicrobial agent quantification*



160 The amount of antimicrobial agent impregnated in the films processed at different tested  
161 conditions was quantified, in duplicate, by UV-Vis spectroscopy (using a spectrophotometer  
162 JASCO, model 530, Japan) measuring at 289 nm, corresponding to the maximum absorption  
163 wavelength of cinnamaldehyde, and using a pre-determined calibration curve. Quantification was  
164 carried out at room temperature ( $\sim 25\text{ }^{\circ}\text{C}$ ) with the films immersed in Milli-Q water (150 mL) for  
165 3 h, which was enough to guarantee that the impregnated antimicrobial agent was completely  
166 released from the films. This method just intended overall quantification of the total amount of  
167 CN impregnated into the cassava starch films for each experimental condition tested. Further  
168 studies should that into account real conditions that may be found considering the envisaged  
169 applications.

170

#### 171 *2.5 Fourier transform infrared (FTIR) spectroscopy*

172 In order to verify the incorporation of cinnamaldehyde in the films, FTIR spectroscopy (Jasco,  
173 model 4200, UK) was performed at 128 scans with a  $4\text{ cm}^{-1}$  resolution, between (500 and 4000)  
174  $\text{cm}^{-1}$ , and using a Golden Gate Single Reflection Diamond ATR accessory. Samples were  
175 analyzed before and after the impregnation experiments.

176

#### 177 *2.6 Scanning electron microscopy (SEM)*

178 Samples were mounted on aluminium stubs, coated with a thin layer of gold (approximately 300  
179 Å) and observed on Scanning Electron Microscope at an accelerate voltage of 5.00 kV (Philips,  
180 model XL-30 FEG) and at an accelerate voltage of 10.00 kV (Jeol, model JSM-5310).

181

#### 182 *2.7 Water vapor permeability (WVP)*

183 The water vapor transmission rate (*WVTR*) was measured by a gravimetric method based on the  
184 ASTM E96/E96M-10 (2010), using the Desiccant Method, with modifications for hydrophilic  
185 edible films, proposed by McHugh, Avena-Bustillos and Krochta (1993). The water vapor

186 permeability ( $WVP$ ) [ $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$ ] was determined as the rate of water vapor  
 187 transmission ( $WVTR$ ) through a unit area of flat material of unit thickness induced by the unit  
 188 vapor pressure difference between two surfaces, under specified humidity and temperature  
 189 conditions. The thickness of the films was measured using a flat parallel surface micrometer  
 190 (MITUTOYO SulAmericana Ltda., model 103-137, Brazil, precision 0.002 mm), at five random  
 191 positions of each sample. A test tube was covered with the film sample and sealed with a holed  
 192 screw cap (circular opening of  $0.6\text{ cm}^2$ ). Silica gel was placed inside the test tube and sodium  
 193 chloride saturated solution (75 % relative humidity, RH) was used in the desiccators to maintain  
 194 the required RH gradient across the film. The system was maintained inside a bath at  $23\text{ }^\circ\text{C}$ . Two  
 195 cells without silica gel were prepared and submitted to the same conditions to account for weight  
 196 changes occurring in the film, since it is a highly hydrophilic material. The RH inside the test  
 197 tube was always lower than outside, and the water vapor transmission was determined from the  
 198 weight gain of the test tube. After steady state conditions were reached (about 2 h), weight  
 199 measurements were made over 48 h at fixed time intervals.  $WVP$  was calculated according  
 200 Equation 1:

$$201 \quad WVP = \left(\frac{w}{\theta}\right) \times \left[\frac{24 \times \delta}{A \times \Delta p}\right] \quad (1)$$

202 wherein:  $WVP$  is the water vapor permeability [ $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{kPa}^{-1}$ ];  $w$  is the weight gain (from  
 203 the straight line) [g];  $\theta$  is the time during which  $w$  occurred [h];  $\delta$  is the average film thickness  
 204 [mm];  $A$  is the test area (cell top area) [ $\text{m}^2$ ] and  $\Delta p$  is the vapor pressure difference [kPa]. All  
 205 samples were evaluated in triplicate.

206

## 207 2.8 Water vapor sorption (WVS)

208 Films were cut into quadrangular ( $1\text{ cm} \times 1\text{ cm}$ ) samples and dried at  $40\text{ }^\circ\text{C}$  until constant weight  
 209 was achieved. Dried samples were then exposed to an atmosphere of 95 % RH (at  $23\text{ }^\circ\text{C}$ ) in a

210 desiccator containing a potassium sulfate saturated solution. Samples were weighed at pre-  
211 determined time intervals and the water vapor sorption loading was calculated as:

$$212 \quad WVS = \left( \frac{w_t - w_0}{w_0} \right) \times 100 \quad (2)$$

213 wherein:  $w_0$  [g] and  $w_t$  [g] are the sample weight at the beginning of the experiment (dried) and at  
214 time  $t$ , respectively. Films impregnated with cinnamaldehyde at different conditions were  
215 submitted to tests in triplicate and non processed films were also tested for comparison.

