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Hydrogels based on ozonated cassava starch: effect of ozone processing and gelatinization conditions on enhancing printability for 3D-printing applications

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Abstract

Ozone is an interesting alternative for modifying starch, as it is considered an emerging and environmentally friendly technology. New applications for food ingredients are receiving attention, such as 3D printing. Consequently, the impact of emerging technologies on new applications must be understood. In this work, cassava starch was modified by ozone to evaluate its printability. Increasing ozonation time produced a starch with higher carbonyl and carboxyl contents, lower pH and molecular size, and gels with different behaviors (stronger and weaker than the native ones, as a function of processing time). The hydrogels obtained were evaluated in relation to pasting and gel properties, including their printability. The effects of starch concentration, gelatinization temperature and storage period were also evaluated. Starch ozonated for 30 min showed the lowest peak apparent viscosity at all the temperatures and starch concentrations evaluated, and provided the strongest gel. Gels produced by native starches and starches ozonated for 30 min showed good printability when the gelatinization temperature used was 65 °C, but up to this temperature, only starch ozonated for 30 min produced gels with good printability. This work highlights that, by using the ozone process to modify starch and varying the process conditions, it is possible to obtain hydrogels with enhanced pasting properties, gel texture, and printability, thereby expanding the potential of starch applications.

Keywords: cassava starch, starch modification, ozone, oxidation, 3D printing.

1 *Hydrogels based on ozonated cassava starch: effect of ozone processing and*
2 *gelatinization conditions on enhancing printability for 3D-printing applications*

3
4 **1. Introduction**

5 Starches are biodegradable biopolymers obtained naturally from different
6 sources, and widely used in different industries, such as foodstuffs, animal feed,
7 chemicals, petrochemicals, textiles, paper, and in other materials. In the search to
8 increase the application potential of starches for different sectors and discover new
9 functionalities, starch modification is conducted using various methods, including
10 chemical, physical, enzymatic and genetic modifications. Ozone technology is
11 considered an innovative chemical treatment for modifying starch, as it is considered a
12 green technology because ozone is rapidly decomposed into oxygen, without leaving
13 residues in food or in the environment [1]. Ozone is a very strong oxidizing agent and is
14 an interesting alternative to the traditional oxidation processes used in the starch
15 industry.

16 Ozone processing can promote starch modification through the cleavage of the
17 glycosidic bonds of both amylose and amylopectin molecules (by decreasing their sizes
18 and distribution), and it also leads to the replacement of hydroxyl groups by carbonyl
19 and carboxyl groups (by changing their charges and chemical affinity), mainly in the
20 amorphous regions of the granules [2]. This process can be used safely in both gaseous
21 and aqueous forms [3].

22 Ozone treatment has shown good results for starch modification and contributes
23 interesting properties for different applications. Chan, Bhat, & Karim (2009) [4] stated
24 that no residues were observed when ozonized starches were introduced into food
25 products. Oladebeye et al. (2013) [5] treated cocoyam and yam starches with ozone, and
26 Klein et al. (2014) [6] worked with cassava starch, although in systems different from
27 that used in the present work. Oladebeye et al. (2013) [5] conducted direct reactions of
28 starch with ozone gas, while Klein et al. (2014) [6] showed that the efficiency of ozone
29 oxidation of starch under aqueous solution is dependent on pH. Amorim et al. (2011)
30 [3] carried out ozone treatment on cassava starch, without observing changes of color;
31 however, they observed changes of odor. Çatal & İbanoğlu (2014) [7] observed that
32 ozone treatment produced a modified wheat starch with higher cooking stability and
33 lower retrogradation tendencies. These authors also reported different pasting
34 properties, as well as structural changes in starch granules. Matta Junior et al. (2019) [8]

35 conducted a mild ozone treatment on cassava starch, resulting in higher clarity, better
36 oven expansion and a softer texture in the biscuits obtained. Castanha et al. (2017) and
37 Castanha et al. (2019) [2,9] studied potato starch processed by ozone. They obtained
38 materials with a low peak apparent viscosity and gels stronger than native starch, thus
39 with characteristics interesting for certain applications, such as the 3D printing
40 highlighted in the present proposal.

41 Among possible applications for starch, 3D printing is gaining attention in the
42 food sector as it can provide new products, such as food structure customization, and
43 customized and digitalized nutrition, thereby broadening the source of food raw
44 materials and shortening the food supply chain [10–12]. According to Azam & Zhang
45 (2018) [13], starch can improve the rheological properties of food matrixes to achieve
46 higher printability for 3D applications and improve their sensory attributes. The
47 literature comprises many works involving the application of native starches for 3D
48 food printing [13–17]. However, the use of modified starches for this application still
49 needs investigation. Besides its application in the food sector, the 3D printing of starch
50 gels has been evaluated and considered for tissue engineering, as a scaffold [18] and for
51 cartilage [19] production. The use of modified starches has not been investigated for 3D
52 printing, though it can expand the range of starch gel applications.

53 Cassava (*Manihot esculenta* Crantz), also called tapioca, yucca or manioc, is a
54 perennial shrub which is currently the sixth most important world food crop, providing
55 sustenance for more than 800 million people in tropical and sub-tropical Africa, Asia
56 and Latin America [20,21]. Cassava presents high tolerance to drought and harsh
57 climatic conditions and around-the-year availability, making it a reliable crop for
58 ensuring food security [22]. Cassava starch, the most essential component of cassava, is
59 commonly used as a thickener and stabilizer in food processing to improve the
60 convenience of the process and quality of final products. It is also used as an additive in
61 the textile and papermaking industries [23]. Another advantage of exploiting cassava as
62 a resource is to greatly increase the choice and diversity of staple foods for a large
63 population, especially as a gluten-free food for people with celiac disease [24].

64 In this context, the aim of this work was to explore the modification of cassava
65 starch by using ozone technology and evaluate the potential of the hydrogels obtained
66 for 3D food printing. To this end, the effect of ozonation was first evaluated in starch
67 structure and properties. Then, the hydrogels obtained from the modified starches were
68 evaluated for their paste and gel properties, including the printability of the gels. The

69 effects of starch concentration, gelatinization temperature and storage period were also
70 evaluated.

71

72 **2. Material and methods**

73

74 ***2.1. Cassava starch and ozone processing***

75 Native cassava starch (Amilogill 1500) was supplied by Cargill Agrícola –
76 Brazil (moisture: 13.2 g/100 g, amylose content: 28.9 g /100 g and amylopectin content:
77 71.1 g /100 g). All the chemicals were of analytical grade.

78 The ozone processing of cassava starch was conducted using a method similar to
79 that described by Castanha et al. (2017) [2], shown in Fig. 1. Ozone processing was
80 carried out in a reactor with cassava starch suspended in distilled water (10 g dry starch
81 / 100 g suspension; 1500 mL) under constant stirring using a magnetic stirrer. Ozone
82 was produced in an ozone generator unit (Ozone & Life, O&G Model RM 3.0, São José
83 dos Campos, Brazil) using the coronal-discharge method from industrial oxygen (95%
84 purity). The ozone-rich gas current was directed to a cylindrical glass reactor (2 L
85 capacity, 56 cm high, and 6 cm internal diameter) via a glass tube ending in a porous
86 gas disperser. The gas flow in the reactor was maintained constant at 1.0 L min⁻¹, and
87 the ozone concentration in the gas current was kept at 43 mg O₃ L⁻¹. The gas flow
88 leaving the reactor was directed to an ozone destructor, which thermally destroys the
89 residual ozone in oxygen. After the ozone treatment, the reactor was supplied with
90 oxygen only for a further 10 min, in order to eliminate the residual ozone. The cassava
91 starch was processed for 15 and 30 min. Native cassava starch, referred to here as “0
92 min” processing, was also considered in the evaluation.

93 After processing, the starch suspension was maintained at rest for decanting, and
94 the supernatant liquid was discarded later. The starch was dried in an air circulation
95 oven (MA 035, Marconi, São Paulo, Brazil) at 35 °C until reaching a moisture content
96 of approximately 12%. The dried starch was then macerated, sifted (250 µm) and stored
97 for further analysis in glass containers.

98

99 **2.2 Molecular characteristics of starch: determination of pH, and carbonyl and**
100 **carboxyl contents**

101 The pH of the starch suspension (10.7 g starch / 100 g suspension) was obtained
102 using a potentiometer (Tecnal, model TEC-5 mode, Piracicaba – Brazil) under constant
103 stirring.

104 Carbonyl and carboxyl contents were obtained according to the method
105 described by Smith (1967) [25] and Chattopadhyay et al. (1997) [26], respectively, with
106 small modifications.

