Carbohydrate Polymers 87 (2012) 61–68

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Determination of the structural changes by FT-IR, Raman, and CP/MAS ^{13}C NMR spectroscopy on retrograded starch of maize tortillas

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 21 June 2011
Accepted 8 July 2011
Available online 19 July 2011

Keywords:
Nixtamalization
Retrograded starch
Spectroscopic methods

ABSTRACT

The nixtamalization, production and storage of tortillas in refrigeration cause several changes on the starch structure, resulting in an increased crystallinity and therefore a higher content of resistant starch. The IR analysis for resistant starch (RS) showed a band at 1047 cm^{-1} associated to the retrogradation process; this band was due to the weakening of the intermolecular H-bonds. These associated together to form ordered regions. The Raman analysis shows a characteristic band at 856 cm^{-1} corresponding to C–C skeletal modes of glucose of α-1,4 glycosidic linkage starches, and a band at 480 cm^{-1} attributed to skeletal vibrations of the pyranose ring in the glucose unit of starches. These changes may be related to the polymerization degree of the starch molecules, as well as to the retrogradation of amylose and amylopectin. The spectrum of ^{13}C CP-MAS/NMR for RS3 supports the results obtained by IR and Raman. Lipidic and proteic groups were observed which may be in the form of complexes with amylose. One can proclaim that the existence of the salt form is induced and stabilized by the interactions dominating the V amylose structure in the solid state.

1. Introduction

Tortillas are extensively consumed in Mexico, with annual sales exceeding $6 billion. They are considered as a good source of calcium and fiber, as well as an important source of protein and starch (Quintanar-Guzmán, Jaramillo-Flores, Mora-Escobedo, Chel-Guerrero, & Solorza-Feria, 2009; www.tortilla-info.com Tortilla Industry Association 05/08/2009). The industrial production of tortillas is based on the traditional nixtamalization process that consists of alkaline cooking of maize with calcium hydroxide, which has been systemized for large-scale production. Small variations in the process significantly affect the quality of end products. The alkaline treatment changes drastically the appearance of the protein bodies in some areas of the kernel. Differences in the size of both nixtamalized and non-nixtamalized starch granules in micrographs from scanning electron microscopy (SEM) have been observed (Mondragón, Mendoza-Martínez, Bello-Pérez, & Peña, 2006; Quintanar-Guzmán et al., 2009).

Starch granules from non-nixtamalized samples usually appear bigger and smoother compared to nixtamalized ones. The structural and physicochemical changes of the grain during processing affect the functional properties of the final nixtamalized product (García-Rosas et al., 2009). The nature of the crystalline form in the starch granule depends on the amylose–amylopectin ratio and any food processing effect which has lead to retrogradation, such as cooking and cooling rich starchy foods. Retrograded starch, also known as RS type 3 (RS3) or retrograded resistant starch (RRS), may be formed in cooked foods kept at or below room temperature (Cummings & Robertfroid, 1997). The nixtamalization and storage conditions of tortillas can significantly influence resistant starch levels of maize processed into tortillas (Mora-Escobedo, Osorio Diaz, Garcia-Rosas, Bello-Pérez, & Hernández-Unzon, 2004).

Gelatinization is a process before the consumption of starch foods that determines the extent of RS formation. When starch granules are fully gelatinized and dispersed, the starch is easily digestible. However, as the gel cools and ages, the polymers again take on a partially crystalline structure (retrograded starch) (González-Soto, Mora-Escobedo, Hernández-Sánchez, Sánchez-Rivera, & Bello-Perez, 2007). The intimate packing of amylose double helices leads to crystal formation, which hinders the accessibility of α-amylase to the glycosidic bonds. In the formation of RS3, the starch granule is completely hydrated and amylose is leached from the granules into solution as a random cool polymer. Upon

Abbreviations: SM, starch of maize; SFT, starch of fresh tortilla; SST, starch of stored tortilla; RS, RS3, resistant starch; RSFTS, RS3 of fresh tortilla starch; RSSTS, RS3 of stored tortilla starch.

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doi:10.1016/j.carbpol.2011.07.011

cooling, the amylose chains begin to re-associate as double helices stabilized by hydrogen bonds.

Other factors that affect starch retrogradation include the structures of amylopectin, the content of amylose and the existence of non-starch components such as protein and lipids. The resistant starch contains molecules with a wide range of molecular weights. A major fraction consists of fragments of retrograded amylose with an average degree of polymerization of 70–80. The structure of retrograded starch is very complex including crystalline and amorphous lamellae (Hoover, 2001; Hoover & Zhou, 2003).

