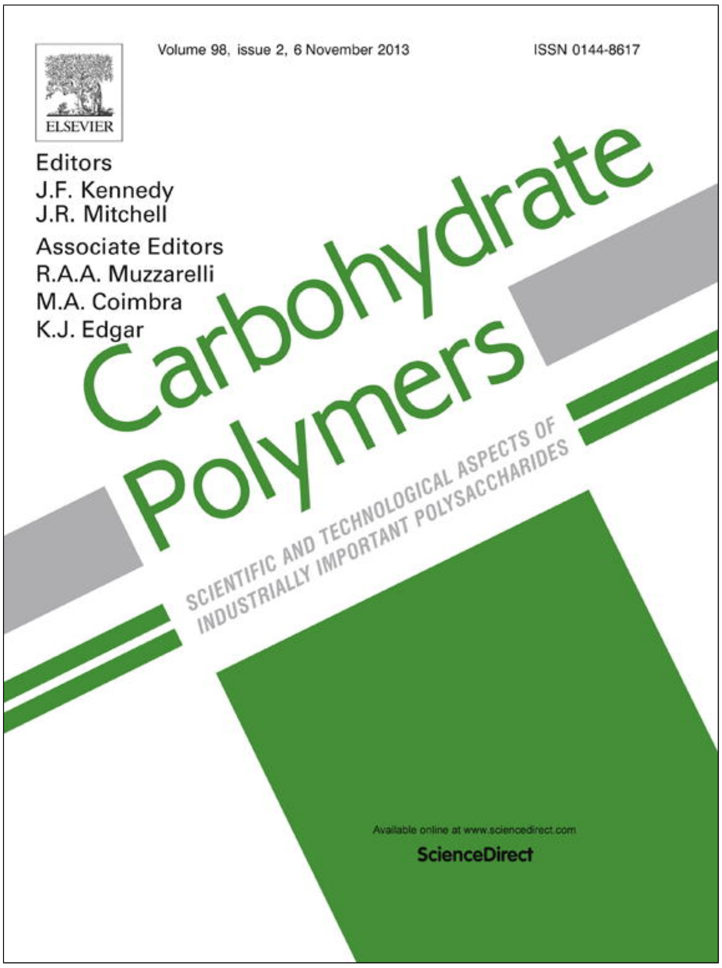
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Evaluation of the mechanical damage on wheat starch granules by SEM, ESEM, AFM and texture image analysis

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ABSTRACT

The effect of mechanical damage on wheat starch granules surface, at a microstructural level, was investigated by scanning electron microscopy (SEM), environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM), and image textural analysis. The SEM and ESEM images of the native sample showed that the starch granules had smooth, flat surfaces and smooth edges. The samples with higher damaged starch content exhibited granular distortion, irregularity and less uniformity. The fractal dimension of contour parameter increased with mechanical damage, indicating that the surface irregularities quantitatively increased due to the damage. The surfaces of damaged granules showed depressions of different shapes and sizes. The roughness parameters and fractal dimension of the surface increased as a result of the mechanical damage. The surface of damaged granules showed higher entropy and lower homogeneity values when damaged starch increased. The results indicated that the mechanical process caused structural modifications at nano level.

1. Introduction

Starch granules consist of two polysaccharides, amylose and amylopectin, and due to the structural arrangement of both macromolecules, the starch native granule is a semi-crystalline material. The short branches of the amylopectin determine the level of crystallinity of the granules. The starch polysaccharides are organized into concentric rings, where thin crystalline lamellae are alternated with less crystalline lamellae. The radial arrangement of amylopectin is the basis of this granular organization (Pérez et al., 2009).

The granular integrity of starch can be affected by the mechanical action of the wheat milling process, thus producing what is called damaged starch. The level of the damage depends on wheat hardness and milling technique. The external surface of starch granules is the first barrier to processes such as hydration and enzyme attack. Starch damage changes the structure of the granule which, in turn, affects the rheological behavior and functional properties of the starch systems (Hoseney, 1994; Barrera, León, & Ribotta, 2012; Barrera et al., 2013). Therefore, the nature of the granule surface and its modifications may have significant effects on the properties of starch.