107 For carbonyl content determination, 2 g of sample was dispersed in 100 mL of
108 distilled water and heated in a bath with boiling water for 30 min under constant
109 stirring. The sample was cooled to 40 °C, the pH was adjusted to 3.2 using HCl solution
110 (0.1 M), and 15 mL of hydroxylamine chloride solution (25 g of hydroxylamine
111 hydrochloride diluted in 100 mL of NaOH 0.5 M) was added. The samples were
112 incubated at 40 °C for 4 h and then the excess hydroxylamine was titrated with 0.1 M
113 HCl until pH 3.2 using an automatic titrator (Plus Titrino 848, Metrohm, Herisau,
114 Switzerland). A blank was made without the sample but following the same procedures
115 used for the samples. The carbonyl content was calculated as the amount of carbonyl
116 groups in relation to 100 units of glucose (CO/ 100 GU) according to Equation 1:

117

$$118 \quad \frac{CO}{100 GU} = \frac{(V_B - V_S) \cdot M \cdot 0.028 \cdot 100}{W} \quad (1)$$

119

120 Where:

121 V_s = the volume of NaOH required for the ozonated samples;

122 V_b = the volume of NaOH used in the blank;

123 M = the molarity of NaOH;

124 W = the sample mass in dry basis.

125

126 To determine the carboxyl content, 2 g of sample was dispersed in 25 mL of HCl
127 solution (0.1 M) in water under stirring (25 °C) for 30 min and centrifuged. The residue
128 was filtrated using a vacuum system with a qualitative filter paper (205 µm), and then
129 washed with 400 mL of distilled water. Next, 300 mL of distilled water was added and
130 the dispersion was heated in a bath with boiling water under continuous stirring for 15
131 min. While it was still hot and under stirring, the sample was titrated with 0.01 M

132 NaOH until reaching pH 8.3 using an automatic titrator (Plus Titrimo 848, Metrohm,
133 Herisau, Switzerland). A blank was made with the non-modified starch but following
134 the same procedures used for the other samples. Carboxyl content was expressed as the
135 amount of carboxyl groups in relation to 100 units of glucose (COOH / 100 GU),
136 according to Equation 2:

137

$$\frac{COOH}{100GU} = \frac{(V_s - V_b) \cdot M \cdot 0.045 \cdot 100}{W}$$

138

139 (2)

140 Where:

141 V_b = the volume of HCl used in the blank;

142 V_s = the volume of HCl required for the sample;

143 M = the molarity of HCl;

144 W = the sample mass in dry basis.

145 The analyses were done in triplicate.

146

147 **2.3 Starch molecular characteristics: Molecular size distribution**

148 The molecular size distribution profile of the native starch (control) and
149 ozonated starches (15 and 30 min) were determined using a gel permeation
150 chromatography (GPC) system, according to Song & Jane (2000) [27], with some
151 modifications.

152 A glass column (diameter: 2.6 cm and high: 70 cm) packed with Sepharose CL-
153 2B gel (Sigma, Sweden) was used. A solution with 0.1 g of starch with 10 mL of
154 Dimethylsulfoxide (DMSO; 90%, Labsynth, Brazil) was prepared. The solution was
155 heated in a bath with boiling water for 1 h and then kept at 25° C for 24 h under constant
156 stirring. An aliquot of 3 mL of this starch solution was mixed with 10 mL of absolute
157 ethanol and then centrifuged (30 min, 3000 g). The precipitated starch was dissolved in
158 boiling distilled water (9 mL) and heated in a bath with boiling water for 30 min. This
159 starch solution (4 mL) was added in the chromatographic column with an eluent
160 solution (25 mmol·L⁻¹ of NaCl and 1 mmol·L⁻¹ of NaOH), at a rate of 60 mL·h⁻¹.
161 Aliquots of 4 mL were collected using a fraction collector (Gilson, model FC203B,
162 Middleton, England) and then analysed by the blue value method [28], using a
163 spectrophotometer at a wavelength of 620 nm (Femto, model 600S, São Paulo - Brazil).
164 Glucose was used as marker to indicate the end of the analysis.

165

166 **2.4 Differential scanning calorimetry (DSC)**

167 The thermal characteristics during starch gelatinization were determined by
168 using a Multi-Cell Differential Scanning Calorimeter (MC-DSC) – (TA Instruments,
169 Landon, UTAH, USA). Before analysis, the samples were weighed in the ampoules
170 (total mass (starch + water) so that each contained 700 mg, keeping the relation of 10.7
171 g starch / 100 g). An ampoule with alumina powder was used as reference and three
172 runs for each sample were analyzed. The MC-DSC heating program consisted in going
173 from 30 to 130 °C at a rate of 2 °C/min. The onset temperature (To), the peak
174 temperature (Tp), the conclusion temperature (Tc), and the enthalpy (ΔH) associated
175 with the starch gelatinization interval were calculated with the aid of the Universal
176 Analyzer software (TA Instruments).

177

178 **2.5 Pasting properties**

179 Starch pasting properties were determined using a Rapid Visco Analyzer - RVA-
180 4 (Newport Scientific Pvt. Ltd., Australia, with Thermocline for Windows software,
181 version 3.0).

182 In the second part of this work (characterization of the modified starches
183 obtained), the standard conditions for starch characterization were employed, using a
184 suspension of 10.7 g starch / 100 g (correct to 14% moisture basis) and following the
185 procedure: temperature held at 50 °C for 1 min, then increased to 95 °C (6 °C.min⁻¹ and
186 maintained for 5 min), followed by cooling to 50 °C (6 °C.min⁻¹), and finally holding at
187 50 °C for 2 min.

188 In the third part of this work (evaluation of the hydrogels obtained), the RVA
189 behavior of starch suspensions was characterized using different concentrations (3.6 and
190 10.7 g / 100 g, correct to 14 % moisture basis) and at different gelatinization
191 temperatures (65, 75, 85, and 95 °C). The procedure consisted in keeping the starch
192 suspension at 50 °C for 1 min, then heating it to the specific gelatinization temperature
193 (at 6 °C.min⁻¹), maintaining it at these temperatures for 5 min, followed by cooling to 50
194 °C (6 °C.min⁻¹), and finally maintaining it at 50 °C for 2 min.

195 The starch concentration of 10.7 g starch/100 g suspension is a standard
196 concentration conventionally used to determine the pasting properties of the starch
197 under standard conditions of analysis. However, the standard concentration used in the

198 RVA assays is higher than the regular concentration of industrial applications,
199 especially for food products. Therefore, the starch concentrations analyzed (10.7 and 3.6
200 g g starch/100 g suspension) were used as a tool to compare the behavior of the samples
201 under different conditions, as the standard conditions may not be representative for all
202 industrial applications.

203

204 **2.6 Gel firmness**

205 The hydrogel firmness (strength) was determined in the different starches and
206 conditions of gelatinization, using the gels obtained after the RVA analysis.

207 Immediately after finishing the RVA analysis, the gels were placed in plastic
208 cups (40 mm diameter × 20 mm height) and stored for different periods (1 and 7 days)
209 in the refrigerator (5 ± 2 °C) before texture evaluation. The cups were kept in a
210 desiccator with water at the bottom to ensure uniform moisture.

211 The samples were then removed from the refrigerator and analyzed immediately
212 to ensure a constant temperature. Gel firmness was determined by a puncture assay
213 using a texture analyzer TA TX Plus (Stable Micro Systems Ltd., Surrey, UK) with a
214 load cell of 50 kgf (490.3 N). The samples were penetrated until a distance of 4 mm
215 using a cylindrical probe (P/0.5R, 0.5 cm of diameter) at $1 \text{ mm} \cdot \text{s}^{-1}$. The equipment
216 measured the force as a function of penetration depth. Gel firmness was evaluated by
217 the energy required to penetrate the material (calculated by the area below the curve
218 force versus distance of penetration).

219

220 **2.7 Gel structure: Optical microscopy**

221 The gels obtained by the RVA analysis were observed using a light microscope
222 (model L1000, Bioval, Brazil; with a 20 W halogen lamp, the magnification of 40 x)
223 and a microscope digital camera of 5.1 megapixels (ECMOS C-Mount, Sony, Japão).
224 The material analyzed consisted of the gels obtained immediately after RVA analysis,
225 diluted with distilled water (1:1, v/v) and colored with Lugol's solution. These samples
226 were placed on a glass slide, covered by a glass cover slip and directly evaluated.
227 Images were obtained after verifying their representativeness.

228

229 **2.8 Hydrogel application: evaluating printability**

230 The gels stored for 1 and 7 days with different starch concentrations (3.6 and
231 10.7 g / 100 g suspension) and gelatinization conditions were placed in a plastic syringe

232 with a die diameter of 1.5 mm and extruded over a glass slide for visual evaluation of
233 the gel's consistency and moldability at room temperature. To ensure the reproducibility
234 and veracity of the procedure, manual extrusion using the syringe was carried out ten
235 times (lines with ~ 6 cm), and photographs were taken to identify the most predominant
236 visual aspect of the extruded material. Although simple, this approach allowed
237 evaluating the printability of the hydrogels obtained (Fig. 9), which is the first step
238 toward 3D printing. Based on this approach, several conditions were selected for 3D
239 printing.