Several studies on molecular changes in starch using different techniques have been reported, such as differential scanning calorimetry (DSC), X-ray diffractometry (XRD) (García-Rosas et al., 2009; Jinsong, Wang, & Yun, 2007; Sevenou, Hill, Farhat, & Mitchell, 2002), near-infrared spectroscopy (NIR) (Bulkin, Kwak, & Dea, 1986; Kazuo et al., 1998; Ramazan & Irudayaraj, 2006; Ramazan, Irudayaraj, & Seetharaman, 2002), Fourier transform infrared (FT-IR), Raman (FT-Raman), ^{13}C CP-MAS/NMR and HNMR nuclear magnetic resonance (Snape et al., 1998; Yuan, Jingmin, & Xiaolin, 2002; Zhang, Golding, & Burgar, 2002). Infrared (IR) spectroscopy has been well established as a useful tool for structure elucidation and quality control in various industrial applications. Raman spectroscopy offers the ability to analyze very small amount of sample non-destructively with high sensibility. Both IR and Raman spectroscopic techniques can help in the understanding of changes on architecture and composition molecular of proteins, carbohydrates, nucleic acids, cell membranes and tissues (Socrates, 2007). Using in combination IR, Raman and ^{13}C CP-MAS/NMR, it is possible to obtain differences among structural macromolecular changes. For example, it is possible to observe differences between crystalline and amorphous substances and also the presence or absence of hydrogen bonding (Socrates, 2007).

In this work, the techniques of the FT-IR spectra, Raman spectra and solid state ^{13}C CP-MAS/NMR spectroscopy, were used as tools to determine the structural changes produced by starch retrogradation occurring in fresh (1 day at $4 \pm 1\,^{\circ}C$) and stored tortillas (10 days refrigerated at $4 \pm 1\,^{\circ}C$).

2. Materials and methods

2.1. Materials

Samples of "maize mate" were collected in Españita, Tlaxcala, Mexico, supplied as a variety of maize (*Zea mays*) obtained by National Institute of Agriculture and Forestry Researches, Tlaxcala, México (INIFAP). This particular maize variety contained a high proportion of starch and protein and it is consumed in the region.

2.2. Tortilla preparation

The traditional method to produce tortillas was applied according to the technique reported by Mora-Escobedo et al. (2004). 1 kg of maize was used in the preparation of nixtamal. Nixtamal was ground into dough (masa) with a commercial machine. The fresh dough was then molded, using a manual machine to make tortillas (approximately of 1.5 mm thickness). Tortillas were baked in a gas fired domestic oven for 1 min per side at $250 \pm 10\,^{\circ}C$, cooled to room temperature, then sealed inside polyethylene bags and stored at $4 \pm 1\,^{\circ}C$ during 1 and 10 days. At the first time a sample of tortillas stored for 1 day at $4\,^{\circ}C$ was separated and lyophilized to analyze, after that the tortillas stored for 10 days were lyophilized as well.

2.3. Isolation of tortilla starch

Starch was isolated from maize, fresh tortillas and stored tortillas by 10 days using the methodology reported by Paredes-Lopez, Schevenin, Hernandez, and Carabez-Trejo (1989). 500 g of maize, fresh tortilla and tortillas stored for 10 days (lyophilized after storage) were ground into powder; then 3 l of 96% ethanol was added to maize and tortillas separately. The suspension was strained through 20, 40, 100 and 200 U.S-mesh screens. The residues retained by each screen were washed with 96% ethanol to recover the starch. Then starch was dried at room temperature for 48 h. It was obtained starch of maize (SM), starch of fresh tortilla (SFT) and starch of stored tortilla (SST).

2.4. Retrograded resistant starch

Resistant starch associated to dietary fiber content, denominated retrograded resistant starch type III (RS3) was prepared according to Mora-Escobedo et al. (2004). This method was described previously by Saura-Calixto, Goñi, Bravo, and Mañas (1993) and Goñi, Garcia-Diz, Maad, and Saura-Calixto (1996). Resistant starch was obtained from three samples; starch from maize (SM), starch from fresh tortillas (SFT), and starch from tortillas stored for 10 days (SST). The samples were suspended and homogenized in 250 ml phosphate buffer (pH 6.0, 55.6 mM), with α-amylase (A-3306, Sigma Chemical Co., St. Louis, MO, USA) (4 g α-amylase/10 g starch) at $96\,^{\circ}C$ for 35 min. Incubation with protease (10 μg) (Sigma P-5380) at $60\,^{\circ}C$, 35 min, pH 7.5 for removal of protein and amyloglucosidase (Sigma A-9913) (10 ml/10 g starch) was added to hydrolyze RS and the mixture was shaken for 35 min at $60\,^{\circ}C$, pH 4.5. Samples were defatted (petroleum–ether extraction) after extraction. Thus samples of resistant starch of maize starch (RSMS), fresh tortilla (RSFTS) and tortilla stored for 10 days (RSSTS) were obtained.

2.5. FT-IR measurements

IR spectroscopy provides information about chemical groups containing highly polar bonds, or bonds whose dipole moment changes during vibration, e.g. the C=O and OH groups. NIR spectroscopy involves overtones and combinations of the fundamental IR absorptions mainly for chemical bonds containing hydrogen atoms like OH and CH. FT-IR spectra were recorded using a FT-IR-Bruker model tensor 27 spectrometer (Madison W), for each spectrum, 32 scans were recorded at room temperature (about $22 \pm 1\,^{\circ}C$) at a resolution of $4\,cm^{-1}$ in a range of 4000–500 cm^{-1}.