216

### 217 *2.9 Water contact angle*

218 Static contact angle measurements were performed using milli-Q water at room temperature (~  
219 23 °C), and using the sessile-drop method (10  $\mu$ L) (Dataphysics Instruments, OCA 20, Germany).

220

### 221 *2.10 Thermal analysis*

222 Thermal events were determined by differential scanning calorimetry (TA Instruments, Q200  
223 MDSC, USA) at the temperature-modulated mode (modulate  $\pm 0.32$  °C every 60 s). Calibration  
224 was performed with indium and sapphire. Non-processed and scCO<sub>2</sub> processed samples (~ 7 mg  
225 weight) were placed in aluminum pans and submitted to a temperature program, under nitrogen  
226 atmosphere in dynamic mode (50 mL·min<sup>-1</sup>). In the first scan, after cooling the sample at  
227 10 °C·min<sup>-1</sup> up to -60 °C, it was submitted to heating at 10 °C·min<sup>-1</sup> until 100 °C. The second  
228 scan was between -60 °C and 250 °C, at the same cooling and heating rates. The glass transition  
229 temperature ( $T_g$ ) [°C] was calculated as the middle point between the onset and end temperatures  
230 caused by the discontinuity of sample specific heat. Two replicate runs were carried out for each  
231 sample.

232

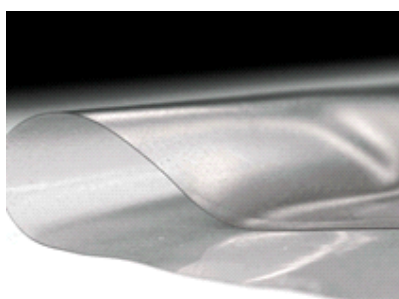
### 233 *2.11 Statistical analysis*

234 Analysis of variance (ANOVA) was applied on the results using the statistical program  
235 Statgraphics Centurion v.15.0 (StatPoint<sup>®</sup>, Inc., USA) and the Tukey test was used to evaluate  
236 average differences (at a 95 % of confidence interval).

237

### 238 **3. Results and discussion**

239 Biocomposite active films produced by the traditional method (casting) were homogeneous,  
240 transparent and flexible, and their surfaces were smooth, without pores and cracks, or insoluble  
241 particles (Fig. 1).



242

243

244 **Fig.1.** Cassava starch film incorporated with antimicrobial agent by casting (photo credit:  
245 Eduardo de Oliveira, FAPESP).

#### 246 *3.1 Antimicrobial agent quantification*

247 The amount of cinnamaldehyde ( $q_{CN}$ ) impregnated into cassava starch films at different process  
248 conditions is presented in Table 1 and is represented in terms of mass of impregnated  
249 cinnamaldehyde per mass of cassava films after impregnation ( $g_{CN}/g_{film}$ ). The values represent the  
250 total released amount for a period of 3 h after immersion of the loaded samples into water. After  
251 this period, samples were immersed in fresh water to guarantee that all the impregnated CN was  
252 released from the films. ANOVA was applied in order to verify the influence of the tested  
253 process parameters (pressure,  $P$ , impregnation time,  $t_i$  and depressurization rate,  $d_r$ ) on the  
254 amount of CN impregnated and indicated that the impregnation time did not significantly  
255 influenced the results ( $P>0.05$ ). The obtained results show that at lower pressure (Table 1) the

256  $q_{CN}$  impregnated into cassava starch films is similar, independently of the experimental  
 257 conditions used, and varied from  $(1.01 \pm 0.31)$   $\text{mg}_{CN}/\text{g}_{film}$  for the higher depressurization rate up  
 258 to  $(1.29 \pm 0.13)$   $\text{mg}_{CN}/\text{g}_{film}$  for the lower depressurization rate. However, this tendency was not  
 259 observed at 250 bar and the highest  $q_{CN}$  value  $(2.50 \pm 0.20)$   $\text{mg}_{CN}/\text{g}_{film}$  was observed with a  
 260 depressurization rate of the  $10 \text{ bar}\cdot\text{min}^{-1}$ , during 15 h of processing.

261 **Table 1.**

262 Total cinnamaldehyde impregnated amount ( $q_{CN}$ ), water vapor permeability ( $WVP$ ) and  
 263 equilibrium water vapor sorption ( $WVS_e$ ) of the biodegradable films impregnated with  
 264 cinnamaldehyde at different process conditions ( $P$ ,  $t_i$ ,  $d_r$ ) of Supercritical Solvent Impregnation  
 265 (SSI) experiments at the temperature of 35 °C.

