Production of resistant starches via citric acid modification: Effects of reaction conditions on chemical structure and final properties

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Abstract

Aiming to contribute to the current knowledge on the impact of reaction conditions on the chemical structure and target properties of starch citrates, in the current contribution different corn starch citrates were prepared by manipulation of reaction time, temperature and citric acid concentration. Modified starches were characterized in terms of chemical structure, morphology, crystallinity, swelling power and resistant starch content. For the first time, total substitution, crosslinking and monosubstitution degrees were quantitatively determined; and the relationship among final chemical structure, reaction conditions and target starch citrates properties was comprehensively analyzed. Products with total substitution values in the range of 0.075-0.24, crosslinking degrees in the 0.005-0.11 interval, and monosubstitution extents within the 0.05-0.12 range, were produced. By proper selection of reaction conditions products with almost 100% of resistant starch were obtained. Results evidenced that starch citrates properties (mainly swelling power and RS content) depend on both chemical structure and the reaction conditions employed. Actually, the reaction temperature set (120°C or 150°C) proved to play a determinant role in the final products properties as evidenced from starch citrates with similar chemical structure and substantially different swelling and digestibility properties.

Keywords: starch; citric acid; esterification; crosslinking; resistant starch

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1. Introduction

Starch is the main energy reserve of plants, where it is deposited in various types of tissues and organs in the form of granules [1]. Depending on their botanical origin, starch granules can vary in shape, size, and composition; but in all cases their main components are two homopolysaccharides: amylose and amylopectin. The former is a water-soluble linear polymer consisting in D-glucopyranose units linked by α-(1→4) bonds, whereas amylopectin has a branched structure consisting of α-(1→4) linked D-glucopyranose units with 5–6% branches linked by α-(1→6) glycosidic bonds to the main chain [2]. Currently, starches and products derived from them find plenty of applications in different industries such as food, paper, textile, detergents, surfactants, fermentation, construction, oil drilling, mineral and metal processing, and bioplastics. In the food industry starches and modified starches are especially valued for their ability to tune the physical properties of many products, in which they are commonly used for gelling, thickening, stabilizing, texturizing, adhesion, moisture-retention, and antistaling purposes [3].

Resistant starch (RS) is the portion of starch that cannot be hydrolyzed by digestive enzymes in the small intestine of healthy people and reaches the large intestine. In the large intestine, RS is fermented by the local microflora, producing short-chain fatty acids, predominantly acetates, propionates, and butyrates; which are recognized for their beneficial properties on the human colon. In the last decades, the nutritional implications of RS in foods have raised considerable interest due to multiple health benefits similar to those attributed to dietary fiber, and to claimed effects on the prevention and control of diabetes, colon cancer, and obesity. Numerous food products enriched with RS (e.g. breads, cookies, muffins, cakes, pizza crusts, tortillas, ice creams, yoghurts, breakfast cereals, pasta and snack products) are already in the market, and they have become popular among consumers demanding for high-quality products with functional health-promoting properties [4, 5].

Although RS is a natural component of some starches, it can also be produced by different heat, chemical and enzymatic treatments of the polysaccharide. Indeed, RS has been classified into 5 subtypes, namely RS1 (physically protected starch), RS2 (raw starch with highly crystalline structure such as B type and high amylose starches), RS3 (retrograded starch), RS4 (chemically modified starch), and RS5 (amylose-lipid-complex starch and maltodextrines).

Among RS sources, of particular interest are chemically modified starches (i.e. RS4) which resist digestion due to the presence of atypical linkages that prevent the access of digestive enzymes [6]. Depending on the type (e.g. esterification, etherification, crosslinking) and extent of chemical modification performed, the hydrolytic action of amylase enzymes on modified starches has been restricted to various degrees [7].

67   Citric acid (CA) is a polyfunctional organic carboxylic acid of natural origin which has been classified

68   as GRAS ("Generally recognized as safe") by the American Food and Drug Administration. CA is

69   extensively used in the food industry as flavoring agent, for pH buffering, to prevent oxidation, and also

70   as emulsifier, texturizer and stabilizer. CA is also used in several cosmetic and pharmaceutical products,

71   in agriculture and in environmental remediation [8]. Starch modification with citric acid was first

72   reported in the mid-20th century, in a reaction performed by heating the mixture of reactants in semi-

73   dry conditions [9]. In this method citric acid is converted to its anhydride which forms ester bonds with

74   the hydroxyl groups of starch. Being CA a polyfunctional molecule, crosslinking (by esterification) is

75   also possible.

76   In the last decade, starch modification with CA (to produce the so-called "starch citrate") has received

77   a renewed interest based on its suitability to produce esterified (a term usually associated with

78   monosubstitution) or crosslinked derivates with tunable physicochemical properties and high resistant

79   starch content using environmentally friendly reagents [10-19]. Even if during the last years other

80   modification methods have been proposed, such as those involving one-step reactive extrusion [15],

81   microwave-discharged cold plasma treatment [17], or sulphuric acid catalyzed reactions in water

82   suspension [18]; most contributions devoted to starch citrates production have relayed on the early

83   described semi-dry method [9]. Up to date, this method has been applied to a wide variety of native

84   starches (e.g. corn, rice, wheat, cassava, potato, sweet potato, banana, lentil, etc.), and also to some

85   chemically and physically modified starches [19], using varying reaction conditions (mainly CA

86   concentration, reaction temperature and time). However, and even if the polyfunctional nature of citric

87   acid may result in monosubstituted and/or crosslinked products, there is a gap in the bibliography in

88   relation to the quantitative determination of the relative contributions of both units to the final citrate

89   starch structure. In this context, in the current contribution crosslinking and monosubstitution degrees

90   of corn starch citrates produced under varying reactions conditions were quantified for the first time;

91   and the impact of the final chemical structure as well as that of reaction conditions on target starch

92   citrates properties was comprehensively analyzed.