2.6. Raman and ^{13}C CP-MAS/NMR measurements

Raman is more sensitive to the fundamental vibrations of less polar molecular groups and bonds like C–C and for symmetric vibrations (Thygesen, Løkkey, Micklander, & Engelsen, 2003). In the Raman spectroscopy analysis, Raman spectra were recorded with a Perkin-Elmer System 2000 FT-IR spectrometer; and for the ^{13}C cross polarization-magic-angle spinning/nuclear magnetic resonance (^{13}C CP-MAS/NMR) analysis. The ^{13}C CP-MAS/NMR spectra were recorded at 75 mHz using a Varian unity 300 mHz, operating at room temperature.

3. Results and discussion

3.1. Analysis of structural changes by infrared spectroscopy (IR) due to nixtamalization process and storage of tortillas

The IR spectra for starch of maize (SM) and starch of fresh tortilla (SFT) showed almost identical characteristic bands, but Fig. 1 shows only the IR spectra of starch of fresh tortilla. The bands are originated mainly from the vibrational modes of amylose and amylopectin. The band at 1600 cm^{-1} is associated to the amorphous

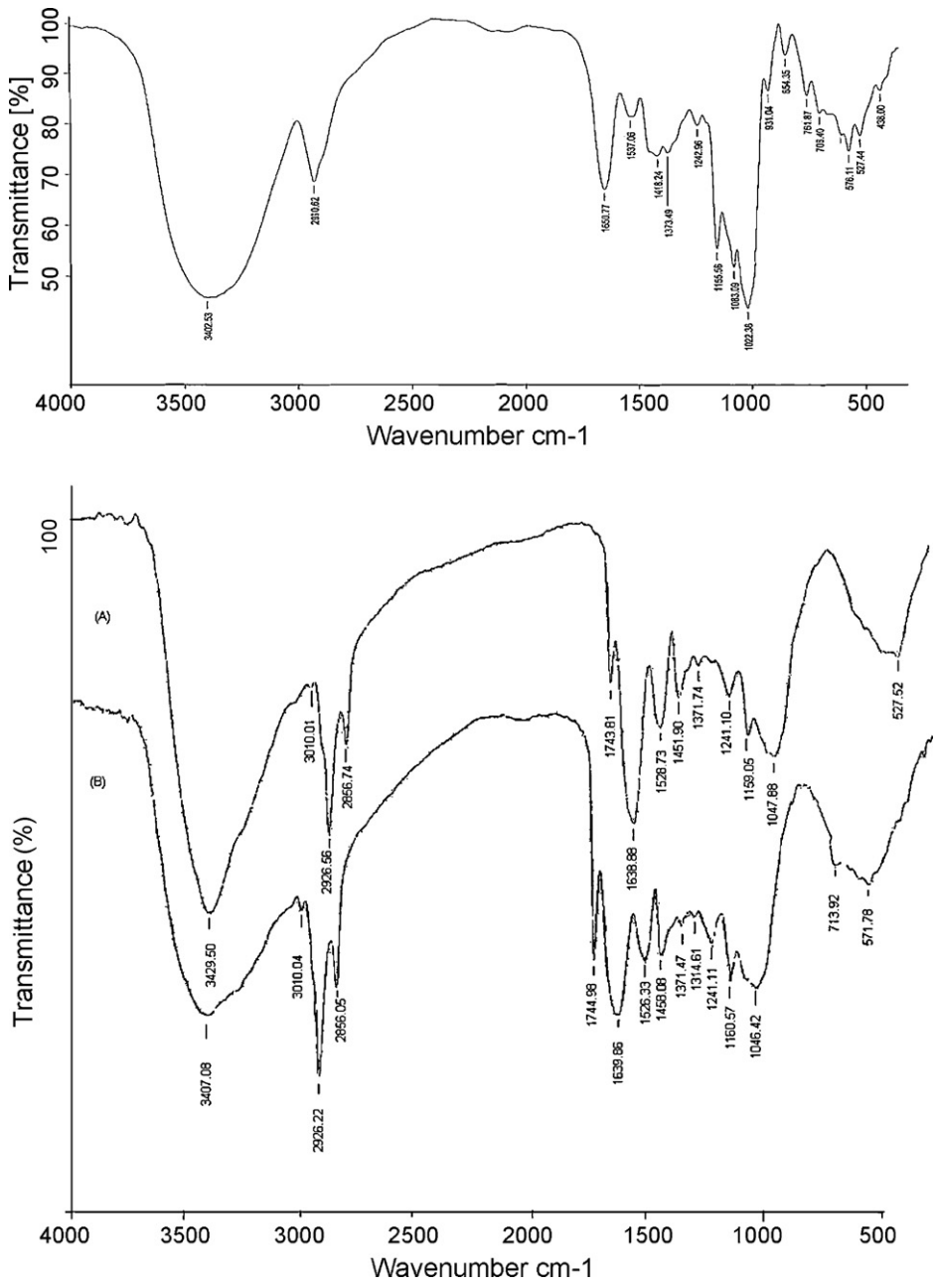

Fig. 1. IR spectrum of starch of fresh tortilla (SFT). (A) IR spectra of RS3 of fresh tortilla starch (RSFTS) and (B) IR spectra of RS3 of stored tortilla starch (RSSTS).

region of starches; the one at 1500 cm^{-1} to the skeletal mode vibration of α-1,4 glycosidic linkage (C–O–C), and the bands at 1022 and 850 cm^{-1} are sensitive to changes in crystallinity. The intensity of the band at 1022 cm^{-1} determines the orientation in intermolecular H-bonding of CH and CH_2 in CH_2OH (Kacurakova & Mathlouthi, 1996; Van Soest, de Wit, & Vliegenthart, 1994).