$P$	$t_i$	$d_r$	$q_{CN}$	$WVP$	$WVS_e$
[bar]	[h]	[ $\text{bar}\cdot\text{min}^{-1}$ ]	[ $\text{mg}_{CN}/\text{g}_{film}$ ]	[ $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$ ]	[%]
150	3	1	$1.29 \pm 0.13$ <sup>1aA</sup>	$5.28 \pm 0.48$ <sup>1aA</sup>	$46.75 \pm 0.98$ <sup>1aA</sup>
150	3	10	$1.01 \pm 0.31$ <sup>1aB</sup>	$5.19 \pm 0.41$ <sup>1aA</sup>	$56.40 \pm 5.19$ <sup>1aB</sup>
150	15	1	$1.19 \pm 0.16$ <sup>1aA</sup>	$5.57 \pm 0.55$ <sup>1aA</sup>	$43.29 \pm 5.43$ <sup>2aA</sup>
150	15	10	$1.22 \pm 0.09$ <sup>1aB</sup>	$5.16 \pm 0.41$ <sup>1aA</sup>	$48.53 \pm 1.41$ <sup>2aB</sup>
250	3	1	$1.37 \pm 0.24$ <sup>1bA</sup>	$6.22 \pm 0.27$ <sup>2bA</sup>	$49.34 \pm 1.35$ <sup>1aA</sup>
250	3	10	$1.74 \pm 0.19$ <sup>1cB</sup>	$6.01 \pm 0.51$ <sup>2bA</sup>	$58.66 \pm 1.82$ <sup>1aB</sup>
250	15	1	$0.99 \pm 0.23$ <sup>1bA</sup>	$5.64 \pm 0.69$ <sup>3aA</sup>	$36.59 \pm 0.71$ <sup>2aA</sup>
250	15	10	$2.50 \pm 0.20$ <sup>1cB</sup>	$4.09 \pm 0.84$ <sup>3aA</sup>	$47.62 \pm 5.94$ <sup>2aB</sup>
Tukey HSD*			0.25	0.62	3.95

266  $P$  pressure [bar]

267  $t_i$  impregnation time [h]

268  $d_r$  depressurization rates [ $\text{bar}\cdot\text{min}^{-1}$ ]

269 \* Means with the same lowercase, in the same column, at the same  $P$ , are not significantly different ( $P < 0.05$ )

270 Means with the same uppercase, in the same column, at the same  $d_r$ , are not significantly different ( $P < 0.05$ )

271 Means with the same number, in the same column, at the same  $t_i$ , are not significantly different ( $P < 0.05$ )

272

273 All these results indicate that the SSI of CN into cassava starch films is mainly dependent on its  
274 solubility in scCO<sub>2</sub> which is higher at 250 bar. According to data previously reported in literature  
275 (Baseri et al., 2010), the solubility of CN increases with pressure about 40 % when the solvent  
276 density increases from 840 kg·m<sup>-3</sup> to 920 kg·m<sup>-3</sup> (values similar those used in this work, which  
277 were 815 kg·m<sup>-3</sup> at 150 bar and 901 kg·m<sup>-3</sup> at 250 bar, both at 35 °C), data taken from NIST  
278 (2011). In addition, at higher pressures, the scCO<sub>2</sub> swelling and plasticization of most polymers is  
279 usually favored and this will also improve the impregnation/loading efficiency (Kazarian, 2000;  
280 Kikic and Vecchione, 2003). During depressurization, the solubility of CN decreases (which may  
281 favor CN-polymer affinity) and scCO<sub>2</sub> changes to a gas, and is vented, leaving the antimicrobial  
282 agent trapped inside the matrix. Moreover, the lower  $q_{CN}$  observed at lower depressurization rates  
283 (for both pressures) is because, at this condition, part of the CN trapped into the matrix re-  
284 solubilize into scCO<sub>2</sub> and escapes with it decreasing the loading efficiency.

285 Finally, and as expected, at higher depressurization rates, the use of higher processing times  
286 improved the sorption capacity of scCO<sub>2</sub> into the matrix and consequently the  $q_{CN}$ . Therefore it is  
287 possible to control the  $q_{CN}$  into cassava starch films by changing the impregnation process  
288 conditions. In this work and for the tested experimental conditions, it was possible to increase the  
289 amount of  $q_{CN}$  from  $(0.99 \pm 0.23)$  to  $(2.50 \pm 0.20)$  mg<sub>CN</sub>/g<sub>film</sub> by increasing the depressurization  
290 rate.

291 The general tendency is that lower pressure and higher depressurization rates lead to lower  
292 impregnated amounts while higher pressure and higher depressurization rates favour CN loading.  
293 However, the results obtained in this work are already promising since it was found that the  
294 maximum  $q_{CN}$  obtained by the incorporation of this antimicrobial agent into the filmogenic  
295 solution of the film produced by casting technique was  $(1.19 \pm 0.02)$  mg<sub>CN</sub>/g<sub>film</sub> (Souza et al.,  
296 2013), indicating that the SSI method can represent an effective alternative if higher impregnated  
297 amounts are required. Moreover, in the same work, authors concluded that samples containing  
298 1.19 mg<sub>CN</sub>/g<sub>film</sub> could inhibit selected fungi (*Penicillium commune* and *Eurotium amstelodami*),