93

94   2.   **Materials and methods**

95   2.1.   **Materials**

96   Commercial corn starch (Buffalo 034010, amylose:amylopectin 25:75 wt.%) was purchased from

97   Ingredion, Argentina, and citric acid ($\geq$ 99.0%) was obtained from Biopack, Argentina. Hydrochloric

98   acid and sodium hydroxide from Merck were used in the saponification and conductometric titration

99   assays. Resistant starch content was measured using the K-RSTAR 09/14 Megazyme kit (Granotec,

100 Argentina), involving pancreatic α-amylase from porcine pancreas (Pancreatin, 3 Ceralpha U/mg) and

amyloglucosidase from *Aspergillus niger* (3300 U/mL on soluble starch at pH 4.5 and 40 °C). Maleic acid ($\geq$ 99.0%, Sigma Aldrich), calcium chloride dehydrate (Stanton) and sodium azide (Stanton) were used in the preparation of the 0.1M sodium maleate buffer used in the resistant starch determination.

2.2.   Chemical modification of starch with citric acid

The methodology used for the modification of starch was adapted from the dry method of Klaushofer, Berghofer and Steyrer [9]. Following a full factorial design, the effect of reaction temperature, citric acid concentration and reaction time on the chemical structure of the products was evaluated. Specifically, 10 g of previously oven-dried (105 ºC, 2 h) native corn starch (NS) were properly mixed with 10 mL of 20% or 40% w/w CA aqueous solutions to meet a final CA concentration of 20% or 40% w/w based on the dry starch weight, respectively. The mixtures were kept at RT for 24 h, and then placed in an air oven at 60ºC for 16 h. The dried samples were later ground and air-dried at the chosen reaction temperatures (120ºC or 150ºC) during the stipulated study time (1, 3 or 5 h). The modified starches (MS) were extensively washed with distilled water and ethanol (96%), dried at 40ºC overnight and grinded to powder. Controls (MS20/0 and MS40/0) were prepared as detailed above but without the heat treatment step at 120ºC or 150ºC. All samples were prepared in duplicate.

2.3.   Characterization of chemically modified starches

Fourier transform infrared spectroscopy (FT-IR): Fourier transform infrared spectra (*FT*-IR) of native and modified starch samples were acquired on a Thermo Scientific Nicolet 6700 spectrometer in absorbance mode. Samples were dried at 105°C for 1 h, mixed with KBr (Grade *FT*-IR 99+%, Thermo Spectra-Tech) at 1:100 ratio, and pressed into a 5 mm disc using a Hand Press accessory of PIKE Technologies. Samples were then scanned 32 times in the range of 4000 to 400 cm^{-1} at a spectral resolution of 4 cm^{-1}. Spectra were normalized using the signal at 1020-1030 cm^{-1}, [20].

Solid-state CP/MAS ^{13}C-NMR spectroscopy: High-resolution ^{13}C solid-state spectra of native and modified starch samples were obtained using the ramp ^{1}H$\rightarrow^{13}$C CP/MAS pulse sequence (cross-polarization and magic angle spinning) with proton decoupling. Data were acquired at RT in a Bruker Avance II-300 spectrometer equipped with a 4-mm MAS probe. The operating frequency for protons and carbons was 300.13 and 75.46 MHz, respectively. Glycine was used as external reference for the ^{13}C spectra and to set the Hartmann-Hahn matching condition in the CP experiments. Depending on the sample, the recycling time varied from 5 to 6 s. The contact time during CP was in all cases 2 ms. The SPINAL64 (small phase incremental alternation with 64 steps) sequence was used for heteronuclear decoupling during acquisition with a proton field H1H satisfying $\omega 1H/2\pi$ = YHH1H = 62 kHz. The spinning rate for all the samples was 10 kHz.

Saponification and conductometric titration: Total substitution, monosubstitution and crosslinking degrees of modified starch samples were determined by quantification of ester and free carboxylic groups present in the starch derivatives. The total ester groups content was quantified by heterogeneous saponification and back titration with HCl [21]. Briefly, previously dried samples (100 mg, 105°C, 2 h) were contacted with 20 mL of ethanol (75%) and heated for 30 min at 50°C. The suspensions were brought to slightly basic pH by addition of a few drops of 0.1 M NaOH using phenolphthalein as indicator, followed by addition of 20 mL of 0.1 M NaOH. Samples were heated again at 50°C for 15 min and then allowed to stand tightly stoppered at room temperature for 48 h. At the end of this period, the excess NaOH present in the flasks was back titrated with 0.1 M HCl.

The content of free carboxylic groups in the derivatives was determined by heterogeneous conductometric titration. Dried starch derivatives samples (250 mg) were suspended in 5 mL of HCl aqueous solution (0.1 M) with vigorous stirring and then titrated with NaOH aqueous solution (0.1 M). Conductivity values were registered each 0.05 mL (conductometer Sper Scientific, model 860033) [22]. Assays were performed in triplicate and the results were presented as mean values ± standard deviation.

X-ray diffraction measurements (XRD): The crystalline structure of dried native and modified starch samples was analyzed using a Philips PW1730/10 automated wide-angle powder X-ray diffractometer operated at 40 kV and 20 mA, with Cu/Kα radiation (λ = 0.154 nm). Diffractograms were recorded in a *2θ* angle range of 10-45° with a scan rate of 0.02° s^{-1} and a step size of 0.02º [23].

Field emission scanning electron microscopy (FESEM): Micrographs of native and modified starch samples were obtained in a Fei Quanta 200 scanning electron microscope equipped with a field emission gun operated at 3 kV. Samples were previously sputter coated with gold to improve the image quality and resolution.

Swelling power: The swelling power of native and modified starch samples was determined by the method described by Garg & Jana [24]. Aqueous suspensions (1 wt%) were prepared in previously weighed centrifuged tubes and heated at 60 °C during 1 h with intermediate vortexing every 5 min. After heating, the samples were quickly cooled to 25 °C and centrifuged at 3000 rpm for 20 min. The supernatants were then carefully removed, and the sediment was weighed (M_1) and dried at 105 °C until constant weight (M_2). Swelling power (g/g) was calculated as the ratio of the weight of the hydrated insoluble starch (g) to the weight of the dried insoluble starch (g) (M_1/M_2). Assays were performed in triplicate and the results were presented as mean values ± standard deviation.

