# STUDIES OF CONFORMATIONAL CHANGES, CRYSTALLINE AND GRANULAR STRUCTURES, AND *IN VITRO* DIGESTIBILITY OF CROSS-LINKED AND METHYLATED CORN STARCHES

*Lilliam Sívoli, Elevina Pérez, Mary Lares and Edgardo Leal*

*SUMMARY*

*Starch granular ultrastructure is an important determinant of its functional properties. Its knowledge, control and application should help to produce a wide range of food products. The goal of the study was to measure the modifications produced by cross-linking and methylation on the conformation and the granular and crystalline structures of corn starch, as well as the effects on the in vitro enzymatic digestibility of starch by α-amylase. Cross-linked and methylated starches were produced from commercial corn starch by low degree substitution (DS) methods. Both native and modified starches were analyzed employing NMR, SEM and X-ray diffraction. The morphologic characteristics, crystalline structure and susceptibility to hydrolysis by α-amylase were dramatically affected by the methylation process, while cross-linked corn starch showed non-significant variations that did not change the type A pattern of the native one. However, the $^{13}C$ CP-MAS NMR spectrum of the cross-linked starch is suggesting a change from a type A to a type B crystalline pattern, while the X-ray diffraction pattern of the methylated starch was completely different from those reported in the literature for any starches. No changes in granular shape were observed (SEM) in the cross-linked starch, while the methylated starch showed larger chunks without granular integrity and with a rough surface due to exo-erosion. It can be presumed that, due to the presence of methyl groups inside the granules, the enzyme-substrate interaction is hindered by steric effects.*

## Introduction

Corn ( *Zea maiz L* ) grows on a wide variety of soil types, from loamy sands to clays to organic soils. It is the main cereal source for many countries around the world. Due to its high starch content it is used, together with cassava, as the main sources of raw material for starch extraction (Thomas and Atwell, 1999).

In turn, starch is an important renewable raw material used in the food, pharmaceuticals and paper industries (Van der Burgt *et al*., 1999). The properties of native starches can be altered by a diversity of physical, as well as, chemical treatments such as oxidation, substitution and cross-linking, among others. These alterations are carried out in order to produce modified starches with the needed properties for industrial uses. Detailed information on the distribution of the substitutions performed can help understand the relation between molecular structure and functional properties (Van der Burgt *et al*., 2000a). Moreover, the study of the effects of the modifications is of interest, as it could give additional experimental information on the structural variety of amylose and amylopectin, and could help elucidate general factors that determine the structure of these carbohydrates. Knowledge of the molecular structure is especially critical for understanding the properties of polysaccharides (Van der Burgt *et al*., 1999, Jhonson *et al*., 2007).

In most chemical modifications of starch, usually referred to as chemical derivatization, the granular form is maintained and hydroxyl groups are partially substituted, yielding ether and starch esters, as well as anionic and cationic starches (Van der Burgt *et al*., 2000b). The number, location and distribution of the substitutions are not expected to occur randomly, in view of the different levels of organization within the starch granule, and they determine the properties of these starch derivatives (Van der Burgt *et al*., 1999). Usually, the modification treatments inhibit retrogradation (Tovar *et al*., 1999a, b) and the formulated products maintain uniformity and appearance for a long time. These modified starches offer advantages over the native starch by diminishing its consistency and increasing its shelf life.

Van der Burgt *et al*. (1999) reported that the crystalline linear side-chains of the amylopectin in methylated starches, which play an important role in the retrogradation of gelatinized starches, contain fewer substituents, than the amorphous branched parts and that they are almost randomly distributed. The same authors (Van der Burgt *et al*., 2000b) also demonstrated that the methylation process does not have any preference for substitution at either branched or linearly linked glucose residues, taking into account the inherently lower amount of substituted sites at branched residues. In order to learn about the relationship between structure and function, it is important to determine the changes in the starch molecules after modification by the derivatization processes and to measure their

**KEYWORDS / Corn Starch / Cross-Linking / Crystalline Structure / Granular Structure / *In Vitro* Digestibility / Methylation /**

Received: 06/06/2008. Modified:10/30/2008. Accepted: 11/10/2008.