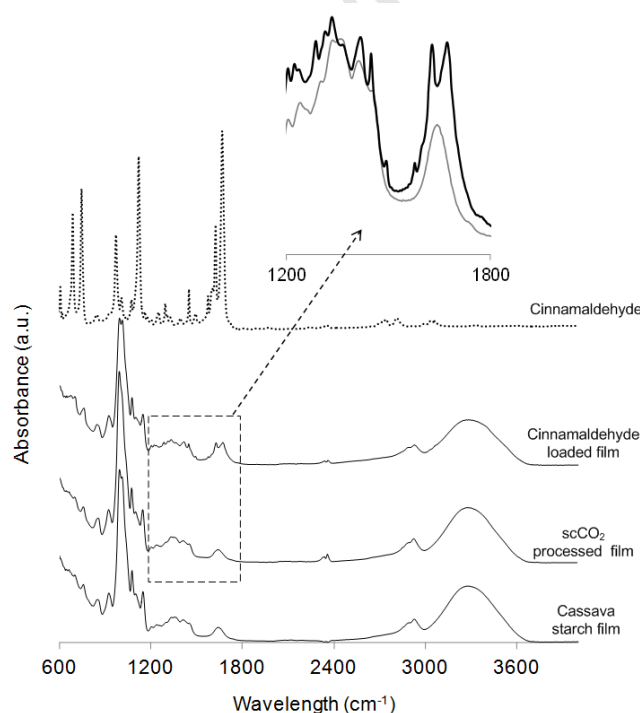
299 commonly found in bread products. In this way, it can be concluded that all conditions studied in  
 300 the present work were able to provide films that are active against the referred microorganisms.

301

### 302 3.2 Fourier transform infrared (FTIR) spectroscopy

303 The incorporation of CN into the films was also confirmed by FTIR-ATR as shown in Figure 2.

304 The spectra of impregnated and of non-impregnated starch based films show significant  
 305 differences in the range  $(1200 - 1400) \text{ cm}^{-1}$  and  $(1600 - 1700) \text{ cm}^{-1}$  attributed to vibrations of the  
 306 aromatic ring and to the aldehyde group of cinnamaldehyde, confirming that the antimicrobial  
 307 agent was effectively impregnated into the films. As expected,  $\text{scCO}_2$  did not induce any  
 308 chemical modification to the matrix and therefore the spectra of non-processed and  $\text{scCO}_2$   
 309 processed cassava starch films are similar.



310

311 **Fig. 2.** FTIR-ATR spectra of cassava starch films impregnated with and without cinnamaldehyde

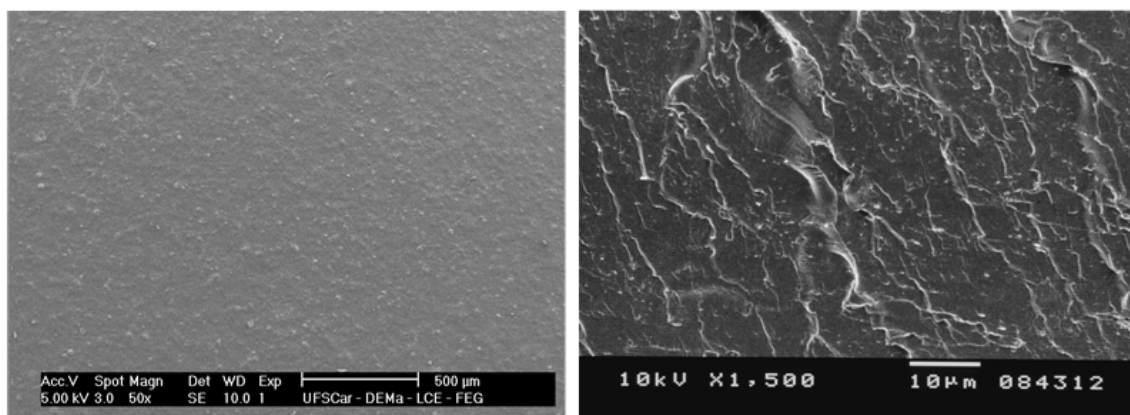
312 processed at 250 bar during 15 h and at a depressurization rate of  $10 \text{ bar} \cdot \text{min}^{-1}$  in comparison

313 with non-processed films and the spectrum of pure cinnamaldehyde.