240 The selected gels were processed in a 3D printer Stampante 3D (3DRAG V1.2,
241 Futura Elettronica, Italy). The gels were produced following the same proportion of
242 10.7 g starch / 100 g. The suspension was stirred and heated until reaching the desired
243 gelatinization temperature, and maintained in this condition for 5 min. The gels
244 obtained were transferred to the printer syringes (60 mL) that were closed using
245 parafilm plastic, and then stored in a refrigerator (5 ± 2 °C) for 7 days. The syringes
246 were then removed from the refrigerator and immediately processed in the 3D printer: a
247 0.8 mm diameter nozzle was used; the nozzle height was set at 18 mm with a speed of
248 20 mm/s and an extrusion rate of 30 mm/s at 20 °C. The physical model (star) was
249 created using the Repetier Host V2.0.1 and Slic3r software (Hot-World GmbH & Co.
250 KG, Willich, Germany) and the dimensions of the star shape were 5 cm x 5 cm x 2 mm
251 (Length x Width x Height).

252

253 ***2.9 Experimental design and statistical analysis***

254 A completely randomized design was applied with four replicates for each
255 processing condition. To evaluate the differences between the materials analyzed, an
256 analysis of variance (ANOVA) and Tukey's test at a 5% significance level were
257 performed with the software *Statistic 13* (StatSoft, USA).

258

259 **3. Results and discussion**

260 ***3.1 Ozonated cassava starch characterization***

261 **3.1.1 Starch molecular characteristics**

262 According to Oladebeye et al. (2013) [5], carbonyl and carboxyl are crucial for
263 starch oxidation, as they are responsible for the depolymerization of starch chains and
264 the conversion of hydroxyl to carbonyl and carboxyl. Therefore, considering that ozone

265 is a recognized oxidizing agent, these functional groups were determined to identify the
266 extension of oxidation in cassava starch by ozone processing.

267 Fig. 2 shows the pH, carbonyl, and carboxyl contents of the cassava native
268 starch ozonated for 15 and 30 min. The pH decreased, and the carbonyl and carboxyl
269 group contents increased as a function of ozonation processing time, confirming the
270 high level of starch oxidation. The reduction of starch pH can be associated with the
271 higher carboxyl content, a functional group acidic nature.

272 In this work, we produced modified starches with two different oxidation levels.
273 This strategy was aimed at extending the cassava starch application as the proportion of
274 carbonyl and/or carboxyl groups present in oxidized starches directly influences their
275 physicochemical properties, potentially fitting the products for different industrial uses
276 [29].

277 According to Wurzburg (1986) [30], two important reactions occur during starch
278 oxidation: (1) the oxidation of hydroxyl groups to carbonyl and carboxyl groups
279 (already mentioned in item 3.1.1), and (2) the de-polymerization of starch molecules by
280 the cleavage of (1 → 4)- α -D and (1 → 6)- α -D glycosidic linkages. In the case of ozone
281 processing, starch de-polymerization is even more substantial and the oxidation of
282 hydroxyl groups to carbonyl and carboxyl occurs simultaneously [2].

283 The ozonated cassava starches obtained in this work showed slightly higher
284 contents of carbonyl and carboxyl groups than the potato starch ozonized for 30 min
285 (0.11 and 0.04 per 100 g glucose unit for carbonyl and carboxyl contents, respectively)
286 in the work of Castanha et al. (2017) [2].

287 The ozone action mechanism on the starch molecule proposed was schematized by
288 Castanha et al. (2017) [2]. Briefly, the starch molecule undergoes two main
289 transformations: (1) the depolymerization of both amylose and amylopectin, resulting in
290 smaller molecules with a different distribution; and (2) oxidation in the hydroxyl groups
291 resulting in carbonyl and carboxyl groups, promoting different interactions between the
292 molecules.

293 In order to evaluate this potential de-polymerization, the starch molecular size
294 distribution was studied by gel permeation chromatography (Fig. 3). Using this
295 technique, the starch molecules are separated and eluted through a column filled with a
296 gel of known porosity to allow separation based on molecular size: the higher molecular
297 weight fractions are eluted first, since they rarely penetrate the pores, while the lowest
298 molecular weight fractions are retained longer in the column [31]. Therefore, as can be

299 seen in Fig. 3, we can observe two peaks: the first consists of molecules of larger size
300 and more ramifications, which can be associated with amylopectins, while the second
301 peak represent molecules of smaller size and a linear structure, which can be associated
302 with amyloses.

303 The first peak of the ozonated cassava starches decreased, while the second peak
304 increased, according to the increase in ozonation process time. Another observation is
305 that the second peak moved to the right (i.e. eluting later) as a function of the increase
306 in ozonation process time. These results indicate the ozone treatment reduced the size of
307 the molecules, attributable to the cleavage of the glycosidic bonds of the ozonated starch
308 molecules [2]. Sandhu et al. (2012) [32] also obtained similar results when treating
309 wheat starch with ozone gas.

310 Once the ozonation process has changed the molecular structure of the cassava
311 starch, the effects on its properties must be evaluated.

312

313 **3.1.2 Pasting properties and gel firmness**

314 The RVA curves, firmness, and images of the gel obtained for the native and
315 ozonated starch for 15 and 30 min are shown in Fig. 4. Table 1 shows the pasting
316 properties of native and ozonated cassava starches. We can observe that the ozonation
317 process affected the pasting properties of the cassava starch differently when different
318 processing times were applied. The peak apparent viscosity of the ozonated starch for
319 30 min decreased significantly when compared with native and ozonated starch for 15
320 min (Fig. 4C). Castanha et al. (2017) [2] attributed this result to the partial cleavage of
321 the glycosidic bonds promoted by the ozone treatment, as observed in Fig. 3. Therefore,
322 the decrease in the apparent viscosity might reflect the weakening of starch granules,
323 implying a reduction of shear resistance to the point where granule integrity cannot be
324 maintained [33]. Chan et al. (2011) [34] also found a reduction of the peak apparent
325 viscosity for tapioca starch processed with ozone in the gaseous phase.

326 The trough, final, and setback apparent viscosities showed similar tendencies to
327 the peak viscosity, decreasing significantly ($p < 0.05$) as the exposure time to ozone
328 increased. On the other hand, the pasting temperature increased significantly as a
329 function of ozonation process time. Castanha et al. (2017) [2] associated this behavior
330 with the presence of smaller size molecules in the ozonated starches (molecular
331 depolymerization), which need more energy to gelatinize than large size molecules.

332 The Relative Breakdown (RBD) (calculated by the ratio between the values of
333 Breakdown (BD) and the Peak Apparent Viscosity (PAV)) is a parameter which can be
334 associated with the facility of starch granule disruption [2]. The ozonated cassava
335 starches showed higher values for the RBD than the native starch. This can be
336 associated with the fact that the ozone treatment confers a new ability of intermolecular
337 association with the polymers due to the replacement of the hydroxyl group by carbonyl
338 and carboxyl groups (Fig. 2) and a change in molecular size distribution (Fig. 3)
339 [2,34,35].

340 Gel firmness was higher for the cassava starch ozonated for 30 min and lower
341 for the cassava starch ozonated for 15 min, when compared with the native cassava
342 starch (Fig. 4B). This result is very interesting since both stronger and weaker gels can
343 be obtained by changing the process conditions, which can cover different industrial
344 applications. This behavior is once again linked to the changes in both molecular size
345 distribution and chemical affinity, including charges (by oxidation of hydroxyl groups
346 to carbonyl and carboxyl), in molecular interaction and gel formation. Specific
347 molecular size distribution results in different rearrangements, with stronger or weaker
348 interactions, as can be see for the difference between the ozonated samples for 15 and
349 30 min. Besides having a smaller molecular size than the other treatments, the starches
350 ozonated for 30 min are composed of a higher fraction of molecules with similar sizes
351 (Fig. 3), which may have promoted better pairing to form the network in gel. This
352 behavior can be observed in Fig. 4(C and D) which shows the visual consistency of
353 these gels and the energy required to deform them, respectively, in line with the results
354 obtained.

355 From these results, we can see that the paste obtained from ozonated starch is
356 less consistent, and the gel obtained from the starch ozonated for 30 min is the
357 strongest, displaying good behavior for a material intended for 3D printing. However,
358 different aspects such as gelatinization temperature and storage time can change the
359 hydrogel's behavior, making it necessary to carry out a further evaluation.

360

361 **3.1.3 Thermal properties**

362 The DSC curves are shown in Figure 6 and the gelatinization properties of the
363 samples (native and cassava starches modified by ozone) are summarized in Table 2.

364 Fig. 6 shows endothermic peaks, indicating the transition from ordered to
365 disordered structures for all the samples. Regarding the gelatinization parameters in
366 Table 2 (T_o , T_p , T_f and gelatinization enthalpy (ΔH)), significant differences ($p < 0.05$)
367 between the control and the ozonated starches were found. Matta Junior et al. (2019) [8]
368 observed similar values for certain gelatinization parameters of native cassava starch
369 ($T_p = 68.1$ °C and $\Delta H = 13.3$ J/g).