Electron microscopy has been the main tool used to study the structural characteristics of starch granules. The architecture of the starch granules, at the higher level of molecular order, has been investigated mainly by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). However, limited applications of atomic force microscopy (AFM) to starch research have been reported. Usually, microscopy techniques provide only qualitative information of the food microstructure, however atomic force microscopy can provide quantitative parameters of the surface roughness through height parameters (R), roughness average (R_a) and root mean square roughness (R_q) descriptors. Because of this, image processing techniques have been developed in order to quantitatively characterize biological surfaces. The microscopy images could be analyzed by means of image processing techniques; and the physical characteristics (size and morphology) and textural properties (roughness and heterogeneity) would thus be obtained (Perea-Flores, Mendoza-Madrigal, Chanona-Pérez, Alamilla-Beltrán, & Gutiérrez-López, 2012; Pérez et al., 2009, Arzate-Vázquez et al., 2012). From image analysis,

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descriptors such as fractal dimension, which provide a numerical parameter of the morphology and texture of objects with complex and irregular structures, and fractal parameters have been applied to explain changes in the structure of food materials during or as a consequence of its processing (Kerdpiboon & Devahastin, 2007; Pérez-Nieto, Chanona-Pérez, Farrera-Rebollo, Gutiérrez-López, & Calderón-Domínguez, 2010; Quevedo, Jaramillo, Díasz, Pedreschi, & Aguilera, 2009). Thus, when microscopy techniques and images analysis are used together, they become an even more powerful tool to evaluate the microstructure and the effects induced by mechanical stress in biomaterials such as starch granules.

Despite several studies have used texture analysis in the food area (Arzate-Vázquez et al., 2012; Basset, Buquet, Abouelkaram, Delachartre, & Culioli, 2000; Fernández, Castillero, & Aguilera, 2005; Jackman & Sun, 2013; Mendoza, Dejmek, & Aguilera, 2007; Pérez-Nieto et al., 2010; Quevedo, Mendoza, Aguilera, Chanona, & Gutierrez, 2008; Quevedo et al., 2009), limited studies of texture image analysis in food materials at microscopic levels have been performed, particularly those related to wheat starch and the effects caused by physical damage. Hence, the aim of this work was to evaluate the effects of the mechanical damage on the surface microstructure of wheat starch granules by means of scanning electron microscopy, environmental scanning electron microscopy, atomic force microscopy, fractal dimensions and texture image analysis.

2. Materials and methods

2.1. Samples

Unmodified (native) wheat starch (Sigma–Aldrich) with moisture and protein contents of 10.7 and 0.2 g/100 g solids, respectively, was used for all assays. Native wheat starch was milled during different times in a Whisper Series Bench Top mill disk (Rocklabs, Auckland, New Zealand) in order to cause a greater rupture of the starch granules. Milling times were chosen to obtain different contents of damaged starch. Temperature was monitored during the milling and kept under 40 °C. Four samples, with different damaged starch content, were obtained: 3.8 (not re-milled in the mill disk), 13.3, 34.1 and 72.9% damaged starch (DS).

2.2. Sample analysis

Damaged starch content was determined according to the AACC 76-30A method (American Association of Cereal Chemists (AACC) AACC, 2000). A fungal enzyme from *Aspergillus oryzae* (A6211, Sigma Chemical Co., St. Louis, MO, USA) was used in this analysis. All experiments were performed by triplicate.