**Lilliam Sívoli**. M.Sc. in Food Science and Technology, Universidad Central de Venezuela (UCV), Venezuela. Professor, UCV, Venezuela.

**Elevina Pérez S**. Doctor in Food Science and Technology and M.Sc. Food Science and Nutrition, University of Wisconsin, USA. Professor, UCV, Venezuela. Address: Instituto de Ciencia y Tecnología de Alimentos, UCV. Apdo. 47.097, Caracas 1041-A, Los Chaguaramos, Caracas, Venezuela. e-mail: pereezee@hotmail.com

**Mary Lares A**. Doctor and M.Sc in Food Science and Technology, UCV, Venezuela. Professor, UCV, Venezuela.

**Edgardo Leal**. M.Sc. in Chemistry. UCV, Venezuela. Professor, UCV, Venezuela.

## ESTUDIOS DE LOS CAMBIOS CONFORMACIONALES, ESTRUCTURA GRANULAR Y CRISTALINA, Y DIGESTIBILIDAD *IN VITRO* DE ALMIDONES DE MAÍZ ENTRECRUZADOS Y METILADOS

Lilliam Sívoli, Elevina Pérez, Mary Lares y Edgardo Leal

*RESUMEN*

*La ultraestructura granular del almidón es un determinante importante de sus propiedades funcionales. Su conocimiento, control y aplicación ayudaría en la producción de una amplia gama de productos alimenticios. El objetivo de este estudio fue medir las modificaciones producidas por el entrecruzamiento y la metilación en la conformación y en las estructuras granular y cristalina del almidón de maíz, así como sus efectos en la digestibilidad enzimática in vitro por la α-amilasa. Almidones entrecruzados y metilados fueron producidos a partir de almidón de maíz comercial por métodos de bajo grado de sustitución. Tanto el almidón nativo como los modificados fueron analizados empleando RMN, MEB y difracción de rayos X. Las características morfológicas, la estructura cristalina y la susceptibilidad a hidrólisis fueron dramáticamente afectadas por el proceso de metilación, mientras que el almidón entrecruzado mostró cambios no significativos que no modificaron el patrón tipo A del almidón nativo. No obstante, el espectro de $^{13}C$ CP-MAS RMN del almidón entrecruzado sugiere un cambio en su patrón cristalino del tipo A al B, mientras que el patrón de difracción de rayos X del almidón metilado fue completamente diferente de aquellos reportados para almidones en la literatura. No se apreciaron cambios (MEB) en la forma de los gránulos del almidón entrecruzado, mientras que el metilado mostró gránulos más grandes sin integridad y con superficie rugosa debida a exo-erosión. Es posible asumir que, dada la presencia de grupos metilo dentro de los gránulos, la interacción sustrato-enzima es obstaculizada por efectos estéricos.*

## ESTUDOS DAS MUDANÇAS CONFORMACIONAIS, ESTRUTURA GRANULAR E CRISTALINA, E DIGESTIBILIDADE *IN VITRO* DE AMIDOS DE MILHO ENTRECRUZADOS E METILADOS