IR spectra of samples of RS3 of fresh tortilla starch (RSFTS) and RS3 of starch of tortilla stored for 10 days (RSSTS) are showed in Fig. 1A and B, respectively. When comparing both spectra, it is possible to find changes in the amplitude in the band at 3400 cm^{-1} (corresponding to the –OH). In both spectra it can be seen other sensitive band at 1047 cm^{-1} which corresponds to retrogradation as Van Soest et al. (1994) reported. This band is a characteristic of crystalline regions of a starch system, being this part more organized than the amorphous region. On the other hand, in Fig. 1A and B the disappearance of the band at 1022 cm^{-1} was observed which is the characteristic of the amorphous region of the starch. Van Soest et al. (1994) reported that this band was sensitive to changes in crystallinity; and it was more pronounced with decreasing crystallinity. Fig. 1A and B shows a 1047 cm^{-1} band, which is more pronounced for RSSTS. This suggests a reduction in amorphousness region or an increase in the organization of the structure. The shift of this peak can be due to the formation of an ordered region of crystalline lamellae' (Tester, Karkalas, & Xin, 2004). This could be associated with the storage time of tortillas and then with the increase of RS3. The bands 1022 and 931 cm^{-1} in Fig. 1 disappeared with the retrogradation process as observed in Fig. 1A and B. Amylase treatment may decrease the proportion of amorphous starch in tortillas, however in Fig. 1A and B it may be observed the appearance of a band at 1047 cm^{-1}, which corresponds to retrograded starch. As the band at 1022 cm^{-1} disappears, the band at 1047 cm^{-1} tends to become better resolved and predominant during retrogradation.

Tortilla resistant starch stored for 10 days shows 1047 cm^{-1} band slightly wider than the resistant starch from fresh tortillas. Several features of enzyme-resistant retrograded starch, however, remain obscured. In particular the logical hypothesis that resistance to amylolysis conferred by double helical crystalline state is difficult to reconcile with the relatively low crystallinity of enzyme-resistant retrograded starch and amylase treated retrograded amylose. However, less double helix aggregates, may also confer resistance to enzyme hydrolysis (Gidley et al., 1995).

Sevenou et al. (2002), Smits, Frank, Johannes, Vliegenthart, and Van Soest (1998), and Van Soest et al. (1994) reported that the ratio of absorbance 1047/1022 cm^{-1} could be used to determine the order in more crystalline regions and it is associated to the degree of retrogradation in starch samples. Other characteristic bands of changes after the process of retrogradation were observed at 2900 cm^{-1}. The presence of one band at 2930 cm^{-1} was observed for starch of fresh tortilla (SFT) and starch of stored tortilla (SST), but since this band was affected by retrogradation, two absorption bands appear afterwards at 2926 and 2856 cm^{-1}, assigned to vibrations of CH_2. The conformational changes due to the phenomenon of retrogradation strengthen this result.

The bands at 1650, 1537, 1418, 1373 and 1242 cm^{-1} also changed with the retrogradation process. In the samples of resistant starch from fresh and stored tortilla starch (RSFTS and RSSTS), additional bands were observed at 1241, 1371, 1451, 1528, 1638 and 1743 cm^{-1}, which were more pronounced. These bands may be assigned to conjugated carbonyl and carboxyl groups and C–O vibrations (Nuopponen et al., 2006; Thygesen et al., 2003). This is an indication of the presence of different macromolecules, such as the band at 1743 cm^{-1} that is associated to lipidic and proteic groups. Thygesen et al. (2003) reported the formation of complexes between lipids and amylose. Lipids may contribute to the stability of the helicoidal structure, as well as encouraging the interactions required for the formation of small crystals of resistant starch. The band at 3010 cm^{-1} indicates the presence of nitrogen from amidic and proteic groups. This is an important finding, since there are no current reports in the literature that associate the structure of resistant starch with lipids and proteins. It is possible that this association takes place only in the resistant starch extracted from tortillas, which were produced using a thermal alkaline treatment.

The peak at 1743 cm^{-1} shown in the IR of the RS3 of tortillas (Fig. 1A and 1B) has been identified as the ester carbonyl group (Hanjun, Mitsunaga, & Kawamura, 2006; Nuopponen et al., 2006; Thygesen et al., 2003). Since a thermal alkaline process is used in the tortilla production, the presence of a peak at 1743 cm^{-1} may be associated to the esterification of maize lipids by $Ca(OH)_2$. Similar results were reported by García-Rosas et al. (2009). Tester et al. (2004) reported that part of the amylose fraction within lipid-containing granules exists as an amylose inclusion complex where the fatty acid chains occupy a hydrophobic core located within the single amylose helix.