2.3. X-ray diffraction (XRD)

X-ray diffraction data were obtained using a diffractometer (X Pert PRO, PANalytical, Holland). The X-ray patterns were taken with Cu kα radiation ($\lambda = 0.154$ nm) and the X-ray tube (Philips PW3830) was operated at 40 mA and 45 kV. The scanning region of the diffraction angle (2θ) was 2–40° with a scanning speed of 0.03°/s. Three peaks with spacing *d* of 5.8, 5.2 and 3.8 Å were analyzed by A-Type crystalline phase identification. The X-ray patterns were adjusted by deconvolution of the peaks and background area. The crystalline and amorphous areas were quantified using PeakFit v4 Software (Peakfit Jandel Scientific, San Rafael, USA). Crystalline peaks were analyzed as pseudo-Voigt form and the amorphous as Gaussian form peaks. Relative crystallinity was calculated as the ratio between the integrate intensity of the crystalline phase and the integrate intensity of the amorphous phase (Ribotta, Cuffini, León, & Añón, 2004).

2.4. Scanning electron microscopy studies

For qualitative observations of microstructural damage on the starch granules surface scanning electron microscopy (SEM) was used. Dry granules were sprinkled onto double-sided tape attached to the specimen stubs and coated with a thin layer of gold (300 Å thickness) using a Pelco 91000 sputter coating system. Samples were then observed using a Supra 55 VP (Carl Zeiss Co., Germany) scanning electron microscope at an acceleration potential of 1 kV. The observations were made using a secondary electron detector "SE" and an "In Lens" detector, characteristic of this microscope. The photographs were taken using an automatic image capture software.

Environmental scanning electron microscopy (ESEM, XL 30, Philips, USA) was used for the quantitative analysis as well as for the observation of the microstructure of starch granules without the dehydration effects and gold coating. This operation mode allows the examination of wet biological specimens without sample preparation. Dry granules were sprinkled onto double-sided tape attached to the specimen stubs. The samples were observed at 25 kV using the gaseous secondary electron detector. The micrographs were captured in 32 bits (RGB) and crops of 712 × 484 pixels were stored in TIFF format. Observations of the samples at magnifications of 400×, 1500×, 2000×, 4000×, 10,000× were obtained for image analysis. Brightness and contrast are the most important variables that must be controlled during the acquisition of images; therefore, the values of these parameters were kept constant for each magnification during the process of image acquisition. Other variables such as acceleration voltage and type of electron detector were also considered for the generation of images when applying this technique.

2.5. Atomic force microscopy (AFM)

The topography of surface granules at micro and nanoscale was analyzed with an AFM, and applying the tapping mode (diMultimode V connected to a diNanoscope V microcontroller, Veeco, USA). It is important to mention that the applied force during the analysis must be kept at a constant value when working with tapping scanning mode. This is performed by controlling the drive amplitude (cantilever oscillation amplitude). At the same time, it is necessary to tune some operational parameters (drive amplitude, amplitude set point, integral gain and proportional gain) so as to adjust the scanned profile and obtain the best quality image.

In order to limit the effect of the probe broad, and based on preliminary studies, silicon probes (MPP-11100-10 model, Brucker, USA) with a resonance frequency of 300–400 kHz (spring constant of 20–80 nm) and a scanning rate of 1 Hz were selected. Dry granules were sprinkled onto double-sided tape attached to the specimen stubs. Seven to nine different granules of each treatment were analyzed (Freeware NanoScope v 7.30, Veeco, USA), obtaining different kinds of images: height (2D, 3D topographic) and amplitude error images. Height topographic images (2D to 3D) give information about lateral (*xy*) and height (*z*) measurements, but the images obtained do not really reflect the shape of the analyzed object. On the other hand, amplitude error images are equivalent to a map of the slope of the sample, displaying its shape more precisely.