Lilliam Sívoli, Elevina Pérez, Mary Lares e Edgardo Leal

*RESUMO*

*A ultra-estrutura granular do amido é uma determinante importante de suas propriedades funcionais. Seu conhecimento, controle e aplicação ajudariam na produção de uma ampla gama de produtos alimentícios. O objetivo deste estudo foi medir as modificações produzidas pelo entrecruzamento e a metilação na conformação e nas estruturas, granular e cristalina, do amido de milho, assim como seus efeitos na digestibilidade enzimática in vitro pela α-amilasa. Amidos entrecruzados e metilados foram produzidos a partir de amido de milho comercial por métodos de baixo grau de substituição. Tanto o amido nativo como os modificados foram analisados empregando RMN, MEB e difração de raios X. As características morfológicas, a estrutura cristalina e a susceptibilidade a hidrólise foram dramaticamente afetadas pelo processo de metilação, enquanto que o amido entrecruzado mostrou mudanças não significativas que não modificaram o padrão tipo A do amido nativo. No entanto, o espectro de 13C CP-MAS RMN do amido entrecruzado sugerindo uma mudança de seu padrão cristalino do tipo A ao B, enquanto que o padrão de difração de raios X do amido metilado foi completamente diferente daqueles relatados para amidos na literatura. Não se apreciaram mudanças (MEB) na forma dos grânulos do amido entrecruzado, enquanto que o metilado mostrou grânulos maiores sem integridade e com superfície rugosa devido à exo-erosão. É possível assumir que, devido à presença de grupos metilo dentro dos grânulos, a interação substrato-enzima é obstaculizada por efeitos estéricos.*

effects on the functional properties and digestibility of the modified starches.

Nuclear magnetic resonance (NMR) is a well-established tool for studying the molecular structure and dynamics of disordered solids such as polymers and bio-materials. The versatility of the technique is such, that new refinements are constantly being developed, resulting in spectra with higher signal-to-noise ratios, better resolution and increased information content (LeBoptlan *et al*., 1998; Li *et al*., 1996). Van der Burgt *et al*. (2000a) have studied the structure of methylated starch using NMR techniques.

X-ray diffraction analysis has been used to reveal the presence and characteristics of the crystalline structure of the starch granule. In native starch, crystal forming zones can be evidenced at the crystalline lamellae. The technique allows defining the types of crystal adjustments, depending on the position of the peak on the diffraction line. For example, an A-type crystal has a major peak at around d-spacing (2θ angle), a doublet at 17º and 18º, and a single peak at 23º (Thitiprapunkul *et al*., 2003). Indeed, crystalline and non-crystalline structures, and the relationship between them, are factors in determining starch properties and have been studied using X-ray diffraction techniques (Matos and Pérez, 2003).

Variations of the *in vivo* and *in vitro* digestibility of starch granules depend on the botanical source (granular size, structure or core, and amylose/amilopectin relationship) and other factors such as location of the granules in the cells, external treatment conditions, modifying agents and conditions of the food processing and storage (Biliaderis, 1991; Ring *et al*., 1998; Tovar *et al*., 1999a).

The goal of this study was to obtain structural and morphometric information about native and chemically modified starches by means of nuclear magnetic resonance, scanning electron microscopy and X-ray diffraction techniques, and to evaluate the susceptibility of the native and modified corn starches to α-amylase digestion.

### Materials and Methods

*Starch materials*

Commercial raw material was provided by INDELMA C.A., Cagua, estado Aragua, Venezuela. Chemical modification of the commercial native starch was carried out at the laboratory.

*Moisture content*

The moisture content of the native and modified starches was measured following the methods described by Whistler and Paschall (1964).

*Starch modifications*

*Phosphate starch*. Corn starch was modified by cross-linking using the method described by Whistler and Paschall (1964). The native starch was phosphated with $Na_3(PO)_3$ at alkaline pH, using low degree substitution (DS), so that the modified starch contained about 0.35% of bound phosphorous (DS= 0.02). The DS for the modified starch samples was determined using the equation for monosodium esters

$$DS = 162P/(3100-102P)$$

where P is the difference in phosphorous content (dry basis) between the chemically modified and the native starch, expressed as percentage (Matos and Pérez, 2003).

*Methylated starch*. Granular native corn starch was methylated in aqueous suspension with dimethyl sulfate to DS values up to 2.0, using the method of partially methylated starch described by Whistler and Paschall (1964) modified as follows.