170 *Resistant starch determination (RS):* RS Megazyme kit (K-RSTAR 09/14) was employed to determine
171 the RS content of native and modified starch samples. Briefly, samples (100 mg) were placed in Falcon
172 tubes and 4 mL of 0.1M sodium maleate buffer solution containing pancreatic α-amylase (10 mg/mL)
173 and amyloglucosidase (3 U/mL) were added. The tightly capped tubes were then incubated horizontally
174 in a thermostatic bath at 37ºC with continuous shaking for 18 h. Enzymatic hydrolysis was terminated
175 by adding 4 to 8 mL of cold ethanol, recovering the resistant starch by centrifugation at 3000 rpm for
176 10-12 min (Tyfon II, Zelian). The precipitate was washed twice with 50% ethanol, followed by
177 centrifugation at 3000 rpm for 10 min, and dried at 45°C to constant weight. All samples were run along
178 with a control sample provided by Megazyme (Resistant starch control, RS=44%, Resistant starch kit,
179 Megazyme), which always showed error values < 2.5%. Assays were performed in duplicate and the
180 results were presented as mean values ± standard deviation.
181
3. Results and discussion

183 Esterification of starch with citric acid may involve just one carboxylic group of the polyacid or two of
184 them, resulting in esterified or crosslinked anhydroglucose units, respectively (**Figure 1**). Therefore,
185 modification of starch with citric acid can lead to complex mixtures.
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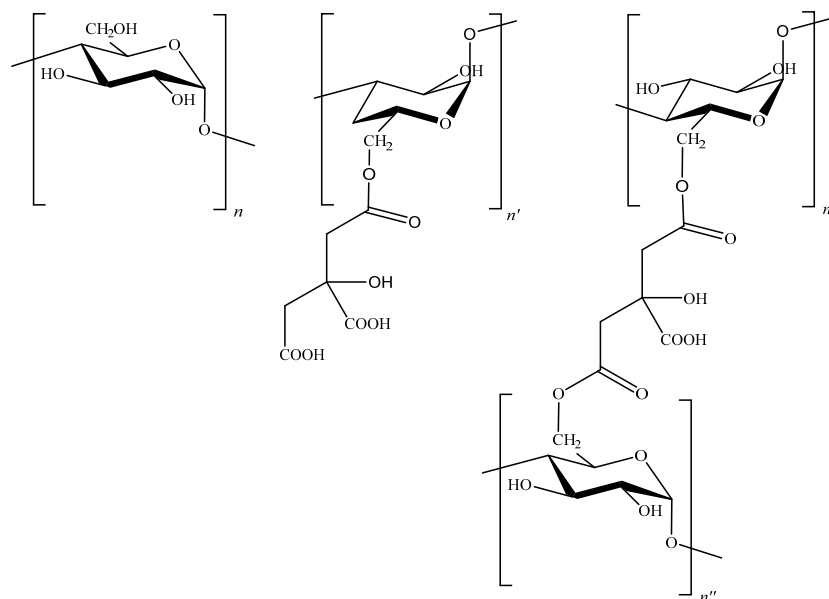
Figure 1. Schematic representation of the expected structural units (the position and number of ester groups in
189   the anhydroglucose unit (AGU) and the carboxylic acid groups of CA involved in the reaction may vary).
190
191 Aiming to contribute to the current knowledge on the impact of reaction conditions on the chemical
192 structure and target properties of starch citrates, herein different corn starch citrates were prepared by
193 manipulation of reaction time, temperature and citric acid concentration according to a full factory

194 design. **Table 1** collects the list of the modified starch samples produced under the reaction conditions
195 detailed.

196

| Sample	CA concentration (%)	Reaction temperature (°C)	Reaction time (h)
MS20/0	20	-	0
MS20/120/1	20	120	1
MS20/120/3	20	120	3
MS20/120/5	20	120	5
MS40/0	40	-	0
MS40/120/1	40	120	1
MS40/120/3	40	120	3
MS40/120/5	40	120	5
MS20/150/1	20	150	1
MS20/150/3	20	150	3
MS20/150/5	20	150	5
MS40/150/1	40	150	1
MS40/150/3	40	150	3
MS40/150/5	40	150	5

197 **Table 1.** Chemically modified starch samples produced.

198

199 **Figure 2** collects the *FT*-IR spectra of native and modified starch samples. The *FT*-IR spectrum of
200 native starch shows the characteristic signals of the main starch groups. Among them, a broad band in
201 the interval of 4000 cm^{-1} and 3000 cm^{-1} assigned to the stretching vibrations of the O-H groups, a signal
202 in the region of 3000 cm^{-1} to 2800 cm^{-1} attributed to the stretching vibration of the C-H bonds, a band
203 around 1645 cm^{-1} assigned to the bending vibration of water molecules absorbed in the amorphous
204 regions of the starch granules, and characteristic starch signals between 1150 cm^{-1} and 950 cm^{-1} assigned
205 to C-O stretching [24-27] are observed.