Scan sizes between 15 μm × 15 μm and 0.25 μm × 0.25 μm were obtained. The fields of view (FOVs) were selected after a preliminary study which showed that at these FOVs the details of images could be observed more clearly without image noise. To elucidate details of the effects of mechanical damage, smaller areas

($1.0\,\mu m \times 1.0\,\mu m$, $500\,nm \times 500\,nm$ and $250\,nm \times 250\,nm$) were also scanned. However, average values of R_a and R_q (roughness parameters) and section analysis were obtained from topographic images scanned at $2.5\,\mu m \times 2.5\,\mu m$, since this scanning area was the largest one that results in data collection without background interference. R_a and R_q values describe the arithmetic average of absolute values of the surface height deviations as measured from the mean plane and the root mean square average of height deviations taken from the mean image data plane, respectively. It is worth mentioning that R_a and R_q values depend on probe broadening, as well as on the scan size and speed, hence it is very important to report the equipment operational conditions at which the test was carried out. All images were collected in RGB color sizing 512×512 pixels in TIFF format. All experiments were performed in ambient air conditions. The height scales of the compared images were the same, in order to compare the numerical information.

2.6. Texture images analysis of microscopy images

The image texture is a characteristic that represents the spatial arrangement of the gray levels of pixels of the image (Du & Sun, 2004; Jackman & Sun, 2013). In order to quantify the microstructure differences between the starch samples, ESEM and AFM images were processed and analyzed using the program freeware ImageJ v 1.45s (National Institutes Health, Bethesda, MD, USA).

Environmental scanning electron microscopy (ESEM) images were processed using image analysis methodology which involves image segmentation, cropping, conversion of the RGB images (32 bits) to gray-scale (8 bits) values, and application of a threshold at gray level 60 to obtain the binary image. The fractal dimension of contour (FDc) of starch granules was estimated by means of a box-counting method with FracLac v2.5e plugin of ImageJ and applying a standard box counting scan tool. To measure the FDc, the outline function of ImageJ was used on each isolated starch granule. This operation eliminates all black pixels except those that form the margin of the granules. The FDc values were used to describe the complexity or roughness of the starch granule contour caused by the milling process. Thus, larger mean FDc values indicate a more convoluted or jagged granule surface (Kenkel & Walker, 1996; Papagianni, 2006).

Texture image analysis was applied to AFM images in order to quantitatively characterize the surface microstructure of starch granules at the different FOVs obtained. The image texture is a characteristic that represents the spatial arrangement of the gray levels of pixels of the image (Du & Sun, 2004; Jackman & Sun, 2013). Atomic force microscopy (AFM) images were converted from RBG color to grayscale images; subsequently, Gray Level Co-Ocurrence Matrix (GLCM) and Shifting Differential Box Counting (SDBC) algorithms were applied to obtain the homogeneity (H), entropy (E) and fractal dimension of the surface (FDs) as textural features, respectively (Arzate-Vázquez et al., 2012; Pérez-Nieto et al., 2010). Homogeneity (H) and entropy (E) parameters were extracted from grayscale images with the *GLCM* plug-in included in the ImageJ software. Gray Level Co-Ocurrence Matrix is a second-order statistic algorithm that compares two neighboring pixels at the same time and compiles the frequency at which different gray-levels can be found within a restricted area. Original GLCM algorithm allows the evaluation of up to fourteen textural parameters (Gosselin, Duchesne, & Rodrigue, 2008; Haralick, Shanmugam, & Dinstein, 1973); however, some of them are redundant. Usually, some parameters are used to better describe the complexity and homogeneity of the images and have a correlation with the visual perception of the textures. Therefore, several reports conclude that only some parameters are relevant for the description of the texture of images (Arzate-Vázquez et al., 2012; Mendoza & Aguilera, 2004; Mendoza et al., 2007; Mery et al., 2010). The fractal dimension of the surface (FDs) was estimated using a *SDBC* plug-in included in the ImageJ software. Fractal descriptors have been used as an efficient parameter to evaluate the irregular morphologies and the roughness of objects and surfaces (Arzate-Vázquez et al., 2011; Gumeta-Chávez et al., 2011; Pérez-Nieto et al., 2010; Quevedo, Carlos, Aguilera, & Cadoche, 2002; Quevedo et al., 2008, 2009). The homogeneity (*H*), entropy (*E*) and fractal dimension of the surface (FDs) values were extracted from $2.5\,\mu m \times 2.5\,\mu m$ 2D topographical images of the surface granules. Homogeneity (*H*), also called inverse difference moment, represents the local invariance of pixels in the field of view; high values can be associated with monotonous or homogenous images. Entropy (*E*) measures the disorder or randomness of images, and it can be used to characterize the image texture. It is an indication of the complexity present within a field of view. Thus, the more complex the images, the higher the entropy values (Haralick et al., 1973; Mendoza et al., 2007).