In summary, four main regions in an infrared spectrum of RS3 of tortilla were identified. A region associated to the skeletal mode of amylose and amylopectin is found at 700 and 400 cm^{-1}. The region between 1022 and 1115 cm^{-1} is attributed to the amorphous and crystalline regions of the starches, respectively. Bands between 1743 and 1638 cm^{-1} correspond to the region of carbonyl and carboxyl groups associated to the lipid and protein molecules present in the sample. The band at 2930 cm^{-1} is due to the change in CH_2 vibrations; and finally the region between 3000 and 3400 cm^{-1} corresponds to the O–H stretch region. Thus, the formation of intra and intermolecular hydrogen bonds is essential to the re-crystallization in the retrogradation process due to the nixtamalization process and storage of tortillas.

3.2. Analysis of structural changes by Raman spectroscopy due to nixtamalization process and storage of tortillas

Raman spectroscopy was used to probe the internal vibrations of molecules of starch of maize (SM), tortillas (fresh and stored) (SFT and SST) and retrograded starch (RSFTS and RSSTS). Raman is more sensitive for the fundamental vibrations of less polar molecular groups and bonds like C–C and for symmetric vibrations. The principal characteristic bands in the Infrared and Raman spectra of native starch are related to the vibration modes of the α-1,4-glycosidic linkage and the C–O–C bond in the glucose ring. For starch, generally the characteristics of Raman bands at 3200, 2900, 930, 856 and 477 cm^{-1} are associated to amylose and amylopectin (Fig. 2). The band at 856 cm^{-1} is in connection with the most intense band at 477 cm^{-1} that is attributed to α-glucose ring (C–O–C) skeletal modes and a further vibration involving the C–O–C ring bond. The band at 1080 cm^{-1} is assigned to a C–O–C stretching vibration, which is sensitive to anomeric configuration (Enrico-Pigorsch, 2009). Amylose and amylopectin can be also successfully analyzed by application of Raman spectroscopy. It has been reported that the structural differences of both starch materials can be detected in the C–H stretching region between 2700 and 3100 cm^{-1}. The bands consist essentially of a C–1–H deformation coupled to a CH_2 vibration (Hartwig & Malgorzata, 2007). Thus, these bands are attributed to the skeletal mode, the C–H and CH_2 deformations, the skeletal mode involving α (1–4) linkage, and the CH_2 deformation (Dupuy & Laureyns, 2002). The band at 930 cm^{-1} is assigned to the symmetric stretching vibration of the α-1,4-glycosidic linkage. The band found at the 1100 cm^{-1} region, which is the characteristic of pyranose sugars, is predicted to be a complex ring-mode in each case. Also, the modes that are dependent upon the crystal structure for amylose are found at approximately 1440, 1340 and 1200 cm^{-1}. These bands can describe a complex of vibrations among CH_2, C–O–H and C–C–H (Schuster, Ehmoser, Gapes, & Lendl, 2000; Socrates, 2007). The Raman bands in 2800 and 3000 cm^{-1} regions are due to C–H stretches, and the band in 1650 cm^{-1} with varying intensities appears to correlate with the amount of amylose present in the maize starches (Chan, Xing, Phillips, & Corke, 2001).As mentioned above for IR, the spectrum for corn starch, fresh and stored tortillas starch showed identical bands, therefore, Fig. 2 only shows spectrum corresponding to tortillas starch. The spectrum shows bands at 3300–3000, 2140–2050 and 1550–1480 cm^{-1} associated to groups of protein because it is known that the proteins can be associated with both the surface and interior of starch granules. The amount depends on the treatment of maize granules and the characteristics of proteins (Debet & Gidley, 2006; Socrates, 2007). The lime is promoting a more ordered structure, due to enhanced protein–starch interactions, or helping the formation of disulphide crosslinks among neighbor polypeptides, resulting in a more ordered structure (Quintanar-Guzmán et al., 2009). These changes reduce the digestibility of the protein, making it less available for proteolytic enzymes.

At first sight, the Raman spectra of the retrograded starch samples (RSFTS, RSSTS) (Fig. 2A and B) displayed significant changes for the bands assigned to the general starch structure. Because of the very low sensitivity of the OH groups to Raman excitation, their bands are practically absent in the Raman spectra. Hence, only the bands of C–H, C–C and C–O bonds of the skeletal structure of the starch molecules are observed. This suggests that the general molecular structure of starch was affected during nixtamalization process and storage of tortillas. New bands appear in the Raman spectra of the retrograded starch of maize and tortillas (Fig. 2A and B) at 1600, 1597 cm^{-1}. These bands are likely due to the nominal C=O stretch and O–H stretch vibrational modes (Chan et al., 2001), perhaps due to the presence of lipid chains associated with