206 On the other hand, the spectra of the modified starch samples show the appearance of a new signal
207 centered at 1736 cm^{-1} associated with the stretching of the C=O carbonyl group of esters, confirming
208 that the esterification reaction took place. The presence of the signal centered at 1736 cm^{-1} has been
209 described in previous contributions dealing with starch modification with citric acid [11, 28, 29].
210 Besides, the intensity of this band increases with reaction time. By the way, the spectra of control
211 samples (i.e., samples contacted with CA but without the heat treatment step at 120ºC/150ºC, MS20/0
212 and MS40/0) does not show any new signal at the mentioned wavenumber.
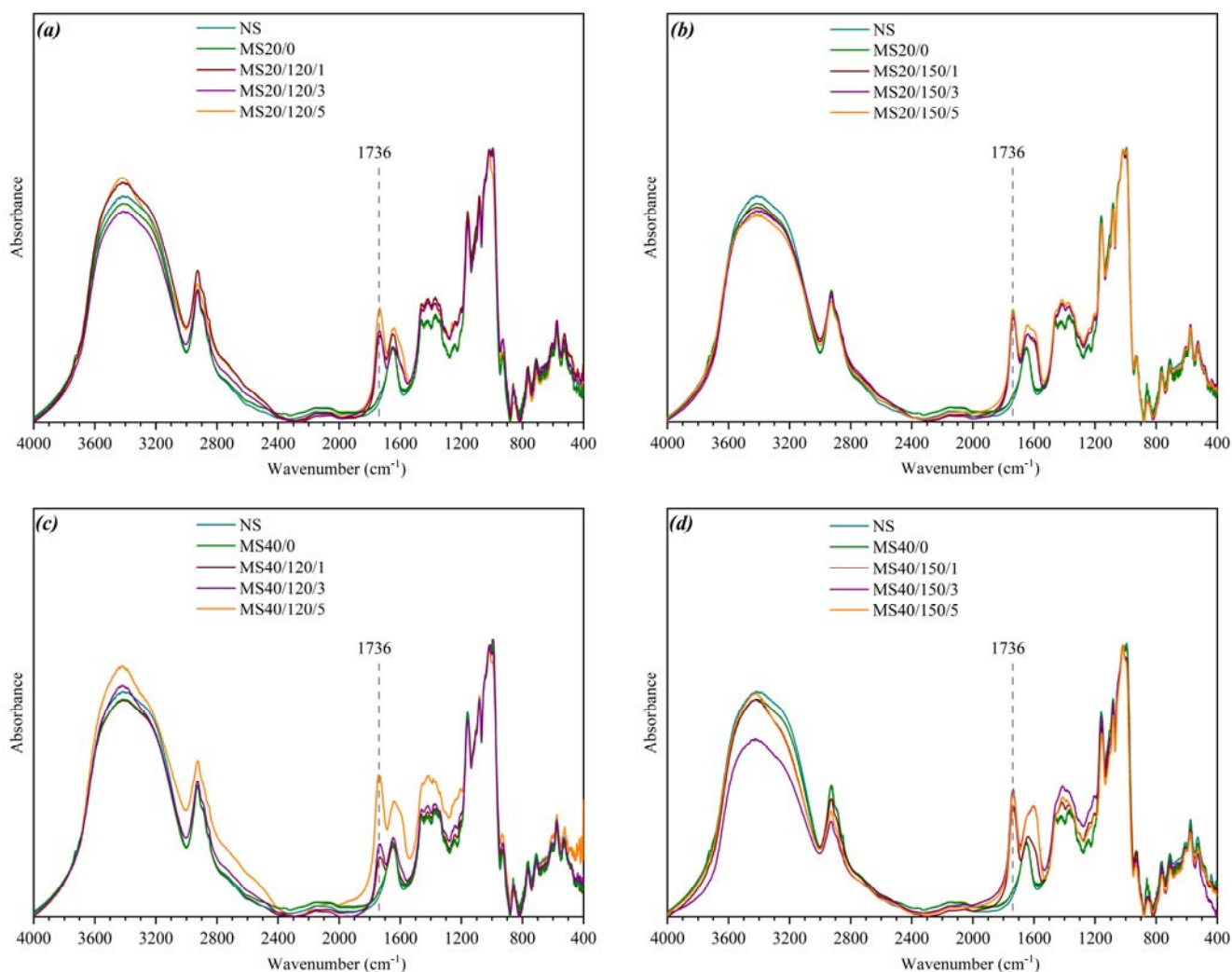
Figure 2. *FT*-IR spectra of native and citric acid modified starch samples.

The extent of starch esterification is generally expressed as the degree of substitution (DS) which is defined as the average number of hydroxyl groups derivatized per AGU. When esterification is carried out with monofunctional acids the DS may be easily determined by saponification considering in the calculus the molecular weight of the esterified AGU. However, in the case of starch modification with polyfunctional acids, monosubstituted and crosslinked AGUs with different corresponding molecular weights may be obtained. So, DS calculation requires the prior knowledge of the relative contribution of each unit to the polymer structure. With this in mind, quantification of the total amount of ester and free carboxylic acid groups was performed.

The total ester groups content was determined by saponification and back titration and correlated to the average structural units (**Figure 1**) using Equation 1, where $[HCl]$ is the concentration ($mol\ L^{-1}$) of HCl solution, ΔV_{HCl} is the difference between the HCl volume (L) consumed by native and modified starches, n_{ester} is the moles number of ester groups in the sample (mol), m_1 is the mass (g) of the starch derivative,

228   u_1 is the average number of hydroxyl groups per AGU involved in monosubstitution, u_2 is the average

229   number of hydroxyl groups per AGU involved in crosslinking, and $\overline{Mr}$ is the average molecular weight

230   ($g\,mol^{-1}$) of the structural units. $\overline{Mr}$ is obtained from Equation 2, where 162 is the molecular weight of

231   the unsubstituted AGU, 174 is the mass added to the molecular weight of AGU per hydroxyl group

232   involved in monosubstitution, and 78 is the mass added to the molecular weight of AGU per hydroxyl

233   group involved in crosslinking.

234

235     $$[HCl] \cdot \Delta V_{HCl} = n_{ester} = \frac{m_1}{Mr}(u_1 + u_2) \tag{1}$$

236     $$\overline{Mr} = 162 + u_1 \cdot 174 + u_2 \cdot 78 \tag{2}$$

237

238   Besides, the content of free carboxylic groups was quantified by conductometric titration and related to

239   the structural unit fractions (**Figure 1**) using Equation 3, where *[NaOH]* is the concentration (*mol L⁻¹*)

240   of NaOH solution, ΔV_{NaOH} is the NaOH volume (*L*) consumed, $n_{carboxyl}$ is the moles number of free

241   carboxylic groups in the sample (*mol*) and m_2 is the mass (*g*) of the starch derivative used in the

242   conductometric titration.

243

244     $$[NaOH] \cdot \Delta V_{NaOH} = n_{carboxyl} = \frac{m_2}{Mr}(2 \cdot u_1 + 0{,}5 \cdot u_2) \tag{3}$$

245

246   Combining Equations (1), (2) and (3), the average numbers of esterified hydroxyl groups per AGU due

247   to monosubstitution and crosslinking (i.e., u_1 and u_2, respectively) were calculated. Then, the total

248   substitution degree (DS) was obtained as the sum of u_1 and u_2. **Figure 3** shows the evolution of the

249   monosubstitution degree, crosslinking degree, and total substitution degree, as a function of reaction

250   time for all combinations of temperature and citric acid concentration assayed.