2.7. Statistical analysis

The data were statistically treated by variance analysis (ANOVA), the means were compared by the LSD Fisher test at a significance level of 0.05, and the relationship between measured parameters was assessed by Pearson's test (significant levels at $p \leq 0.05$) using the Infostat Statistical Software (Di Rienzo et al., 2009).

3. Results and discussion

3.1. X-ray diffraction

In order to obtain starch samples with different levels of damaged starch, these were milled at different periods of time in a disk mill. As expected, the damaged starch content increased with the milling time for each sample. The effects of the mechanical damage on the properties of starch samples, measured by X-ray diffraction, are shown in Fig. 1. The diffraction patterns presented by all starch samples correspond to the A-type diffraction pattern assigned to cereal starches. X-ray peak intensities of samples decreased with the level of damaged starch. Biliaderis (2009) showed that the overall loss in order depends on the severity of the milling conditions and milling time, as measured by X-ray diffraction, DSC or solid-state ^{13}C NMR. The total mass crystallinity grade (TC) of starch, determined from the separation and integration of the areas under the amorphous and crystalline peaks (Ribotta et al., 2004), decreased as the content of damaged starch increased. The total mass crystallinity grade (TC) of native starch was 39.3% and it was reduced to 35.4%, 31.2% and 12.7% for 13.3 DS, 34.1 DS and 72.9 DS samples, respectively. The results indicated that the damaged granule lost crystallinity by mechanical action. Similarly, Morrison, Tester, and Gidley (1994) reported that mechanical damage to starch granules caused progressive losses of order. They observed that the crystallinity determined by X-ray diffraction and the double-helix content of the amylopectin decreased with the increase of damaged starch. These authors propose that a new amorphous material is formed, which should be distinguished from the amorphous starch in native granules.

The loss in crystallinity was consistent with the decrease in the gelatinization enthalpy found in a previous study (Barrera et al., 2013).

3.2. Scanning electron microscopy studies

Scanning electron microscopy (SEM) micrographs of native and damaged starch granules are shown in Fig. 2. Large lenticular and small spherical granules, which correspond to A- and B-type granules, respectively, could be observed. In the native sample, the granule surfaces were smooth and flat, although some ridges, that