370 The T_o , T_p , and T_f increased as the ozonation processing time increased, which
371 indicates the ozone processing resulted in starch with higher thermal stability. The
372 results obtained in this study suggest that the process of gelatinization, which involves
373 the destruction of starch crystallite and the loss of helical conformation [5], was affected
374 by the ozonation. Regarding the peak temperature (T_p), the values and behavior were
375 similar to those found for the pasting temperature obtained in the RVA analysis (Table
376 1). The enthalpy of gelatinization represents the amount of thermal energy involved in
377 the gelatinization process and can provide an indirect measure of the number of
378 crystalline regions [36,37] and the structure of double helices of the starch broken
379 during heating [38]. The smallest gelatinization enthalpy value (ΔH) found for the
380 starch ozonated for 30 min clearly corroborated the hypothesis that in addition to the
381 depolymerization of starch chains, some double helices disassociated within the
382 crystalline and non-crystalline regions of the starch, leading to a decrease in crystallinity
383 [36,37].

384

385 ***3.2 Effect of gelatinization temperature, starch concentration and storage time on*** 386 ***hydrogel properties***

387 **3.2.1 Pasting properties**

388 Fig. 6 shows the RVA curves for the different starch concentrations at
389 gelatinization temperatures of 65, 75, 85, and 95 °C.

390 We can observe in Fig. 6 that the starch concentration at 3.6% suspension resulted
391 in much lower apparent viscosity (~ 20 x) than when using a 10.7% suspension, as
392 expected. This behavior is related to the smallest interaction between the swollen
393 granules and the continuous phase, as usually occurs at low concentrated suspensions.
394 As the concentration increases, starch–starch interactions become dominant and a larger
395 number of water molecules are immobilized, resulting in an additional increase of
396 apparent viscosity [39].

397 The difference of the apparent viscosity between the modified starches in the
398 lowest starch concentration was not as evident as in the highest concentration. For the
399 starch concentration of 3.6%, the ozonated starches always presented a lower apparent
400 viscosity than the native one, followed by the cassava starch ozonated for 15 min and
401 for 30 min. For the starch concentration of 10.7%, the cassava starch ozonated for 15
402 min showed results similar to those of the native starch when gelatinized at 85 and 95
403 °C.

404 By analyzing the sample's behavior at different gelatinization temperatures, it
405 can be observed that there is an increase in the apparent viscosity when the
406 gelatinization temperature changes from 65 to 75 °C. However, it did not vary
407 significantly between 75 and 95 °C, except for the starch ozonated for 15 min at the
408 higher starch concentration, which showed an increase in apparent viscosity when the
409 temperature changed from 75 to 85 °C. When the starch granules under suspension are
410 heated, the apparent viscosity increases due to the gelatinization process, which occurs
411 through the absorption of a large amount of water, resulting in swelling and increased
412 granule size [40]. The notable change of viscosity between 65 and 75 °C can be
413 associated with the temperature zone for which the starches present the highest increase
414 in swelling power, though for temperatures above 75 °C there were no significant
415 changes to this property.

416 Fig. 7 shows the optical microscopy images used to understand how the starch
417 granules are changed to a gel during the RVA analysis.

418 According to Atkin et al. (1998) [41], starch gelatinization is accompanied by
419 water uptake, swelling, the irreversible disruption of internal molecular order through
420 the dissociation of double helices and the melting of crystallites, the leaching of
421 polysaccharides, the disintegration of swollen granules, and the formation of a paste or
422 gel.

423 At 65 °C, the microscopies of all the starch gels showed swollen granules for
424 both starch concentrations. At 75 °C, native and cassava starch ozonated for 15 min
425 showed apparently swollen starch granules but cassava starch ozonated for 30 min
426 showed less defined granule shapes (less marked surfaces), indicating a more significant
427 breakdown of these structures.

428 At the lowest starch concentration at 85 and 95 °C, the cassava starches
429 presented only “ghosts”; “Ghosts” is name given to “empty shell” fragments of starch
430 granules found in the medium [42]. At the highest starch concentration, at 85 and 95

431 °C, native starch still presented apparent swollen starch granules but the ozonated
432 cassava starches presented only “ghosts”. The same behavior was observed by Castanha
433 et al. (2019) [9], when comparing native potato starch with ozonated potato starches.
434 These authors explained that unmodified granules require more energy to disrupt the
435 bonds of the crystalline region, a finding confirmed by the lower RBD value calculated
436 for the native starch when compared with the ozonated starches. This means that native
437 starches show greater resistance to starch granule disruption.

438 For all the samples, the increasing temperature was positive for the granular
439 rupture and greater amylose leaching, mainly between 65 and 75 °C. As can be seen for
440 the pasting properties, the transition from 65 to 75 °C was more decisive for granule
441 swelling and rupture.

442 Therefore, we obtained starches with different rheological behaviors that may
443 lead to diverse applications. In the case of 3D food printing, the ideal viscosity of the
444 material should be low enough to permit easy extrusion through a small nozzle and high
445 enough to be cohesive without deformation of the layers deposited previously [43].
446 Thus, the starch ozonated for 30 min can be a good alternative to form a gel with lower
447 viscosity at the temperatures evaluated.

448

449 **3.2.2 Gel firmness**

450 This criterion is used to evaluate the strength of the hydrogels produced after
451 RVA. After the starch undergoes gelatinization in the presence of water, the subsequent
452 cooling causes retrogradation, i.e. a process in which the disordered macromolecular
453 components of starch are reorganized (amylose or amylopectin) [44]. Thus, gel firmness
454 depends on the reorganization and network of these structures.

455 Fig. 8 shows the strength of the different hydrogels obtained. Firstly, it is
456 important to compare the order of magnitude of axis y for the starch suspension of 3.6
457 g/ 100 g and 10.7 g /100 g. By increasing the starch content 3 times, a significant
458 increase (~ 2 until 40 x) in gel strength was reached for all the starches (native and
459 ozonated). The higher starch concentration led to an increase in the number of starch
460 molecules per unit volume, and increased the probability of intermolecular hydrogen
461 bonding, leading to a more compact network structure with increased gel strength [15].

462 The storage period had a small effect on the gel strength, which can be
463 interesting when considering industrial product storage. On the other hand, the effect of

464 gelatinization temperature was considerable (although to different extents as a function
465 of the different treatments).

466 For the gels produced with the lowest starch concentration (3.6%), the increase
467 in gelatinization temperature had a small effect on gel strength, with the exception of
468 the native starch from 75°C to 85 °C, which increased ~30%. On the other hand, by
469 increasing the gelatinization temperature, stronger gels were obtained at 10.7%, in
470 particular in the case of the cassava starch ozonated for 30 min. The strongest gel was
471 obtained with 10.7%, with ozonation for 30 min and gelatinization at 95 °C. Its strength
472 was ~4 times higher than the stronger gel obtained from the native starch (gelatinization
473 at 65 °C) and ~10 times the gel obtained from the native starch at the same temperature.
474 This indicates that a specific molecular size distribution and charges were reached in
475 this treatment to ensure better rearrangement, promoting better pairing to form the
476 network in the gel. It is worth mentioning that this result is remarkable from the
477 industrial standpoint.

478 Finally, using three variables: starch type, starch concentration and gelatinization
479 temperature, we observed different behaviors of the gel strength obtained, thus
480 extending the possible applications of gels. Their printability was then evaluated.

481

482 **3.2.3 Evaluating the printability of the gels**

483 Printability, or the potential of a material to be used for printing, requires that the
484 dimensional stability of the material in question prevents it spreading on surfaces, and
485 that it is capable of supporting its own weight during layer-by-layer deposition [11].

486 The gels produced with the lowest starch concentration (3.6 %) were not able to
487 form a straight line when extruded and spread on a surface by a syringe. Therefore, the
488 evaluation was conducted with the gels produced with the highest concentration of
489 starch (10.7%). Fig. 9 shows images of the simulated printing with the gels stored for 1
490 and 7 days at this concentration, considering different ozonation times and
491 gelatinization temperatures.

492 In Fig. 9, we can see that it was possible to extrude all the starch gels produced
493 in a straight line and with a defined shape. However, it can be noticed that the line
494 produced by the cassava starch ozonated for 15 min resulted in less defined and regular
495 lines, for all the gelatinization temperatures employed. On the other hand, the gels
496 produced with the native starch and starch ozonated for 30 min show a regular structure,
497 especially the ozonated starch. Furthermore, the gels stored for 7 days show a more

498 regular structure than gels stored for 1 day. Indeed, these results agree with the gel
499 strengths obtained (and also demonstrate the important of evaluating gel strength with
500 respect to printability).