Native corn starch (300g) was shaken in 800ml of distilled water, in a water bath (28 ±2°C) to form a smooth paste. The paste was transferred to a vessel containing a vigorously stirred solution of 330g of crystalline barium hydroxide in 800ml of hot water. The mixture was heated to boiling and 94.7ml of pure dimethyl sulfate were added drop by drop while stirring continuously. The mixture was kept at 45°C for 24h and then centrifuged at 2500rpm during 15min to decant the excess barium sulphate. The precipitate was re-suspended in water at 55°C and centrifuged at 2500rpm during 15min, repeating this step as necessary, so as to eliminate the precipitated barium sulphate. The residue was extracted twice with 100ml of chloroform and the chloroform extracts evaporated to dryness under reduced pressure. This procedure left a residue which was powdered and dried at 50°C under reduced pressure.

*Nuclear magnetic resonance (NMR)*

All NMR spectra were obtained with a Bruker AM300 (Bruker Instrument, Mountain View, CA, USA) equipped with a 7mm rotor, to CP/MAS. The acquisition parameters used were spin velocity 3500Hz, temperature 300°K, contact time 2ms, a 4sec 90° pulse and scanning number 128. All spectra were replicated at least twice (Choi and Kerr, 2003).

*Scanning electron microscopy (SEM)*

Starch samples were sprayed on a metal plate previously covered with double-sided adhesive tape and shadowed under vacuum with gold-palladium. The starch granules were examined with a scanning electron microscope (Hitachi S-2400) at 20kV accelerating voltage (Matos and Pérez, 2003).

*X-ray diffraction*

X-ray diffraction patterns were obtained with a Phillips diffractometer using monochromatic cobalt radiation, 31kV, 26mA, 4sec time constant and 1cm/min chart speed. Difractograms were recorded at $2\theta = 4$ - 30° at a scan rate of 1°/min (Zobel, 1988). The samples were measured on wet basis, moisture contents being given below.

*Starch α-amylolysis*

The degree of hydrolysis of both native and modified starches were assessed following the method described by Holm *et al.* (1985) and modified by Tovar *et al.* (1992), using type B pancreatic α-amylase. Wheat starch was used as a reference. The starches (native and modified) were not gelatinized previous to the hydrolysis.

*Statistical analysis*

Mean and standard deviation were calculated, using the statistical package SPSS version 8.0 (1997).

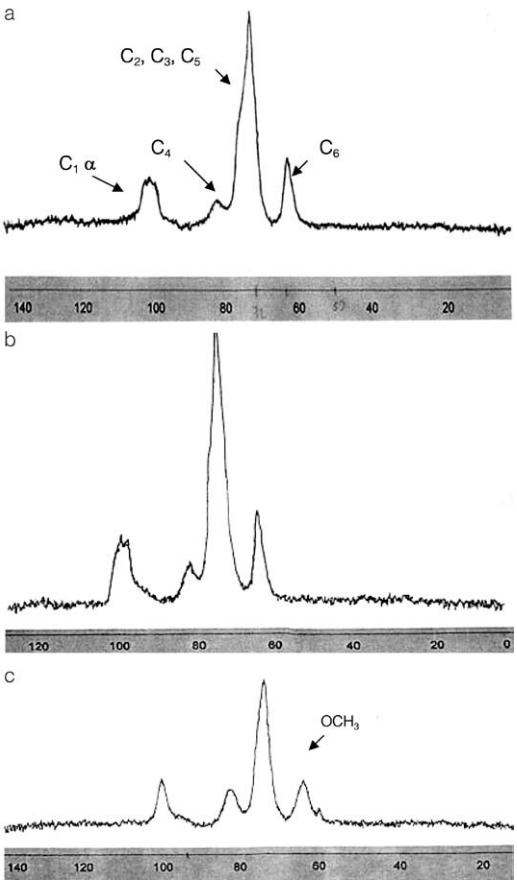

Figure 1. $^{13}C$ CP-MAS spectra of native (a), cross linked (b) and methylated (c) corn starches.