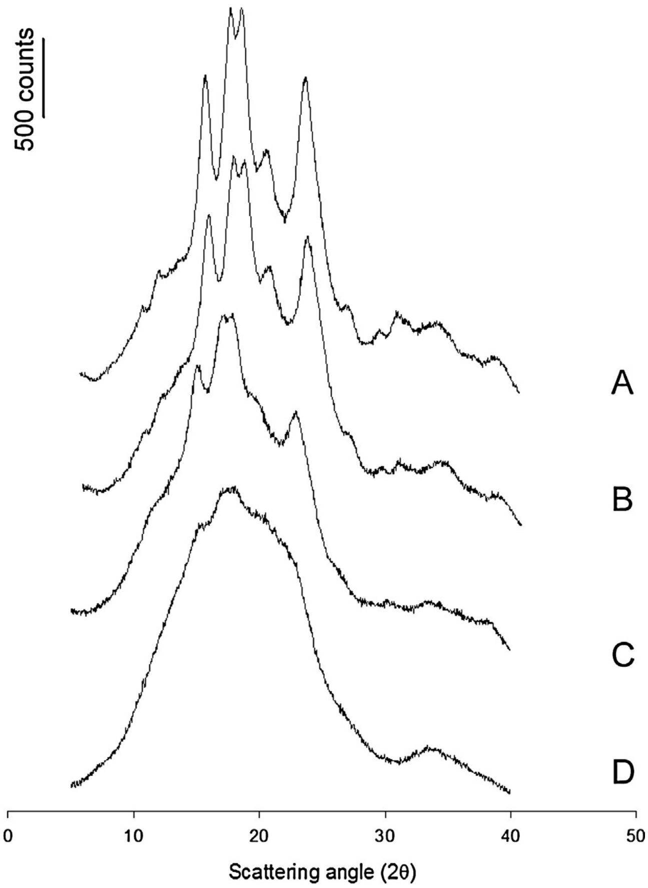

Fig. 1. Effects of starch mechanical damage on X-ray diffraction properties of starch samples. (A) Native starch, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS.

appear to mark sites where granules were in close contact with each other (Tester, Morrison, Gidley, Kirkland, & Karkalas, 1994), could be observed. The surface of starch granules changed from slick to rough, a flaky surface was observed. Tester et al. (1994) also observed rough granular surfaces, many clumps formed by distorted granules and very few undamaged granules after the ball milling process.

Environmental scanning electron (ESEM) micrographs of starch granules are shown in Fig. 3. As is the case with SEM images, the ESEM micrograph of the native sample (Fig. 3A) shows A- and B-type granules with smooth, flat granule surfaces and smooth edges. In this image (Fig. 3A), some ridges could be identified in the surface granules as observed in SEM photograph (Fig. 2). The qualitative examination of micrographs showed that mechanical damage produced distorted, less uniform and irregular granules, which can be attributed to the harmful effect of the milling process (Fig. 3B–D). The degree of distortion and irregularity of granules gradually increased with the mechanical damage. The effects of damage were similar for A- and B-type granules.

The Environmental scanning electron microscopy (ESEM) technique has advantages such as fast scanning speed, and it is possible to observe samples in their native state using the environmental mode. Nevertheless, one disadvantage that this type of microscopy presents is that the morphological analysis obtained is only qualitative in character, but through image analysis quantitative information could be extracted from ESEM images. Consequently, a quantitative characterization of the degree of jagged and irregular boundaries of the granule was developed. Fig. 4 shows surface boundaries of native and modified granules obtained from isolated granule ESEM images. The observation of the boundaries of starch granules with different degrees of damage showed clear differences among samples, being the most uneven samples those with the highest level of damaged starch. The fractal dimension of contour (FDc) parameter of starch granules significantly increased with the mechanical damage (Table 1), which can be linked to the changes observed in shape and surface irregularity of starch granules (Fig. 4). These higher values of FDc can be associated with irregular contours and complex surfaces, as put forward by Pérez-Nieto et al. (2010) and Kerdpiboon and Devahastin (2007). The contour and surface irregularities and deformation observed in the starch granules were produced as consequence of friction phenomena and surface erosion.

3.3. Atomic force microscopy (AFM)

Fig. 5 shows atomic force microscopy (AFM) topographic images, 2D and 3D plots and the sections analysis of the starch granules surface. Native granules (Fig. 5A) appeared very similar to the images of native granules obtained by different authors (Krok, Szymońska, Tomasik, & Szymoński, 2000; Szymońska, Krok, Komorowska-Czepirska, & Rebilas, 2003). The native granule surface looked smooth and polished; although