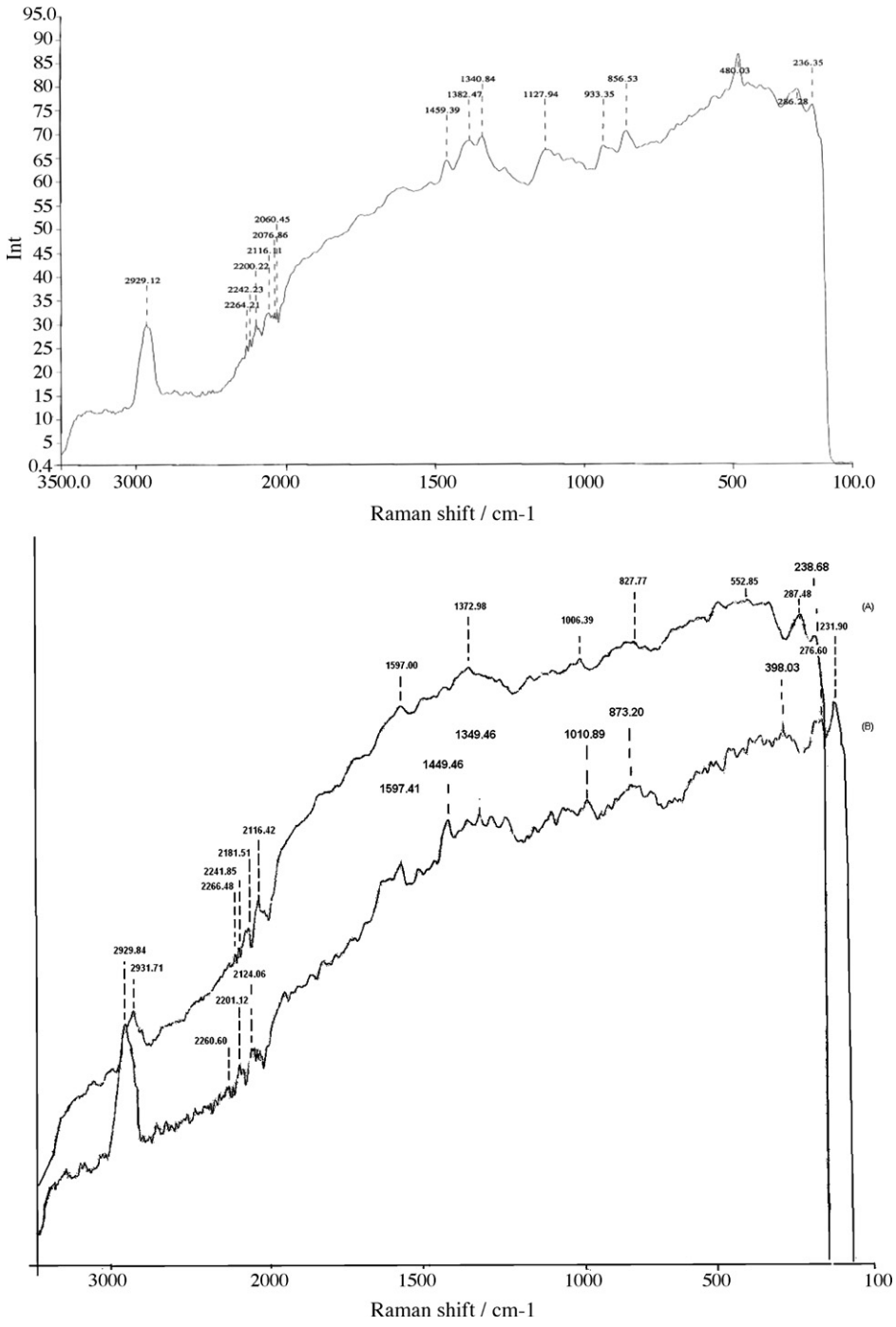

Fig. 2. Raman spectrum of starch of fresh tortilla (SFT). (A) Raman spectra of RS3 of fresh tortilla starch (RSFTS) and (B) Raman spectra of RS3 of stored tortilla starch (RSSTS).

resistant starch, since Thygesen et al. (2003) reported that the bands between 1600 and 1700 cm^{-1} (C=O stretch) indicate the presence of a lipid group. Valor-Reed (2002) found that nixtamalization process favors the neutralization of free fatty acids, giving calcium salts, being fatty acids responsible for the binding of calcium. Furthermore, we can infer the existence of saponified lipids, which are induced and stabilized by interactions (Zabar Shiran, Katz, Eyal, & Bianco-Peled, 2009).

It was found that the bands at 1450 cm^{-1} indicate the presence of protein, which was associated to the ratio between 3100 cm^{-1} and 3300 cm^{-1} (Enrico-Pigorsch, 2009). On the other hand, the band at 1010 cm^{-1} (coupled C–C and C–O vibrations) shows the interactions between starch polymers and protein (Hing-Wan, Siu-Mei, Phillips, & Ching-Yung, 2009; Tester, Rabiah, Bernd, & Harald, 2007) (Fig. 2B). Taking into account these results, it can be said that in addition to the resistant starch interaction with lipid molecules, there is also an association with proteins. It is important to emphasize that Raman spectroscopy could provide a better resolution the presence of resistant starch-associated proteins.