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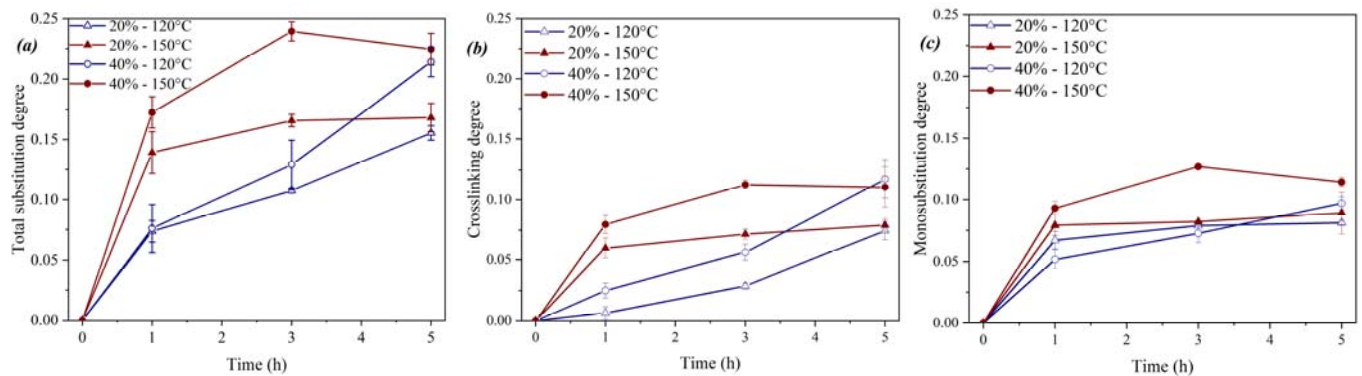
253   **Figure 3**. a) Total substitution degree, b) crosslinking degree, and c) monosubstitution degree of modified

254   starches as a function of reaction time, reaction temperature and CA concentration.

Under the set of conditions assayed total substitution degrees in the range of 0.075-0.23 were attained. Results included in **Figure 3a** indicate that the total substitution degree increased with reaction time, reaction temperature and CA concentration. Esterification reactions at 150°C were faster than those performed at 120°C, requiring 3 and 5 hours, respectively, to attain similar total substitution degrees. Besides, these values were highly dependent on the CA concentration used. Temperature, CA concentration and reaction time had a similar effect on the evolution of the crosslinking degree (**Figure 3b)**. On the other hand, under the conditions chosen, monosubstitution extent was less influenced by reaction variables (**Figure 3c)**.

The modification of starch upon treatment with CA was also confirmed by CP/MAS ^{13}C-NMR. **Figure 4** shows the spectra of native and representative modified starch samples obtained at 120°C and 150°C. Native starch spectrum shows signals for the carbons of the glucopyranose unit in the expected intervals: 90-105 ppm (assigned to C-1), 65-80 ppm (C-2,C-3,C-5), 79-87 ppm (C-4) and 56-65 ppm (C-6) [30, 31]. On the other hand, in agreement with previous contributions dealing with the citric acid treatment of starches from other botanical sources [11, 32], the spectra of the CA-treated samples show extra signals which confirm that chemical modification occurred. There are two new overlapped signals in the 170-185 ppm interval which can be assigned to the carbonyl groups of esters and free acid groups resulting from monosubstitution and crosslinking reactions. Besides, a new resonance located within the 35-50 ppm interval associated with the methylene group of citric acid is also observed [11, 33].
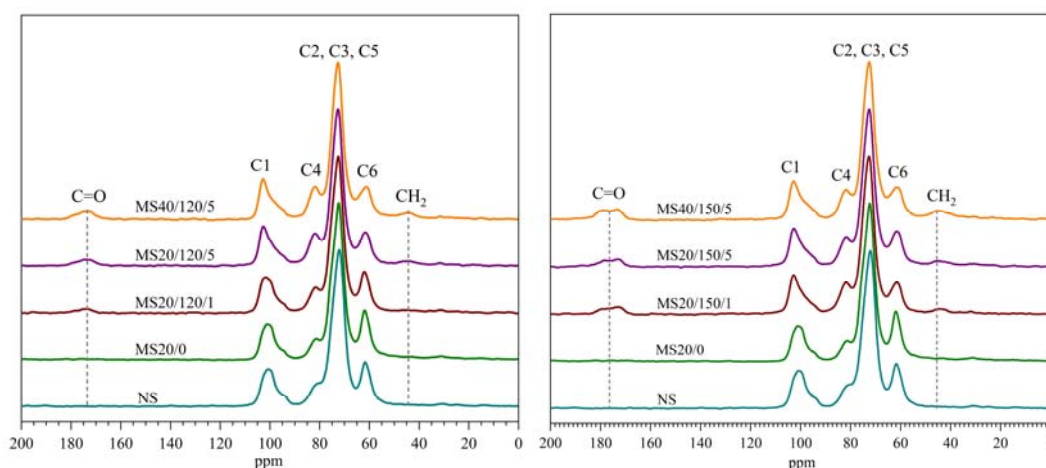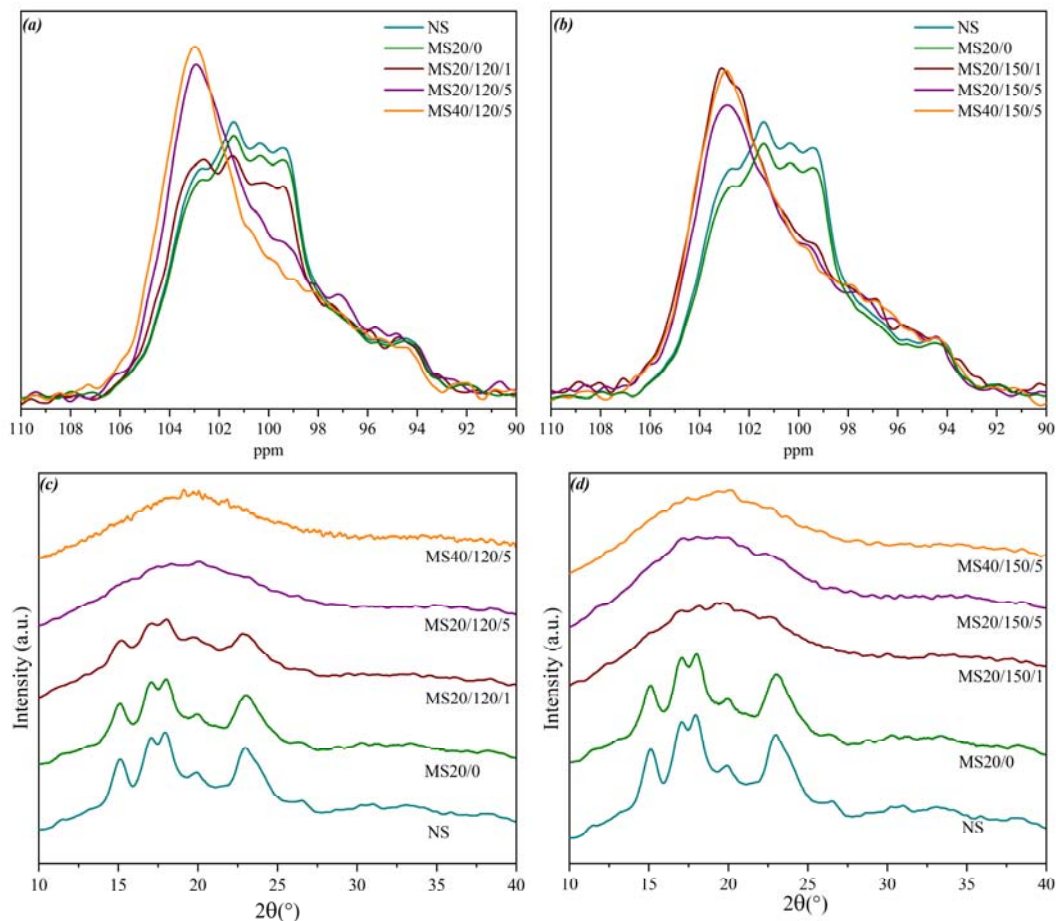