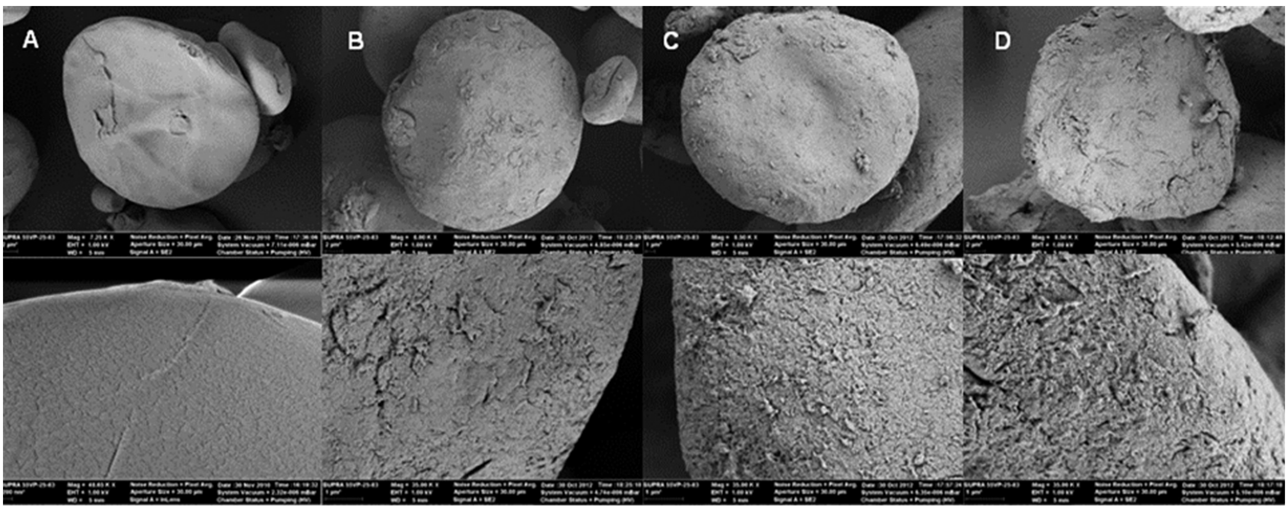

Fig. 2. Scanning electron micrographs of native and damaged starch granules. (A) Native starch, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS. The images magnification was approximately 8 kx for the whole granules and 40 kx for the micrograph near of the granules surface.