## Results and Discussion

*Starch moisture content*

The moisture content was 11.47% in the case of the native starch, while for the phosphated and methylated starches it was 6.87% and 6.71%, respectively.

*Nuclear magnetic resonance (NMR)*

NMR spectra of native, cross-linked and methylated corn starches (Figure 1) showed signals between 50 and 110ppm. In native starch (Figure 1a) the signal at 59-61ppm corresponds to that of $C_6$, as indicated by Atichokudomchai *et al.* (2004), who reported a signal at 58-65ppm for $C_6$. The strong signals 72-80ppm match the resonance signals of the $C_2$, $C_3$ and $C_5$ internal carbons of the glucose chain. $C_2$, $C_3$ and $C_4$ are the reactive carbons from the anhydroglucose unit. The $C_4$ resonance appeared as a weak peak at ~82ppm. Finally, the region around 100ppm corresponds to the anomeric carbons $C_1(\alpha)$ according to Li *et al.* (1996) and Atichokudomchai *et al.* (2004). The crystalline structure of starch has been demonstrated by the shape of the $C_1$ resonance line (Li *et al.*, 1996). Morgan *et al.* (1995) reported that the $C_1$ carbon atoms show chemical shifts characteristic for each of the three types of crystalline conformation. The $C_1$ resonances of the native starch are triplets (~102, 101 and 100ppm) and relate to the double helices symmetry, because the repeated unit is a maltotriosyl unit, in the A form (Buléon *et al.*, 1998). In Figure 1a the $C_1$ carbon atom appears as an incipient cluster of three peaks. It corresponds with the X-ray pattern of conformation A, in harmony with reports by Morgan *et al.* (1995), Li *et al.* (1996) and Buléon *et al.* (1998).

Figure 1b shows the NMR spectrum of cross-linked corn starch. As can be noted in the figure, the changes are most evident in regions from 80 to near 110rpm, which correspond to $C_4$ internal carbons of the glucose chain, and to $C_1$ or

anomeric carbons. The $C_4$ resonance in the region ~82ppm of the cross-linked starch is higher than that of the native counterpart, probably due to changes in crystalline conformation produced by the thermal treatment. The $C_1$ resonance in the region ~100ppm is characterized as a doublet splitting, as the B form crystalline structure, and is also higher than the native counterpart. No substitution was observed over the branched regions, as expected. An increment was also evident in the height of the signals from $C_6$ and the $C_2$, $C_3$ and $C_5$ internal carbon regions of the glucose chain.

The $^{13}C$ CP-MAS NMR spectrum of the methylated starch (Figure 1c) shows an incipient signal at 61ppm, which indicates the presence of the methyl (-$OCH_3$) groups in the anhydroglucose chain branched region. It is also observed that the region above 100ppm is narrower and lower than in the spectra of the other two samples, which could be due to the change at its anomeric carbon. The intensity of the signal in the region from 80 to 82ppm, corresponding to the $C_4$ carbon, is higher and more conspicuous than in the native starch. Also, the branched region (60-40ppm) is altered, probably due to a preference for the substitution sites at branched glucose residues (Van der Burgt *et al.*, 2000b).

On the other hand, the changes in resonance reflect the structural transition from crystalline to amorphous state due to the temperature