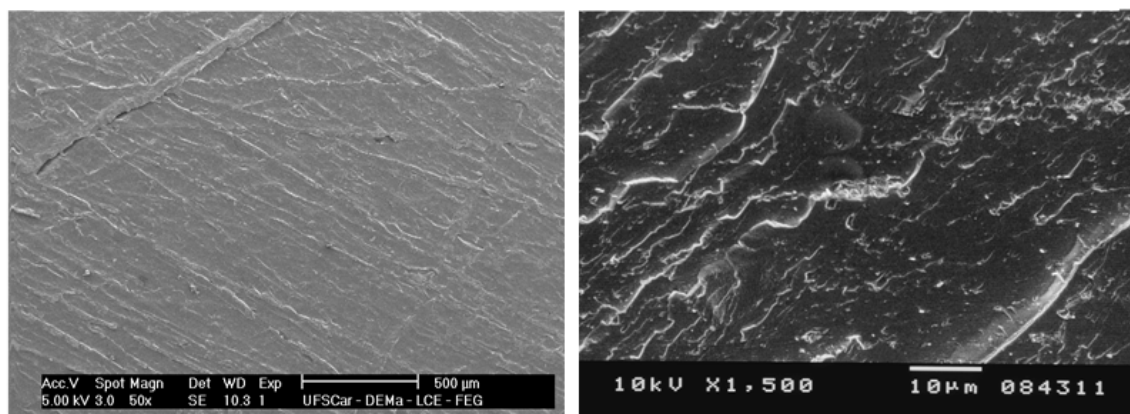
## 314 3.3 Scanning electron microscopy (SEM)

315 It was also observed by SEM that the process do not alter/damage the polymer structure. The  
 316 surface and cross-section images of non-processed and CN impregnated films are shown in  
 317 Figure 3, where it is possible to observe some changes in the film surface, probably due to  
 318 deposition of CN that may occur during depressurization, but the inexistence of bubbles or any  
 319 other deformation in both cases.

Cassava starch films processed with scCO<sub>2</sub> at 250 bar for 15 h and higher depressurization rate (10 bar·min<sup>-1</sup>)



Cassava starch films loaded with cinnamaldehyde at 250 bar for 15 h and higher depressurization rate (10 bar·min<sup>-1</sup>)



320

321 **Fig. 3.** SEM micrographs for non-loaded (top) and cinnamaldehyde-loaded films at 250 bar  
 322 during 15 h and higher depressurization rate (10 bar·min<sup>-1</sup>) (bottom). Left side figures represent  
 323 surfaces while right side figures represent cross sections.

324

## 325 3.4 Water vapor permeability (WVP)



326 ANOVA applied on the water vapor permeability (*WVP*) results presented in Table 1 indicated  
327 that only the interaction of pressure (*P*) with impregnation time (*t<sub>i</sub>*) influenced significantly this  
328 property. At 3 h of *t<sub>i</sub>* the *WVP* increased as pressure increased, whereas at 15 h of *t<sub>i</sub>* the *WVP*  
329 decreased as pressure increased. Films processed with scCO<sub>2</sub> without cinnamaldehyde at 150 bar  
330 (Table 2) present *WVP* values that varied from  $(11.64 \pm 0.50) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$  to  $(13.01 \pm$   
331  $0.44) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$ . These values are similar to those obtained for films processed with  
332 scCO<sub>2</sub> without cinnamaldehyde at 250 bar (Table 2) that varied from  $(12.17 \pm 1.04) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$   
333  $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$  to  $(13.03 \pm 1.19) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$ . For comparison, ANOVA indicated that the  
334 impregnation processing significantly decreased the *WVP* from average value of  $(5.40 \pm 0.65)$   
335  $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$  for films with cinnamaldehyde, to  $(12.63 \pm 0.49) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$  for  
336 scCO<sub>2</sub> processed films without cinnamaldehyde. These results indicate that the treatment with  
337 scCO<sub>2</sub> caused an increase in *WVP* values, which was equal to  $(10.09 \pm 0.35) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$   
338  $\text{g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$  before processing. This may be probably due to the formation of micro-porosities (not  
339 detected by SEM) in the polymer structure caused by the dissolution/release of scCO<sub>2</sub> into/from  
340 the film, originating materials that are more permeable to the water vapor.

341 As already mentioned, ANOVA applied on these results indicated that the tested process  
342 conditions did not significantly affect ( $P>0.05$ ) the *WVP* of films impregnated with  
343 cinnamaldehyde. However, a significant and positive influence of the process on the *WVP* of the  
344 films was observed after cinnamaldehyde incorporation by SSI since impregnated films present  
345 values that are almost half that observed for films without impregnation. It can also be noticed  
346 that the different cinnamaldehyde loaded amounts resulted in a significant decrease in *WVP* of  
347 the films. The *WVP* of the films loaded with the antimicrobial agent by the conventional  
348 methodology was equal to  $(9.78 \pm 0.45) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{day}^{-1}\cdot\text{kPa}^{-1}$ , which is almost 140 % higher  
349 than the value obtained by the SSI method (at 250 bar, 15 h of impregnation and 10 bar.min<sup>-1</sup> of  
350 depressurization rate). This is a very interesting result for food packaging applications for which

351 it is important to maintain *WVP* as low as possible, or at least to reduce moisture transfer between  
 352 food and atmosphere (Souza et al., 2012).

353 **Table 2.**

354 Water vapor permeability (*WVP*) of biodegradable films submitted to Supercritical Solvent  
 355 Impregnation (SSI), with and without antimicrobial agent, according to pressure (*P*), time of  
 356 impregnation (*t<sub>i</sub>*) and depressurization rate (*d<sub>r</sub>*).