501 At a gelatinization temperature of 65 °C, the gels produced by native cassava
502 starch and cassava starch ozonated for 30 min resulted in good candidates for use in 3D
503 printing. For process temperatures up to 75 °C, the cassava ozonated starch for 30 min
504 was the best candidate for 3D printing.

505 To evaluate if the ozone treatment really improves the printability of these
506 hydrogels, 3D stars were printed with selected gels. Fig. 10 shows images of the stars
507 produced based on gels obtained from the control (native) starch and those processed
508 for 30 min with ozone, at gelatinization temperatures of 65 and 95 °C, and a storage
509 period of 7 days in a refrigerator (5 ± 2 °C).

510 Fig. 10 shows that when the gels were produced using the lowest gelatinization
511 temperature (65 °C), it was possible to print stars with both gels. However, it is notable
512 that the star produced with the ozonated starch showed better resolution (well-defined
513 angles). For the gels produced using the highest gelatinization temperature (95 °C), the
514 native starch did not present good printability and spread over the surface. On the other
515 hand, the hydrogels produced with starch ozonated for 30 min continued to exhibit good
516 printability, resulting in a star with better resolution when compared with the others
517 printed in this work.

518 Consequently, this work successfully developed ozonated starches for use as
519 ingredients with enhanced properties for 3D printing.

520

521 **4. Conclusion**

522 This work demonstrated that ozone treatment is a relevant strategy for modifying
523 cassava starch intended for 3D printing. The effect of ozonation processing was
524 evaluated on the starch biopolymer, paste and gel properties. Then, the obtained
525 hydrogels were evaluated by also considering different starch concentrations,
526 gelatinization temperatures and storage times. The results obtained were correlated, in
527 order to better understand the changes made to the paste, gel strength, structure and
528 printability properties of the ozonated starches, when compared to the native one. This
529 work also revealed that starch concentration was an important parameter for the gelling
530 stage and that the gelatinization temperature was changed for the native and ozonated
531 starches. Ozone processing promoted starch oxidation, reduced starch molecular size

532 (depolymerization), and the starch ozonated for 30 min exhibited a lower peak apparent
533 viscosity. The lowest starch concentrations studied were not capable of forming
534 consistent gels with the requisite characteristics of printability. On the other hand, the
535 highest starch concentration formed stronger and more printable gels. The stabilization
536 of the gels for 7 days was found to be preferable, resulting in a lower propensity for
537 spreading and better consistency thanks to the retrogradation of starch biopolymers. The
538 gels produced by native and starches ozonated for 30 min presented good printability
539 when the gelatinization temperature used was 65 °C, but up to this temperature, starch
540 ozonated for 30 min produced more printable gels. The gels produced by starch
541 ozonated for 15 min did not present good printability. Finally, ozone processing can be
542 an alternative for obtaining starches with new and potentially interesting properties,
543 thereby expanding the potential of using cassava starch in 3D printing.

544

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554

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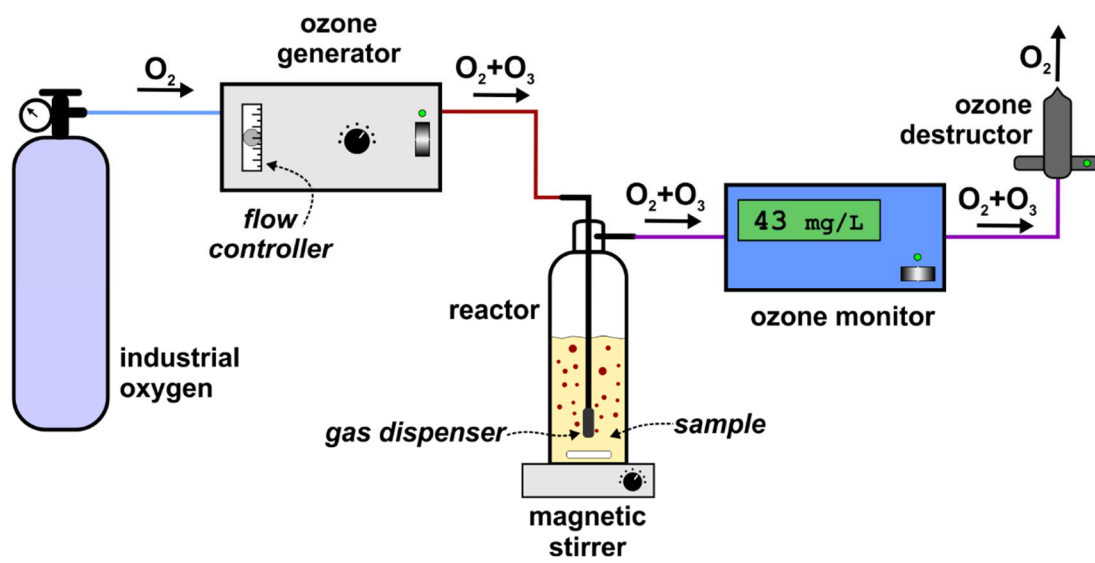


Figure 1. Schematic representation of the ozone processing system.

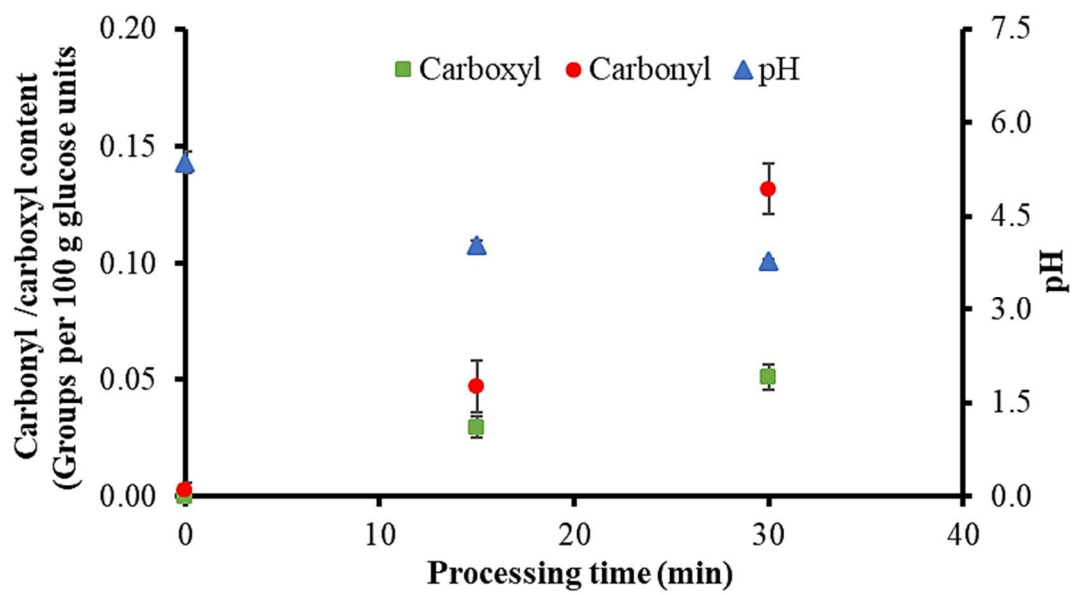


Figure 2. pH and contents of carbonyl, and carboxyl groups of control (native) and cassava starches ozonated for 15 and 30 min. Carboxyl groups in native starches are considered as negligible and thus zero. The dots are the mean values and the vertical bars are the standard deviations.

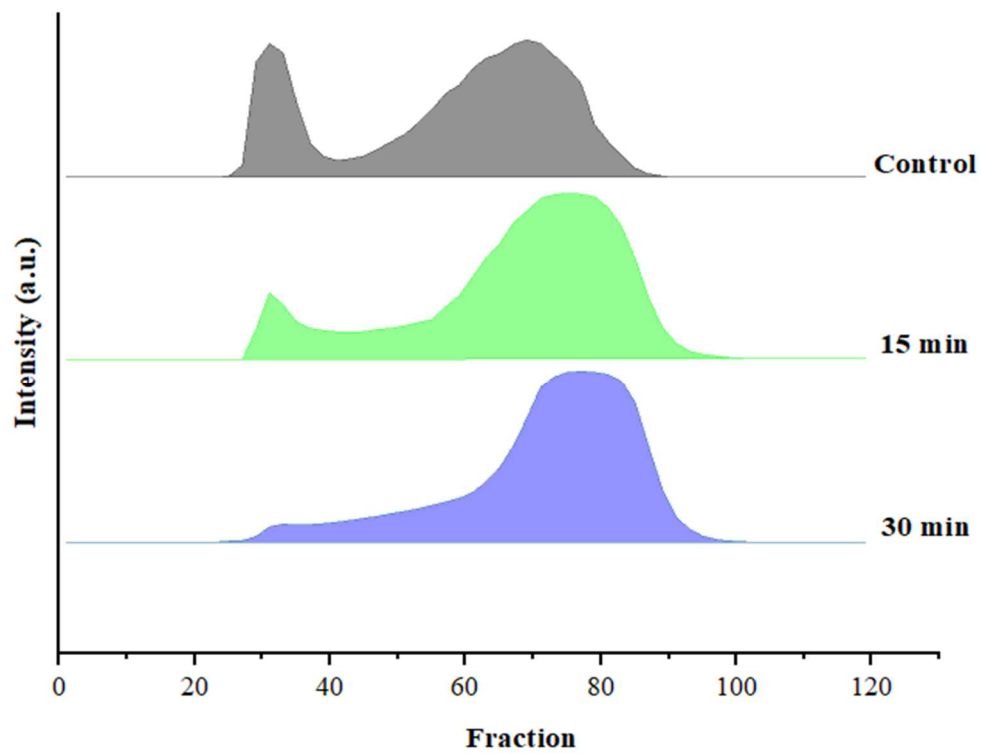


Figure 3. Molecular size distribution determined by the blue value (BV) during gel permeation chromatography of control (native) and cassava starches ozonated for 15 and 30 min.