Figure 4. Representative CP/MAS ^{13}C-NMR spectra of native and citric acid modified starch samples.

Besides, the analysis of the C-1 signal in solid state CP/MAS ^{13}C-NMR spectra gives information of the changes in the ordered structure of starch samples resulting from chemical modification. In the spectra of native starch, control samples, and -less defined- in the spectrum of the sample with the lowest total substitution, crosslinking and monosubstitution degrees shown in Figure 5a and b (i.e. MS20/120/1), a C-1 signal with three maxima centered at ~99, 100 and 101.5 ppm is observed. In the literature devoted

to the study of the molecular organization of starches by CP/MAS ^{13}C-NMR spectroscopy, the multiplicities and the chemical shifts assigned to C-1 atoms have been correlated for a long time to the glycosidic conformation and polymorphism of starch [34-37]. In particular, the triplet for C-1 observed in Figure 5a and b for the mentioned samples is well-stablished that is typical of the double helical conformation of A-type polymorphism characteristic of cereal starch [37-39], which adopts a 2-fold packing symmetry resulting in three inequivalent residues per turn in the C-1 region [35, 36, 40-42]. In contrast, in the spectra of the samples with higher total substitution, crosslinking and monosubstitution degrees (Figure 5a and b) the C-1 signal is shifted to lower field (larger chemical shifts), and the original triplet with maxima centered at 99, 100 and 101.5 ppm is replaced by a broad C-1 signal characterized by a unique maximum at 103 ppm, which is characteristic of amorphous starch samples [37, 39, 43, 44]. The previous indicates that chemical modification induced a reduction of starch crystallinity, which may be associated with the partial replacement of hydroxyl groups by more voluminous ester groups which limit the formation of inter and intramolecular hydrogen bonds, resulting in the progressive destruction of the semicrystalline structure of starch [45]. The increase in the intensity of the C-4 signal centered at 82 ppm previously associated with amorphous content in starch samples [35-37, 39, 42, 46, 47] also points towards a decrease in the contribution of ordered arrays of double helices as a consequence of chemical modification.

Figure 5. Representative C-1 signal in CP/MAS ^{13}C-NMR spectra (*a* and *b*) and XRD diffractograms (*c* and *d*) of native and citric acid modified starch samples.

Figure 5c and 5d collects the X-ray diffraction patterns of native and representative of modified starch samples. The diffractograms of native starch and the control samples exhibit diffraction peaks centered at 2θ values of 15°, 17°, 18°, 20° and 23°, which are characteristic of the type A diffraction pattern of cereal starch [38]. In accordance with CP/MAS ^{13}C-NMR spectra analysis, the X-ray diffractograms of modified starches also show that samples crystallinity was reduced with the evolution of reaction due to the progressive destruction of the semicrystalline structure of starch [45].

Figure 6 collects the micrographs of native and CA treated corn starch samples. Native starch granules show polyhedral shapes with diameters between 7 and 25 μm and smooth surfaces. A few granules present small pores that can be attributed to the natural processes that occur within the plant, to those that occur during the mechanical treatment to obtain the granules, and/or to the action of amylases [48]. The control samples show granules similar to native starch ones, with no evidence of damage to the particles or traces of citric acid. On the other hand, samples treated with CA at 120°C and 150°C show rough/eroded surfaces or inward collapse of some granules. These results are consistent with other