		<i>WVP</i> [g·mm·m <sup>-2</sup> ·day <sup>-1</sup> ·kPa <sup>-1</sup> ]					
<i>P</i> [bar]		150			2		
<i>t<sub>i</sub></i> [h]		3		15		3	
<i>d<sub>r</sub></i> [bar·min <sup>-1</sup> ]		1	10	1	10	1	10
	with	5.28±0.48 <sup>aA</sup>	5.19±0.41 <sup>aA</sup>	5.57±0.55 <sup>aA</sup>	5.16±0.41 <sup>aA</sup>	6.22±0.27 <sup>aA</sup>	6.01±0.51 <sup>aA</sup>
	without	13.01±0.44 <sup>aB</sup>	12.81±1.07 <sup>aB</sup>	11.64±0.50 <sup>aB</sup>	12.96±1.13 <sup>aB</sup>	12.57±0.86 <sup>aB</sup>	12.88±1.24 <sup>aB</sup>

357 *P* pressure [bar]

358 *t<sub>i</sub>* impregnation time [h]

359 *d<sub>r</sub>* depressurization rates [bar·min<sup>-1</sup>]

360 \* Means with the same lowercase, in the same row, are not significantly different (*P*<0.05)

361 Means with the same uppercase, in the same column are not significantly different (*P*<0.05)

362

362  
363 The decrease in *WVP* data observed for films impregnated by SSI may be due to: *i*) the  
364 homogeneous dispersion of CN through the matrix including surface, as indicated by SEM,  
365 which acts as a barrier against water vapor; *ii*) possible rearrangement of starch polymeric chains  
366 induced by the SSI process and; *iii*) loss of glycerol during the system depressurization,  
367 considering its solubility in scCO<sub>2</sub> that varies from 44 g·m<sup>-3</sup> of CO<sub>2</sub> at 150 bar to 57 g·m<sup>-3</sup> at 250  
368 bar (Sovová, Jez, & Khachatryan, 1997). Previous data reported in the literature showed that the  
369 *WVP* of cassava starch based films significantly decreases when lower amount of glycerol is  
370 added as plasticizer (Souza et al., 2012).

371 Taking into account that the CN impregnated films developed in this work envisage food  
372 applications as packages it is important to guarantee that the proposed alternative processing  
373 methodology do not alter the water barrier properties of cassava starch films, namely their water  
374 vapor sorption and permeability capacities. These values may vary significantly depending on the  
375 specific application however typical values range between  $(3.81 \pm 0.58) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{kPa}^{-1}$  and  
376  $(7.81 \pm 0.58) \text{ g}\cdot\text{mm}\cdot\text{m}^{-2}\cdot\text{d}^{-1}\cdot\text{kPa}^{-1}$  (Souza et al., 2012).

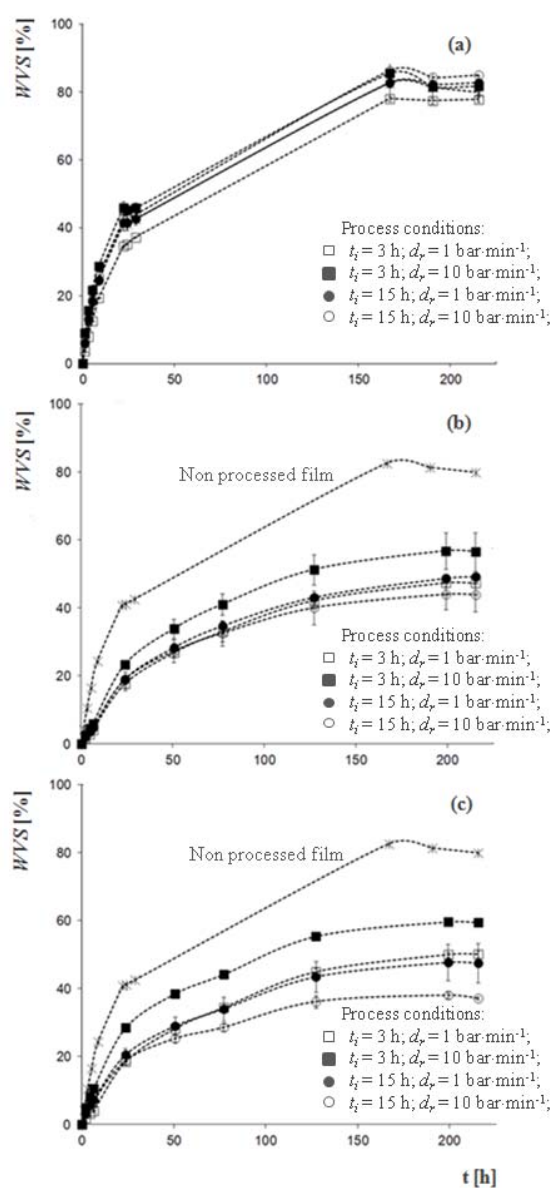
377

### 378 3.5 Water vapor sorption (WVS)

379 Water vapor sorption curves (*WVS*) of the films with and without cinnamaldehyde are shown in  
380 Figure 4. It can be observed that films processed by SSI presented lower water vapor sorption in  
381 comparison to the non-processed ones.