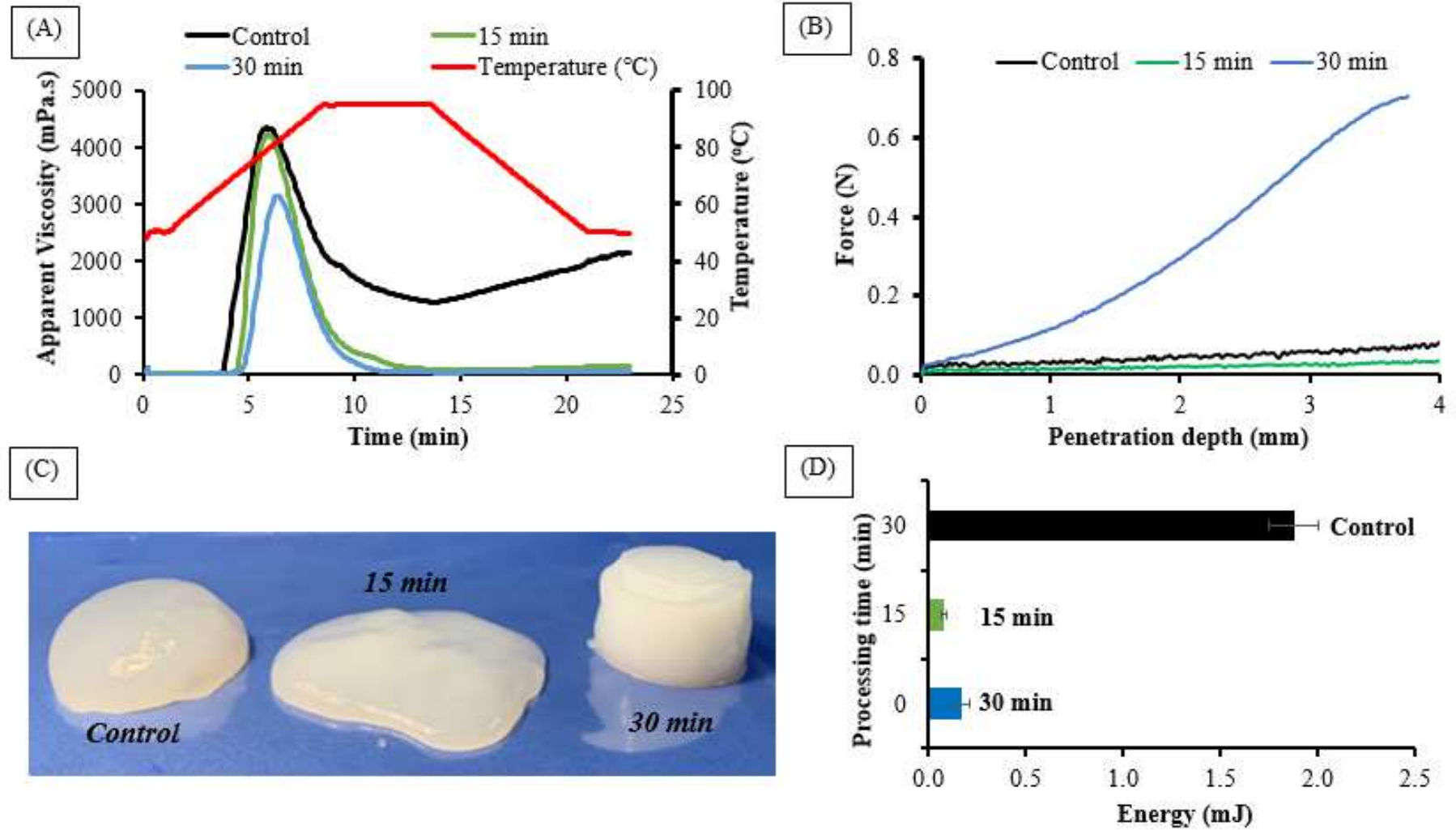


Figure 4. (A) RVA curves, (B) Gel firmness, (C) images of the gels obtained, and (D) the energy required in a puncture assay of the control (native) and cassava starches ozonated for 15 and 30 min gels (10.7 g starch / 100 g suspension)

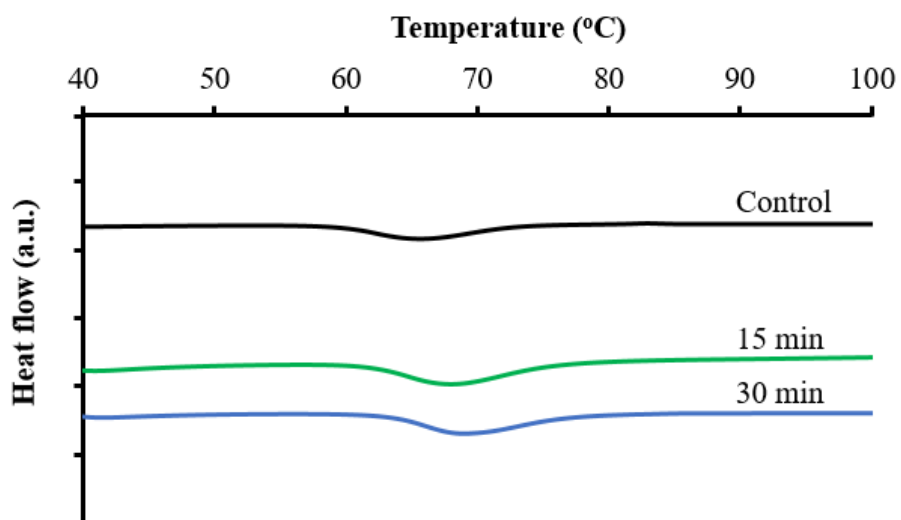


Figure 5. DSC representative curves of the gelatinization properties of the native and ozonated starch samples, in arbitrary units (a.u.) of heat flow.