contributions where the treatment with CA induced deformations in the granules, collapse and occasional development of grooves on their surface [14, 29]. Reaction temperature showed a prominent effect on samples morphology, with whole individual granules and several agglomerates of fused granules observed for reactions performed at 120°C, and individual granules -some with doughnut-like appearance- detected for reactions carried out at 150°C. This shape has been previously reported for starch treated with CA, mainly for large granules [32, 38]. In reference to the effect of CA concentration, in samples treated with 40% CA a greater presence of broken or fissured granules is observed. It has been reported that high concentrations of the acid can cause an increase in the erosion of the granule due to the possibility of hydrolysis and the concomitant enlargement of the channels present in the granule [11].
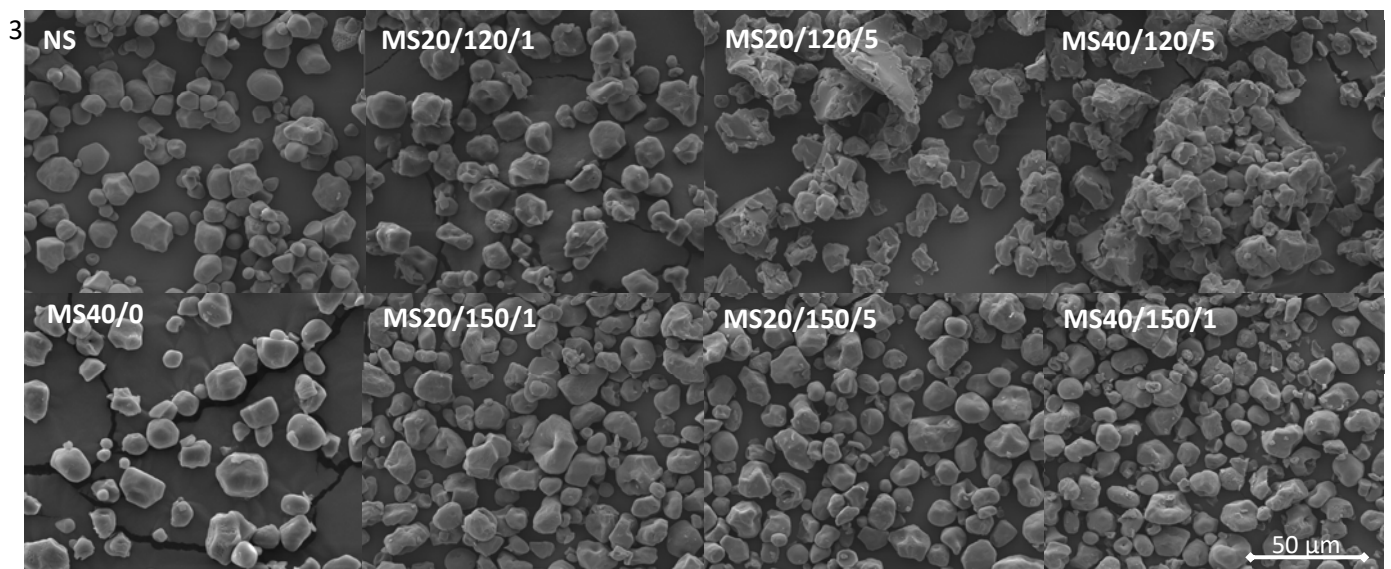
**Figure 6**. Representative FESEM micrographs of native and citric acid modified starch samples.

Figure 7 summarizes the results of the swelling power values of native and modified corn starch samples as a function of total substitution, crosslinking and monosubstitution degrees. The measured swelling power for native starch at 60°C was 3.1 g/g, a value in agreement with previous reports for corn starch assayed under similar conditions [24]. All modified starches showed swelling power values higher than that of native starch, a behavior aligned with the disruption of hydrogen bonds between the macromolecules due to the introduction of ester groups described above, which facilitates the entry of water into the granules [49].

In reference to the effect of total substitution, crosslinking and monosubstitution degrees on the swelling power of the starch citrates produced, a global analysis of results evidences a diminishing trend with the enhancement of the total substitution degree (**Figure 7a**), a behavior mainly attributed to the impact

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341 of the increment of the crosslinking degree (**Figure 7b**), since monosubstitution degree does not seem
342 to play a role in the swelling power of the granules (**Figure 7c**). Crosslinking has been reported to
343 promote a more compact network and reinforce the granular structure of starch leading to products with
344 lower swelling power, as well as greater resistance to shear stress, temperature and acid conditions;
345 higher gelatinization temperatures (or even inability to gelatinize), and reduced tendency to solubilize
346 [11, 14, 50, 51].

347 However, it is worth noting that swelling power showed a particularly strong dependence on reaction
348 temperature, with significantly lower values for the reactions performed at 150°C regardless the total
349 substitution, crosslinking and monosubstitution degrees achieved. In fact, all modified starches
350 produced at 120°C show swelling power values 160%-230% higher than that of native starch, whereas
351 modified starches produced at 150°C show much lower increments in this value (15%-75%) with
352 respect to native starch. The previous suggests that besides its effects in the evolution of total
353 substitution, crosslinking and monosubstitution degrees (**Figure 3**), temperature could have also
354 promoted tridimensional changes in the modified starch granules that further conditioned their swelling
355 behavior. Accordingly, and considering that granules swelling is essential for gelatinization, different
356 from the products of reactions performed at 120°C, none starch citrate produced at 150°C gelatinized
357 after 30 min at 96°C.

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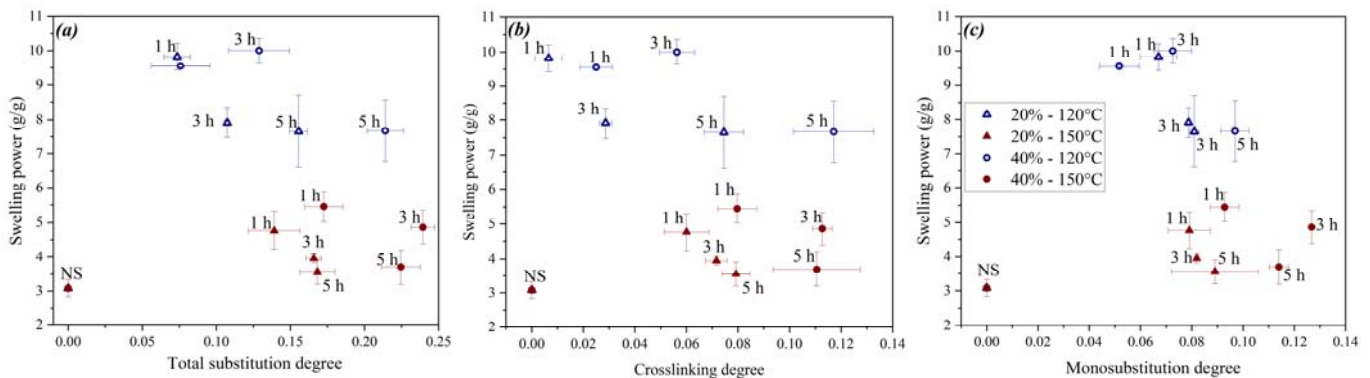
360 **Figure 7**. Swelling power at 60°C of native starch and citric acid modified starches.