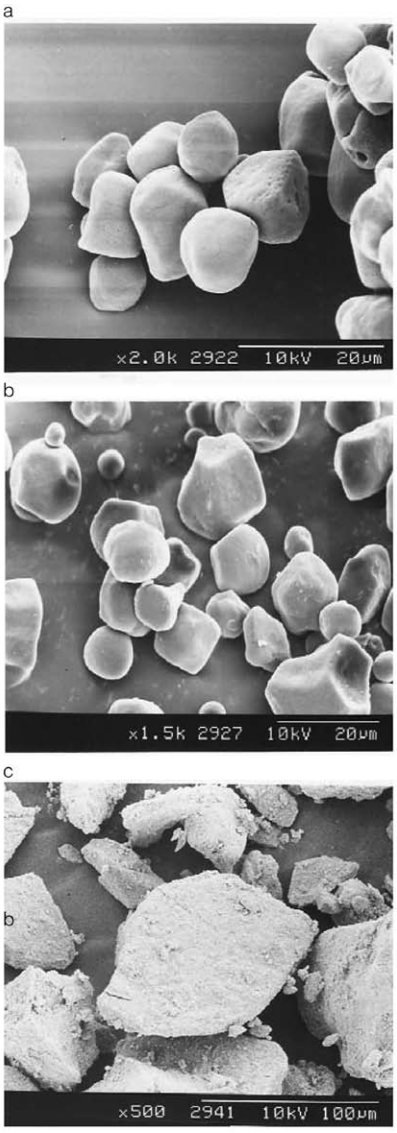


Figure 2. Scanning electron micrographs of native corn starch at x 2.0k (a), cross linked corn starch at x 1.5k (b), and methyled corn starch at x 500k (c).

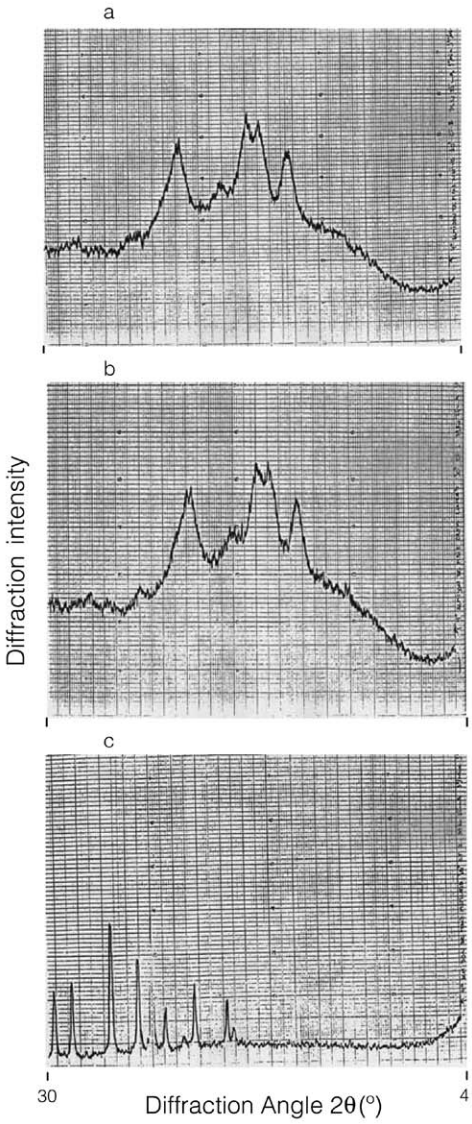


Figure 3. X-ray diffraction of native corn starch (a), crosslinked corn starch (b), and methyled corn starch (c).

(Li *et al.*, 1996). The resonance from the amorphous domain appeared between the $C_4$ and $C_1$ regions. The crystalline regions are narrower than amorphous regions.

*Scanning electron microscopy (SEM)*

The appearance of the starch granules before and after modification is shown in Figure 2. That of native corn starch is similar to that reported in the literature (Thomas and Atwell, 1999). Both native and cross-linked starches show granules of various types, round and polyhedral shaped. These findings suggest that there are no changes in the shape of corn starch granules due to modification by cross-linking, because the treatment did not produce gelatinization of the starch. Similar results were reported by Matos and Pérez (2003) for cassava starch. The surfaces of the native and phosphate granules are smooth, with some surface pores. In contrast, the methylated starch shows larger chunks without granular integrity, as compared to the native and cross-linked counterparts and, also, a rough surface due to exo-erosion.