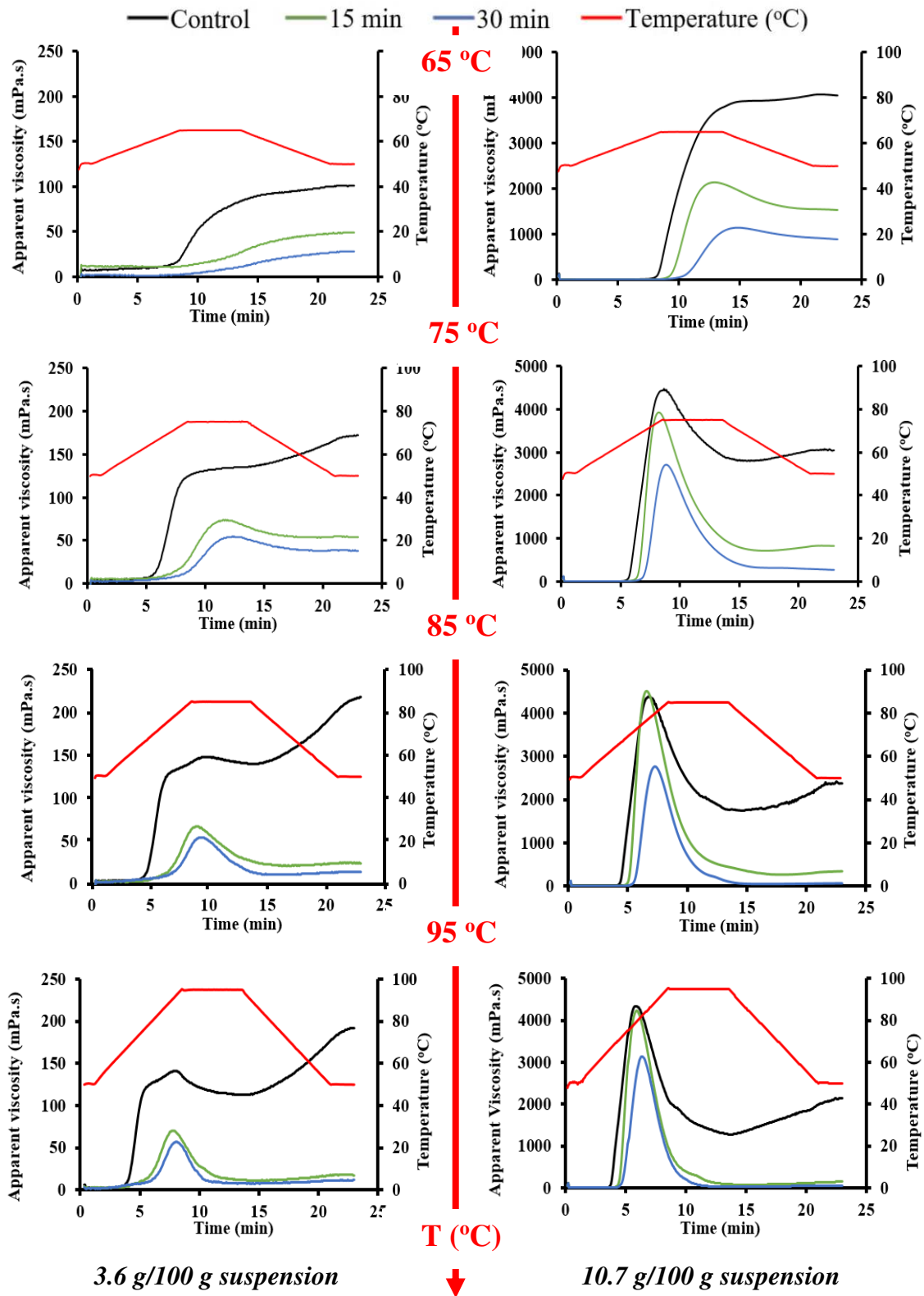
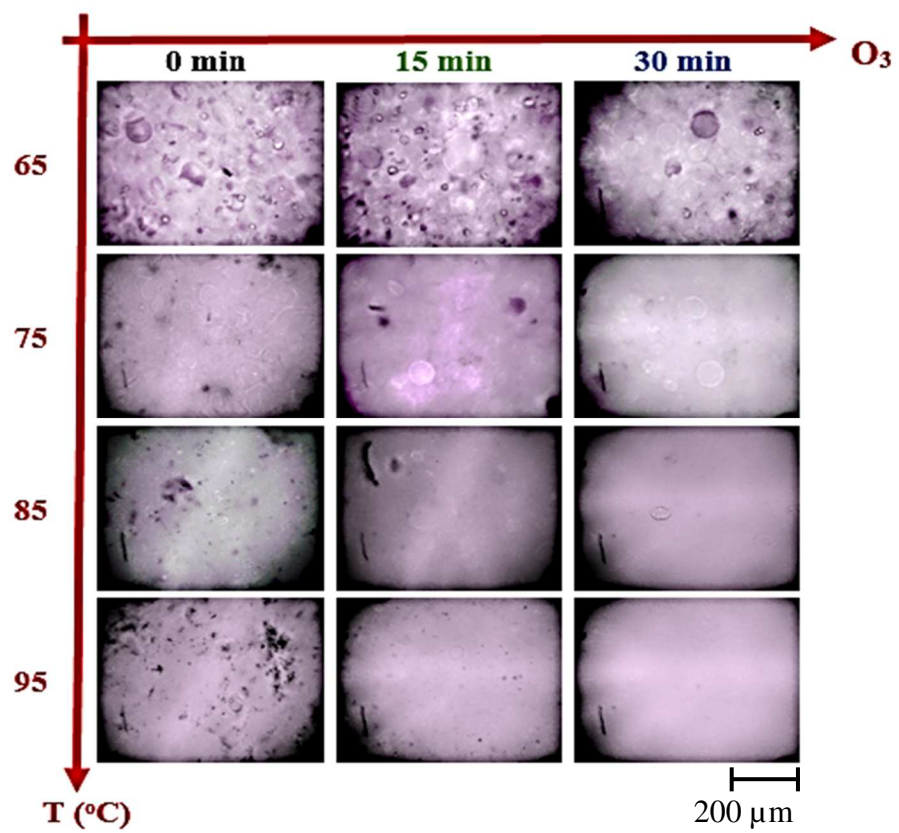
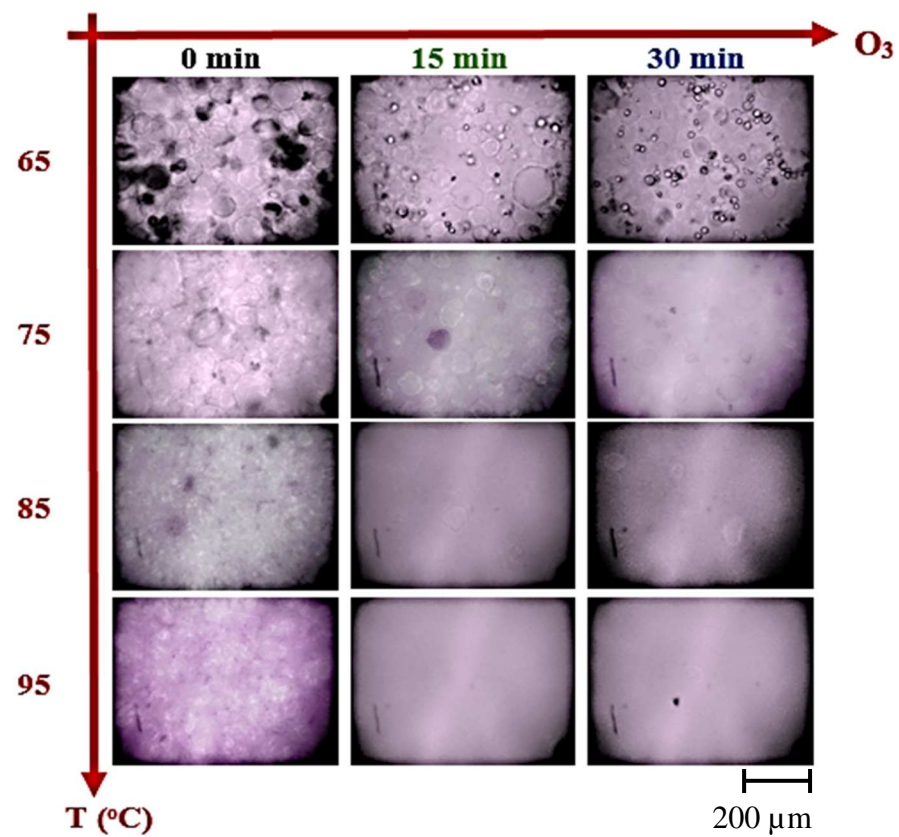


Figure 6. RVA curves for the different starch concentrations at the gelatinization temperatures: 65, 75, 85, and 95 °C.



3.6 g/100 g suspension



10.7 g/100 g suspension

Figure 7. Optical microscopy for the gels produced after the RVA analysis.