361

362 The results of the resistant starch content of native and modified starch samples are shown in **Figure 8**.
363 Results illustrate the suitability of the methodology used to produce starch derivates with high RS
364 content. Within the assay intervals chosen, the three reaction variables evaluated (temperature, time and
365 CA concentration) proved to play a significant role on the modulation of the RS content of the modified
366 samples produced. For all variables assayed their increase led to higher RS contents. However, this

367 increment cannot only be attributed to the effect of these reaction variables on the chemical structure of
368 the starches, since products with similar substitution pattern herein obtained showed substantially
369 different RS contents depending on the reaction temperature used. In fact, RS values attained at 120°C
370 were in the 0-40% range, whereas in reactions performed at 150°C RS values in the ≈50-100% interval
371 were achieved. At each reaction temperature, the RS content did increase with the total substitution,
372 crosslinking and monosubstitution degrees. The additional impact of reaction temperature on the RS
373 content of starch citrates with similar degree of substitution was also observed by Kapelko-Żeberska et
374 al. [10].

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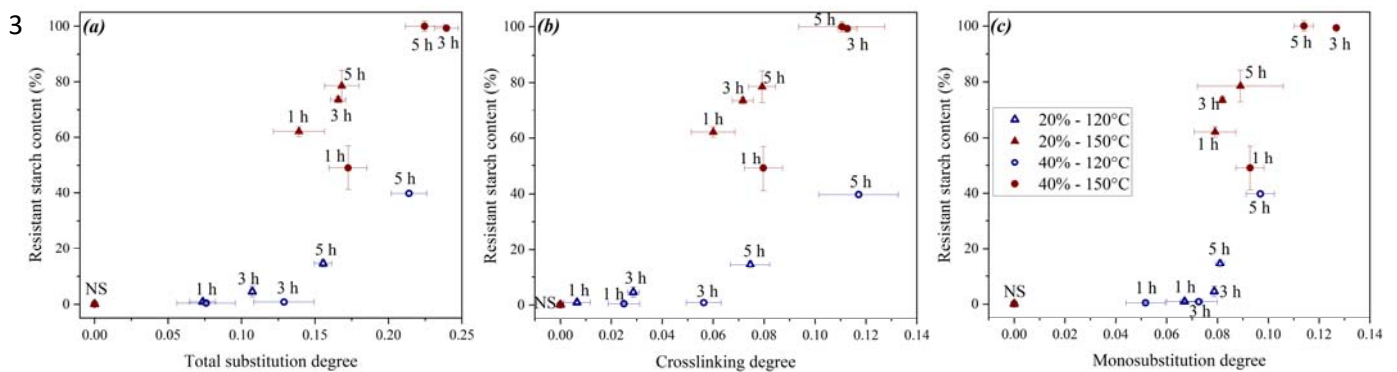
Figure 8. Resistant starch content of native starch and citric acid modified starches.

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378

379 The resistance to amylolysis of the starch citrates can be explained in terms of the high specificity of
380 digestive enzymes which do not hydrolyze the new bonds present in the modified starch samples.
381 Besides, both the substituents introduced into the polysaccharide and crosslinking restrict the formation
382 of the enzyme-substrate complex and thus make the neighboring bonds also resistant to enzymatic
383 degradation [14]. Furthermore, crosslinking limits the movement of enzymes through the pores and
384 channels of the granule surface [7, 11, 19]. Last but not least, tridimensional changes in the modified
385 starch granules promoted by their heating in the reaction medium may also restrict the formation of the
386 enzyme-substrate complex, increasing the RS content of modified starches. This last effect was
387 particularly evident for starch citrates produced at 150°C.

388

389 **4. Conclusion**
390 In the current contribution chemical modification of corn starch with citric acid under varying reaction
391 conditions was assayed, and the products obtained were characterized in terms of chemical structure,
392 morphology, crystallinity, swelling power and resistant starch content. The results obtained showed that
393 within the intervals herein assayed reaction time, temperature and citric acid concentration were suitable
394 variables to modulate the extent of chemical modification, the substitution pattern, and the resulting

395 properties of starch citrates, including RS content. Noteworthy, proper selection of reaction conditions
396 (i.e. 150°C, CA 40%, at least 3h) led to the obtention of modified starches with RS contents close to
397 100%.

398 On the other hand, for each set of reaction conditions assayed, the total substitution, crosslinking and
399 monosubstitution degrees achieved were quantified for the first time, and results were comprehensively
400 analyzed to determine whether the differences in the evaluated starch citrates properties could be
401 attributed to their final chemical structure. A global analysis of results evidenced a reduction of the
402 swelling power together with an increase in the resistance to amylolysis of starch citrates as higher total
403 substitution degrees were attained, a behavior mainly attributed to the impact of the increment of the
404 crosslinking degree. However, results clearly evidenced the determinant role of reaction temperature
405 which surpassed its influence on the reaction evolution. Actually, regardless the total substitution degree
406 and the relative contribution of crosslinked and monosubstituted units to the final polysaccharide
407 structure, starch citrates with lower swelling power and higher resistant starch content were obtained in
408 reactions performed at 150°C.

409 Overall, results show that, under the reaction conditions intervals assayed in this contribution,
410 production of citric acid modified starches with high resistant starch content mainly rely on the use of
411 sufficiently high reaction temperatures.

412

416

417 *Conflict of interest*

418 The authors declare no conflict of interest.

419

420 **5. References**

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