*X-ray diffraction pattern*

Figure 3 presents the X-ray diffraction patterns of native and modified starches. Native starch (in a) shows a type A pattern, which is characteristic of cereal starches (Thomas and Atwell, 1999). The cross-linked starch (in b) maintains the crystalline type A-pattern, with insignificant variations that do not change the original pattern. These variations could explain the result found by NMR, which suggests a B type crystalline conformation. The small variations could be due to the introduction of phosphate groups inside the crystalline lamellae. On the other hand, the marked change observed after modification by methylation (Figure 3c) represents a different structure, not found in the reviewed literature.

*Starch digestibility*

The degree of hydrolysis of native and modified starches by α-amylase, expressed as the percentage of starch that was hydrolyzed, is shown in Figure 4. It can be seen that the degree of hydrolysis *in vitro* of the native and cross-linked corn starch is similar to that shown by the native wheat starch used as a reference up to 60min. A plateau is reached at less than 40% hydrolysis after 30min of digestion. These results agree with those reported by several authors, who maintain that these types of modification do not af-

fect the hydrolysis significantly (Wooton and Chaundry, 1979, Ostergrad *et al*., 1988; Hung and Morita, 2005). As pointed out before, these starches were not gelatinized previous to the hydrolysis, a fact contributing to a lower degree of hydrolysis than that found in gelatinized corn starch, of 66.6% at 15min and 73.7% at 60min (Laurentín *et al*., 2003). Except for methylated starch, all the starches were rapidly hydrolyzed in the initial 5min and thereafter the rate of hydrolysis decreased significantly.

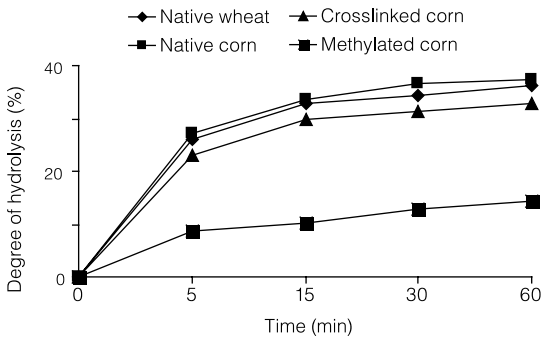

Figure 4. α-amylolysis of native and modified starches.

However, it can be noted in Figure 4 that the modifications alter the degree of hydrolysis of the starches and also that the methyl starch is severely affected as compared with cross-linked corn starch, which is affected in a minor proportion. It can be presumed that the low degree of hydrolysis shown by the methylated starch could be due to the presence of methyl groups within the granules, hindering the enzyme-substrate interaction by steric effects.

## Conclusion

Changes in the conformation of corn starches were demonstrated by the $^{13}C$ CP-MAS NMR spectra. The changes are indicative of the degree of substitution and the effect of temperature modification on the crystalline conformation. The $^{13}C$ CP-MAS NMR spectrum of native starch is compatible with a crystalline type A-pattern, as is reported for cereals. However, the cross-linked starch $C_1$ resonance in the spectrum region ~100ppm is characterized as a split doublet, similar to the B form of the crystalline structure reported for non-cereal starches. Despite of this, the X-ray diffraction pattern for both native and cross-linked starches reveal them as type A starch. There are more evident changes in the methylated starch spectrum, where the presence of -OCH3 groups near the $C_6$ terminal can be clearly observed. The cross-linking modification does not alter the granular structure of the starch, but methylation does alter it, the granular integrity being lost. The cross-linked corn starch shows a similar degree of hydrolysis by α-amylase to that of the native corn starch. As a result, they should have similar digestibilities when cooked and ready to eat. In turn, methylation reduces the starch bio-availability by hindering enzymatic digestion. All of these characteristics must be considered when using these modified corn starches.


### ACKNOWLEDGEMENTS

Published as a paper based on research conducted under projects Nº: PCP-2007002000 and 200701146, supported by the Fondo Nacional de Ciencia, Tecnología e Innovación (FONACIT) and Misión Ciencias, respectively.


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