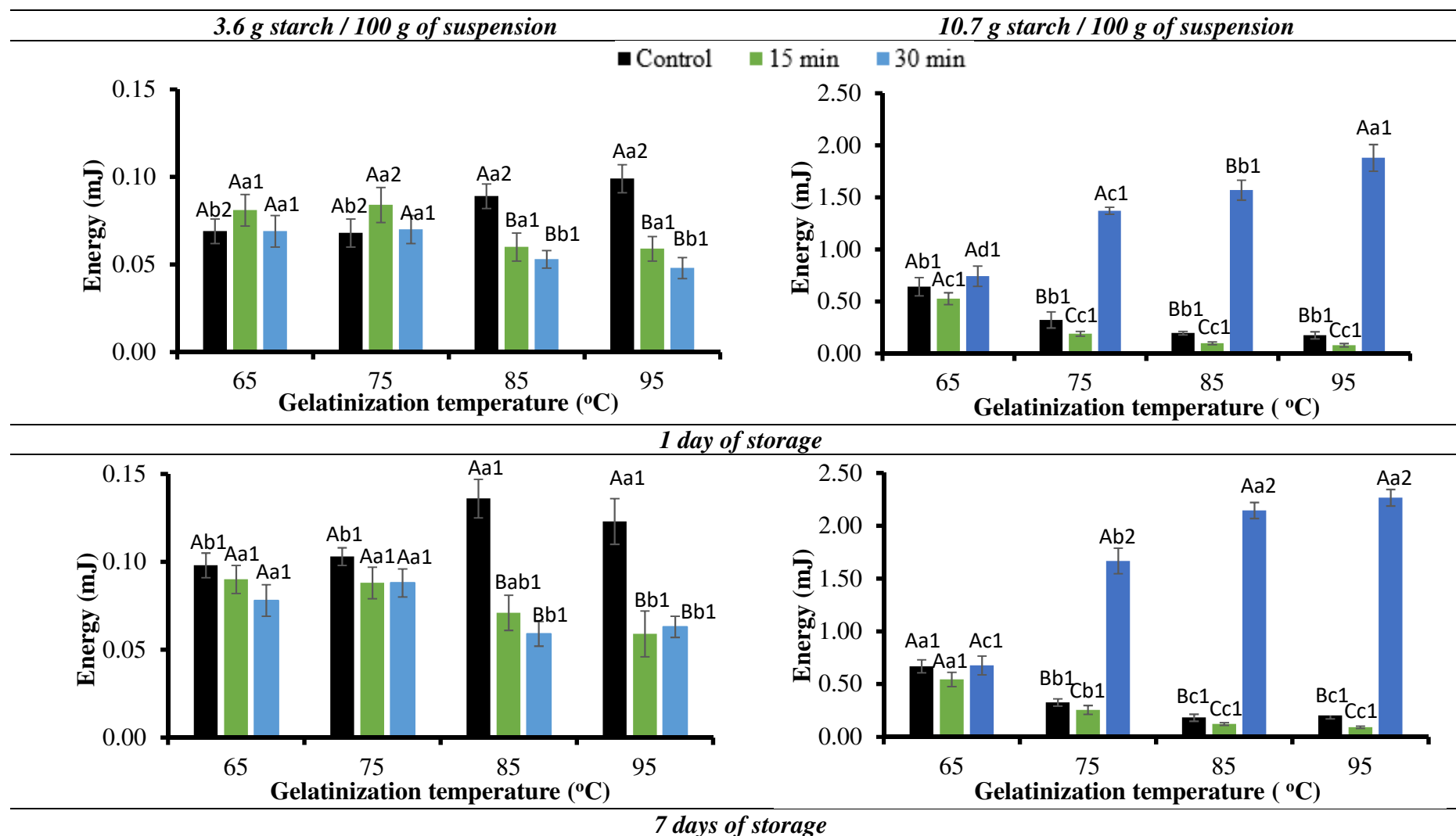


Figure 8. Strength of the hydrogels obtained: effect of ozone processing, gelatinization temperature and storage time. (A – C: different large caps indicate significant differences between the different starches at the same gelatinization temperature, as revealed by Tukey's test, $p < 0.05$; a - c: different small caps indicate significant differences between the same starches at different gelatinization temperatures, as revealed by Tukey's test, $p < 0.05$; 1 - 2: different numbers indicate significant differences between the same starches at the same gelatinization temperature but with different storage periods, as revealed by Tukey's test, $p < 0.05$).

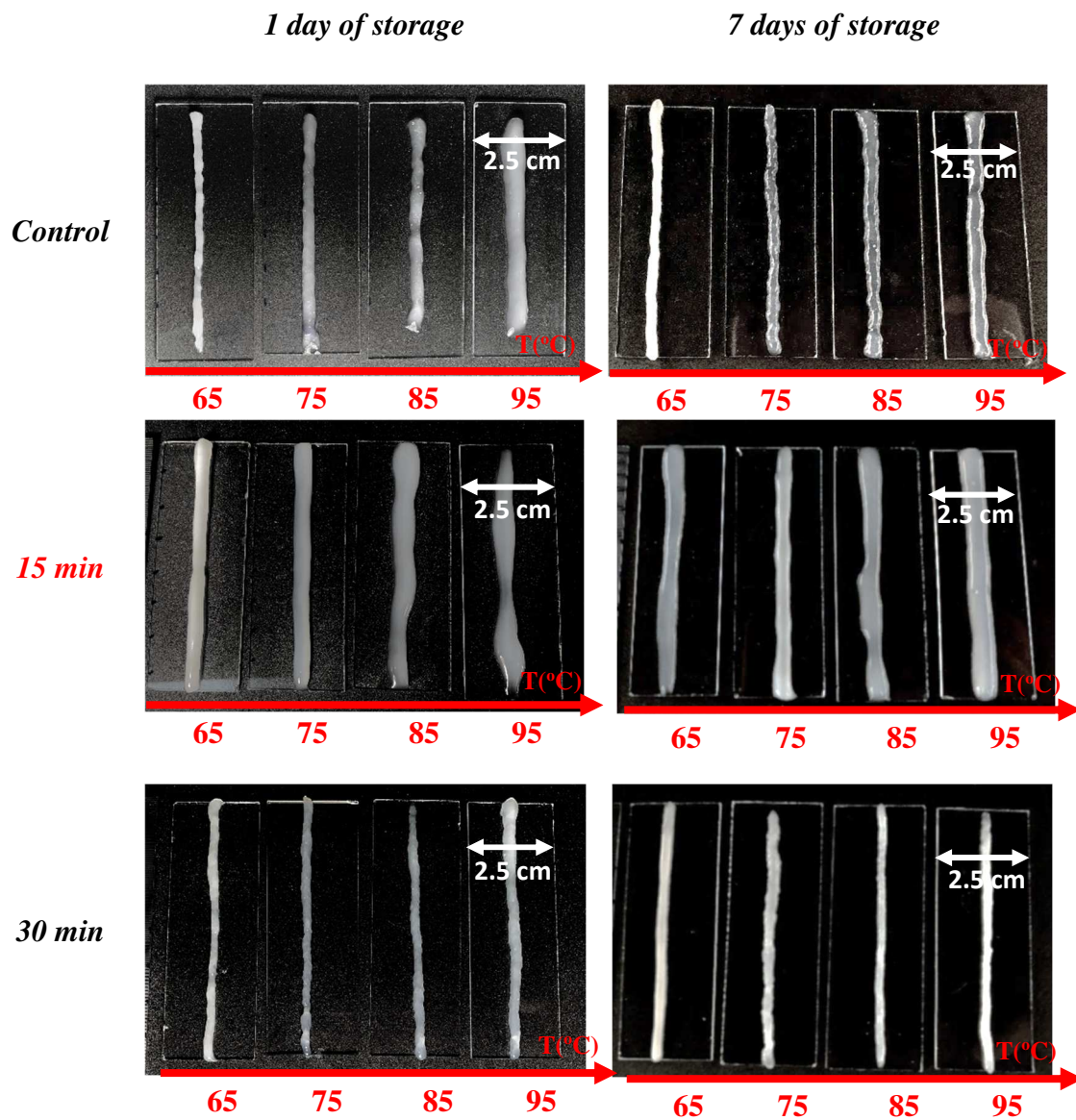


Figure 9. Images of the simulation of gel printability. The images were taken for storage periods of 1 and 7 days with starch concentration of 10.7 g / 100 g suspension.





Gelatinization temperature (°C)	Control	Ozone (30 min)
65		
95		

Figure 10. Images of the stars obtained by a 3D printer of cassava starch gels: control (native starch) and starch ozonated for 30 min, produced at gelatinization temperatures of 65 and 95 °C, and stored for 7 days at 5 °C before printing.

Table 1. Pasting properties of native and ozonated cassava starches (average \pm standard deviation)

Processing Time (min)	PAV (mPa.s)	TAV (mPa.s)	RBD (mPa.s)	FAV (mPa.s)	SB (mPa.s)	PT (°C)
0	4308.50 \pm 61.39 ^a	1264.25 \pm 19.07 ^a	70.54 \pm 0.96 ^c	2111.50 \pm 71.71 ^a	847.25 \pm 56.96 ^a	65.99 \pm 0.79 ^c
15	4314.60 \pm 106.45 ^a	90.40 \pm 14.12 ^b	97.89 \pm 0.90 ^a	164.80 \pm 21.86 ^b	74.60 \pm 10.85 ^b	70.14 \pm 0.26 ^b
30	3414.60 \pm 167.05 ^b	40.40 \pm 1.52 ^c	99.03 \pm 1.07 ^b	64.60 \pm 2.88 ^c	24.20 \pm 1.92 ^c	71.47 \pm 0.37 ^a

Peak Apparent Viscosity (PAV), Through Apparent Viscosity (TAV), Relative Breakdown (RBD), Final Apparent Viscosity (FAV), Setback (SB) and Pasting Temperature (PT).

a - c: different small caps in the same column indicate significant difference among treatments, as revealed by Tukey's test, $p < 0.05$.

Table 2. Gelatinization properties of native and ozonated cassava starches
(average \pm standard deviation)

Processing time (min)	To (°C)	Tp (°C)	Tf (°C)	ΔH (J/g)
0	55.30 \pm 0.40 ^c	65.77 \pm 0.08 ^c	81.14 \pm 0.56 ^b	12.61 \pm 0.22 ^a
15	56.41 \pm 0.46 ^b	67.98 \pm 0.26 ^b	81.57 \pm 0.30 ^b	12.22 \pm 0.32 ^a
30	59.36 \pm 0.62 ^a	69.06 \pm 0.26 ^a	84.26 \pm 0.79 ^a	11.03 \pm 0.29 ^b

a - c: different small caps in the same column indicate significant difference among treatments, as revealed by Tukey's test, $p < 0.05$.

To = onset temperature, Tp = peak temperature, Tf = final temperature and ΔH = enthalpy