382 ANOVA indicated that impregnation time ( $t_i$ ) and depressurization rate ( $d_r$ ) influenced  
383 significantly this property (Table 1). As impregnation time increases the *WVS* decreases, whereas  
384 at the same  $t_i$ , it increases with the depressurization rate. It can be observed in Figure 4a that the  
385 *WVS* of the films processed with scCO<sub>2</sub> at 250 bar achieved the equilibrium after about 9 days  
386 (220 h), presenting values between (78 and 85) % for all the tested conditions. A significant  
387 difference ( $P > 0.05$ ) was observed for films impregnated with CN, which present lower

388 equilibrium water vapor sorption ( $WVS_e$ ) that ranged from (44 to 57) % for films processed at 150  
 389 bar (Figure 4b) and from (37 to 59) % for those processed at 250 bar , after 9 days (Figure 4c).  
 390 These values correspond to a decrease of the  $WVS_e$  by (30 to 50) % depending on the  $scCO_2$   
 391 processing conditions and when compared to non-processed and non-loaded films . These results  
 392 showed that the impregnation/deposition of cinnamaldehyde into the films increased their  
 393 hydrophobicity and, consequently, decreased their capacity to adsorb water, which is a significant  
 394 improvement considering the envisaged applications.



395

19

396 **Fig. 4.** Water vapor sorption loading of scCO<sub>2</sub> processed films at 250 bar (a) and films  
397 impregnated with cinnamaldehyde at 150 bar (b) and at 250 bar (c).

398

### 399 3.6 Water contact angle

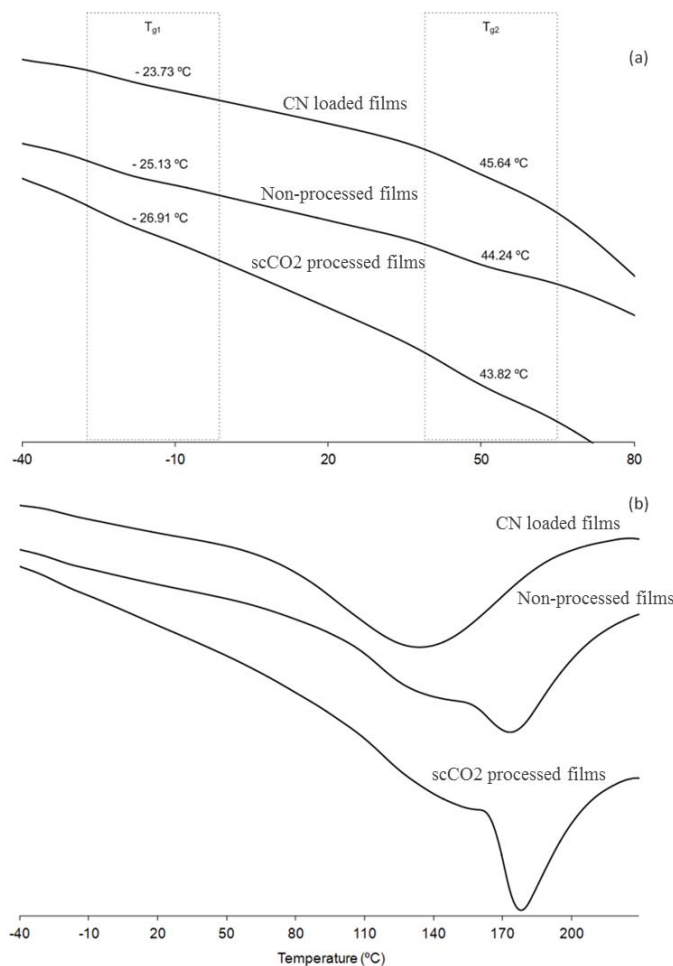
400 The higher hydrophobicity of the films impregnated with CN was confirmed by measuring their  
401 surface water contact angles, which were equal to  $(55.1 \pm 2.5)^\circ$  and  $(76.8 \pm 2.1)^\circ$  for non-  
402 processed and CN impregnated samples, respectively.