Fig. 2B shows Raman spectra of RSSTS, in which the changes on the intensity of the band 2900 cm^{-1} can be observed. These may be attributed to the variation of the ratio amylose/amylopectin, as well as to the presence of the amylose/lipid complex. The formation of such complex may begin with the opening of the glycosidic bonds of the pyranose ring, or with the formation of glycosidic intermolecular bonds able to interact with non-carbohydrate molecules (Kacurakova & Mathlouthi, 1996).

The band at 480 cm^{-1}, which is associated to the vibrations of the glucose structure in the pyranose ring, tends to disappear along the retrogradation process as it can see in Fig. 2A and B. Fechner, Siegfried, Kleinebudde, and Reinhard (2005) mentions that the position of this band changes in intensity as a function of the amount of amylose present in the starch.

Others bands that decrease in intensity with the retrogradation phenomena were 1459, 1127 and 856 cm^{-1}. The 1127 cm^{-1} band was attributed to the contribution of two main vibrational modes C–O stretching and C–O–H deformation. The band at 1459 cm^{-1} was attributed to the CH_2 deformations, while the bands between 1100 and 800 cm^{-1} were attributed to C–C skeletal modes of α-glucose of α-1,4 glycosidic linkage starches. These bands are associated to crystallinity of starches (Van Soest et al., 1994), since they could be linked to structural changes of the amylopectin due to its smaller helical chain lengths, which accelerate the crystallization of this polymer.

Raman spectra should contribute also to a better understanding of the molecular mechanisms involved in the retrograded process for starches. This analysis showed that bands in the 480-cm^{-1} skeletal-mode region and the 2900-cm^{-1} C–H stretching-mode region are completely sensitive to the retrogradation process. These results help to a better understanding of the molecular mechanisms involved in the retrograded process of starch by the nixtamalization process, elaboration, and storage of tortillas.

3.3. Solid state ^{13}C CP-MAS/NMR

The signals that appear in the ^{13}C CP-MAS/NMR spectra for SM, SFT and STS were similar; therefore only one spectrum (Fig. 3) for SFT is showed. Three dominant signals into 103 ppm are observed, which correspond to the anomeric carbon C-1 for α-glucose. A carbon chemical shift for starch has been identified in 106–96 ppm for C-1, in 70–73 ppm for C-2, C-3 and C-5, in 79–83 ppm for C-4, and 59–62 ppm for C-6 (Baik, Dickinson, & Chinachoti, 2003; Primo-Martin, Nieuwenhuijzen, Hamer, & Van Vliet, 2007; Smits et al., 1998; Tavares, Bathista, Silva, Filho, & Nogueira, 2003; Zhang et al., 2002). However, Primo-Martin et al. (2007) reported that the multiplicity of the C-1 position of the glucose units gives information on the crystallinity of starch and the double helix symmetry. An A-type crystal presents three peaks at 102, 101 and 100 ppm, whereas in a B-type conformation, the C-1 resonance exhibits two peaks at 101 and 100 ppm, this corresponds to the non-identical sugar residues of amylose and amylopectin. Baik et al. (2003) and Therien-Aubin, Florence, Wilms, Zhua, & Marchessault (2007) show that the broad peak of C-1 at 103 or 104 ppm corresponds to a typical single helix organized in a V-type crystalline phase or dispersed in an amorphous phase. Therefore, for the three samples of starches (SM, SFT and STS) it is observed that non-crystalline domains and V-type single helices exist, since the C-1 peak between 101 and 106 ppm is associated with single helices in amorphous zones. The broad peak at 72 and 76 ppm corresponds to carbons 2, 3 and 5 of the glucose unit in a poorly ordered system. The peak between 81 and 85 ppm corresponds to C-4. The peak at 82 ppm is characteristic only for amorphous starches and the peak at 62 ppm is of glucose C-6. The significant variation in chemical sifts indicated different helical forms in resonances for C-1 and C-4. Paris, Bizot, Emery, Buzare, and Buleon (2001) reported that the peak at 83 ppm is assigned to the partial fraction of C4 carbons exhibiting V type single helical conformations which are known to dominate in the amorphous state.

Fig. 3A and B shows the spectra of resistant starch ^{13}C CP-MAS/NMR, for samples RSFTS and RSSTS. The spectra show a multiplicity in the resonance peak for C-1 to 104 and 102 ppm, which is due to a simple helix organized in a crystalline phase, or