403

### 404 3.7 Thermal analysis

405 The effect of the process on the glass transition temperature ( $T_g$ ) of the films (and indirectly on  
406 their flexibility) was accessed by DSC analysis. Data represented in Figure 5a (thermogram  
407 recorded during the first heating) permits to identify two glass transition temperatures around  
408  $-25^\circ\text{C}$  and  $45^\circ\text{C}$ , with the lowest probably indicating phase separation between the starch-rich  
409 and the glycerol-rich phase, as previously reported by Forssell, Mikkilti, Moates and Parker  
410 (1997). The second  $T_g$  is between the average values previously reported by the authors (Souza et  
411 al., 2012) probably because of the larger amount of glycerol that was used to compensate possible  
412 glycerol removal during scCO<sub>2</sub> processing. These  $T_g$  values are identified for all samples  
413 indicating that processing did not significantly affect the thermal properties of the films. This  
414 relatively high  $T_g$  values, considering the final application, and the tenuous transitions detected  
415 are because samples were stored at relative humidity of  $\sim 20\%$  before measurements to reduce  
416 the plasticizing effect of water (Mali, Sakanaka, Yamashita, & Grossmann, 2005; Perdomo et al.,  
417 2009). Data recorded during the second heating run (Figure 5b) shows that the melting  
418 temperatures ( $T_m$ ) increased from  $173.16^\circ\text{C}$  for the non-processed and non-impregnated samples  
419 to  $177.63^\circ\text{C}$  for the scCO<sub>2</sub> processed sample (at 250 bar during 15 h and with highest  
420 depressurization rate of the  $10\text{ bar}\cdot\text{min}^{-1}$ ) and decreased to  $133.69^\circ\text{C}$  for the sample impregnated  
421 with CN at the same experimental conditions. These results show that scCO<sub>2</sub> processing did not

422 significantly affect the melting temperature of the sample and induced a slight decrease (~ 12.5  
423 %) of the associated enthalpy,  $\Delta H_m$  (that changes from 77.6 J·g<sup>-1</sup> to 67.9 J·g<sup>-1</sup>). This decrease  
424 indicates that the process may induce a reorganization of the polymer chains meaning that  
425 dissolved CO<sub>2</sub> besides induce swelling of the amorphous structure, may also lead to a reduction  
426 of the size of the crystalline domains. This information is consistent with that previously reported  
427 by Muljana, Picchioni, Heeres and Janseen (2009) when studying the effect of scCO<sub>2</sub> on the  
428 gelatinization of potato starch. When comparing with CN loaded samples, a significant decrease  
429 in the  $T_m$  is observed (~ 40 °C) which may indicate that the presence of CN interrupted the  
430 rearrangement of polymer chain due to the bulky benzene structure of the molecule. In this case,  
431 the melting endothermic peak seems to be completely overlapped with that corresponding to  
432 water evaporation and, therefore,  $\Delta H_m$  was not calculated in this case. However it is important to  
433 refer that the peak correspondent to bounded-water evaporation (that was observed at 132.95 °C  
434 as confirmed by TGA, data not shown) appears at a lower temperature since loaded samples  
435 absorb lower water amounts as previously discussed.



436

437 **Fig. 5.** DSC thermograms of cassava starch based films obtained during the first (a) and second  
 438 (b) heating steps impregnated with cinnamaldehyde in comparison with non-processed films and  
 439 scCO<sub>2</sub> processed films at the same experimental conditions (pressure of 250 bar during 15 h and  
 440 at the highest depressurization rate of the 10 bar·min<sup>-1</sup>).

441

#### 442 4. Conclusions

443 The main objective of this work was successfully achieved since biocomposite films based on  
 444 cassava starch were incorporated with an antimicrobial agent (cinnamaldehyde) using  
 445 supercritical solvent impregnation originating active films with good properties for the envisaged  
 446 applications. The highest CN impregnated amount ( $2.49 \pm 0.30$ ) mg<sub>CN</sub>/g<sub>film</sub> was obtained at  
 447 higher pressure (250 bar), higher impregnation time (15 h) and at higher depressurization rate

448 (10 bar·min<sup>-1</sup>) indicating that the solubility of CN in scCO<sub>2</sub> is the main factor ruling the  
449 impregnation process. However all the other tested conditions permitted to impregnate an amount  
450 of CN that previously proved to inhibit *P. commune* growth. Moreover it was found that the best  
451 impregnation condition also lead to a significant decrease in the films water vapor permeability  
452 that changed from (10.09 ± 0.35) g·mm·m<sup>-2</sup>·day<sup>-1</sup>·kPa<sup>-1</sup> for the non-processed films to (4.09 ±  
453 0.84) g·mm·m<sup>-2</sup>·day<sup>-1</sup>·kPa<sup>-1</sup>, for CN loaded films. The equilibrium water vapor sorption capacity  
454 of the loaded films was also significantly decreased due to an increase of the surface films  
455 hydrophobicity. The results establish that films based on plasticized cassava starch reinforced  
456 with clay nanoparticles and incorporated with cinnamaldehyde can be considered as an  
457 interesting biocomposite alternative packaging material. All together these results are  
458 encouraging and suggest that SSI may be an interesting alternative to process cassava starch  
459 based films when compared with conventional methods.

460

461



461

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466

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## Highlights

- Development of biodegradable film with cinnamaldehyde.
- Supercritical Solvent Impregnation (SSI) to produce active films.
- Total cinnamaldehyde impregnated depends on CO<sub>2</sub> solubility.
- Water vapor permeability of the films decreased by SSI.

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