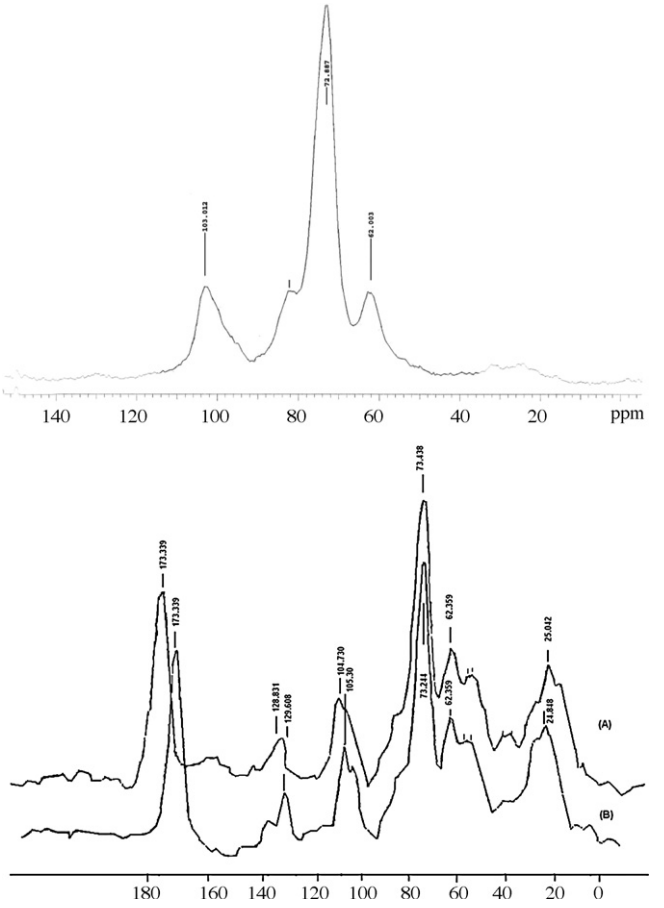

Fig. 3. CP/MAS ^{13}C NMR spectrum of starch of fresh tortilla (SFT). (A) CP/MAS ^{13}C NMR spectra of RS3 of fresh tortilla starch (RSFTS) and (B) CP/MAS ^{13}C NMR spectra of RS3 of stored tortilla starch (RSSTS).

dispersed in an amorphous phase (Paris, Bizot, Emery, Buzare, & Buleonb, 1999; Paris et al., 2001; Primo-Martin et al., 2007; Tavares et al., 2003).

The spectrum of retrograded starch (RSFTS and RSSTS) presents two peaks in C-1 (100 and 105 ppm), the deformation of three peaks in C-6, between 47 and 62 ppm, and a decreased peak corresponding to C2, C3 and C5 at 73 ppm. The presence of multiplicity in the signal is observed in the range of 20–42 and 173 ppm. The signal at 173 ppm corresponds to carbon atoms of a carboxyl group of acetyl groups present in the structure (Nuopponen et al., 2006; Zhang et al., 2002). High resolution solid state ^{13}C CP-MAS/NMR gives characteristic spectra for ordered helices and non-ordered chains, which can be used to estimate the double helix content into solid starch samples. The same technique can also be used to assess whether any lipid component is present within an amylose single helix (Gidley et al., 1995). However, Morrison, Tester, Gidley, and Karkalas (1993) show evidence for some B-crystalline polymorph also obtained from the ^{13}C CP-MAS/NMR spectra, which was consistent with a mixture of double helices and V-type glycosidic conformations, with only a small proportion of non-ordered regions. The presence of these groups has been observed in the IR spectrum, and is related to the formation of the calcium salts obtained from the reaction of lipids and calcium hydroxide used in the nixtamalization process.

Another sign of importance that appears in the ^{13}C CP-MAS/NMR spectrum for RSSTS is observed between 20–35 ppm and 129 ppm, which corresponds to the presence of carbon associated to proteins and amino acid groups of the types aliphatic and aromatic. A rapid removal of protein is shown by the decrease of the peak

at 37 and 45 ppm in the ^{13}C CP-MAS/NMR spectra. Presumably protein and lipid must be associated; since protein extraction facilitates lipid extraction (Tester et al., 2007). Even though Baldwin (2001) reported the presence of lipid–amylose and lipid–protein complexes associated with lipids on granule surfaces, these may be also present in the retrograded starch.

The results obtained using the techniques described above for the analysis of changes in maize starch during processing and storage of tortillas show structural changes (intermolecular between amylose–amilopectin, amylose–lipid or amylose-protein complexes) when comparing the maize starch and resistant starch of stored tortillas.

These techniques showed changes in the crystallinity of starch, which may be due to the intermolecular rotational mobility of the C–H and CH_2 groups, as well as the pyranose groups from starch amylose and amylopectin. On the other hand, it is possible that the intermolecular interaction with lipids and protein prevails in the retrograded starch, and that these interactions are closely related to the nixtamalization and tortilla production processes.

4. Conclusions

The techniques of IR, Raman and ^{13}C CP-MAS/NMR spectroscopy showed structural changes associated to the retrogradation process of starch due to tortillas storage. The spectrum of IR to RS3 showed changes observed in the bands of pyranose, OH and CH_2 groups. The appearance of the band at 1047 cm^{-1} and the disappearance of the band at 1022 cm^{-1} are fully due to retrogradation, which is associated with the recrystallization of starch. Other bands of importance observed in retrograded starch were the presence of amino and carboxyl groups. Through this technique could be observed differences between resistant starches with and without storage. Both Raman spectroscopy and ^{13}C CP-MAS/NMR support these findings. The peaks observed in the spectra for resistant starch obtained from stored tortilla for 10 days were more pronounced, which indicate that storage has a strong contribution to the formation of RS3.

Acknowledgments

A.F.M. thanks to Instituto Tecnológico del Altiplano de Tlaxcala, M.J.E. to Instituto de Química, UNAM, R.M.E. to E.N.C.B., I.P.N. and COFAA, I.P.N.

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