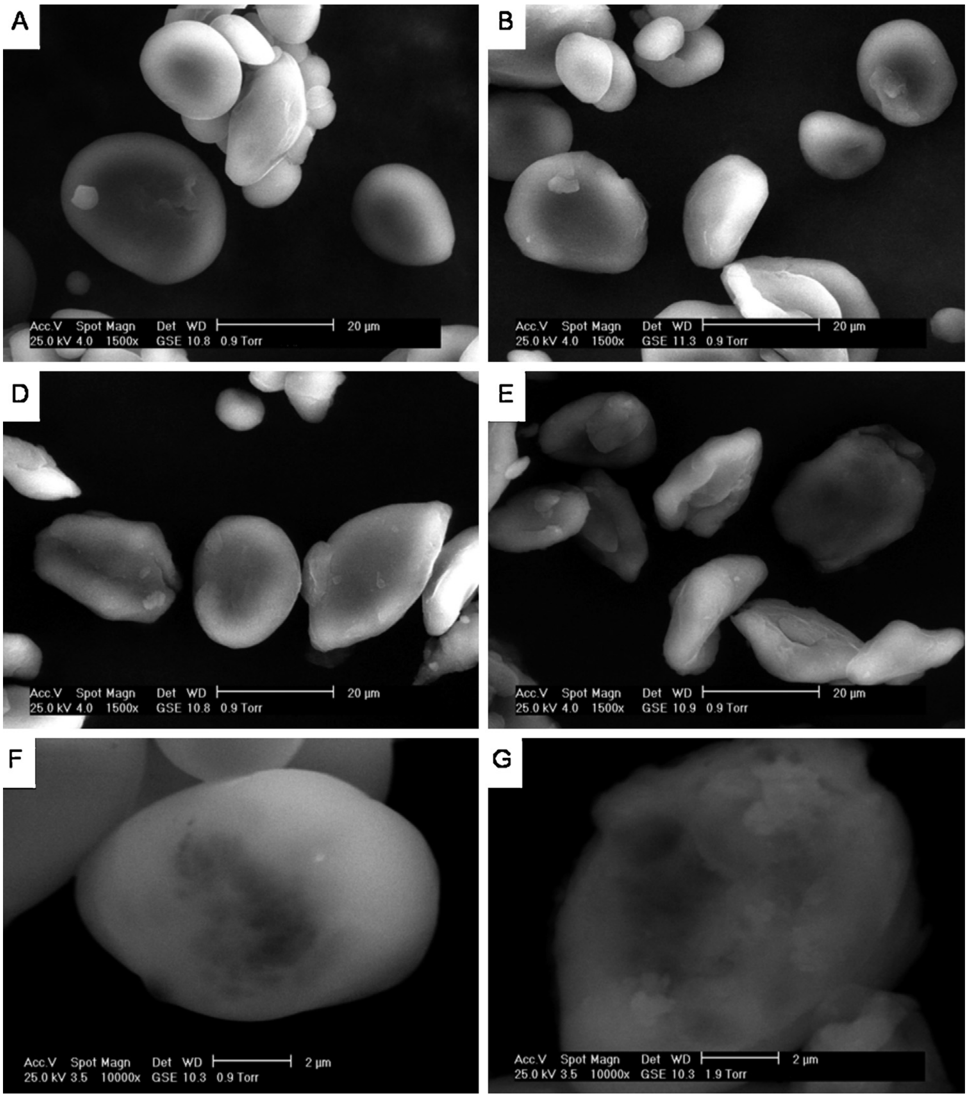

Fig. 3. Environmental scanning electron microscopy micrographs of native and damaged starch granules. (A) and (F) Native starch, (B) 13.3 DS, (D) 34.1 DS and (E) and (G) 72.9 DS.

some ridges, that appear to mark sites where granules were in close contact with each other (Tester et al., 1994), were observed.

As shown by the environmental scanning electron microscopy (ESEM) technique, the surfaces of damaged granules were distorted, less uniform and irregular, with many depressions of different shapes and sizes, and these changes gradually increased with the damaged starch level.

The height profile (section analysis) of starch granule surfaces confirmed the dimension of superficial irregularities, which gradually increased with the damaged starch level.

The AFM allowed obtaining the roughness average (R_a) and root mean square roughness (R_q) (which are closely related to each other) of the starch granule surfaces (Table 1).

Fig. 6 shows images that illustrate the surface roughness of native and damaged starch granules. Native starch surface showed

Table 1
Damages starch content, fractal dimensions and textural parameters of starch granules.

Parameters	Native 3.8 DS	13.3 DS	34.1 DS	72.9 DS
FDc	1.019 ± 0.005a	1.02 ± 0.006a	1.029 ± 0.012b	1.036 ± 0.006b
R_q	14.2 ± 2.7a	36.5 ± 2.8b	44.9 ± 6.8b	58.2 ± 8.0c
R_a	11.3 ± 2.3a	28.9 ± 1.6b	34.9 ± 4.7b	46.5 ± 7.5c
H	0.724 ± 0.044b	0.684 ± 0.063ab	0.586 ± 0.06a	0.592 ± 0.025a
E	5.94 ± 0.1a	6.19 ± 0.25ab	6.44 ± 0.28b	6.44 ± 0.06b
FDs	2.047 ± 0.038a	2.069 ± 0.027a	2.131 ± 0.017b	2.134 ± 0.02b

Different letters within columns mean significant differences at $p \leq 0.05$.
DS: damaged starch content (%).
Fractal dimension of contour (FDc) were obtained from ESEM topographic images by means texture image analysis.
Root mean square roughness (R_q) and average roughness (R_a) were obtained from AFM topographic images (2.5 µm × 2.5 µm).
Homogeneity (H), entropy (E) and Fractal dimension of the surface (FDs) were obtained from AFM topographic images (2.5 µm × 2.5 µm) by means texture analysis.

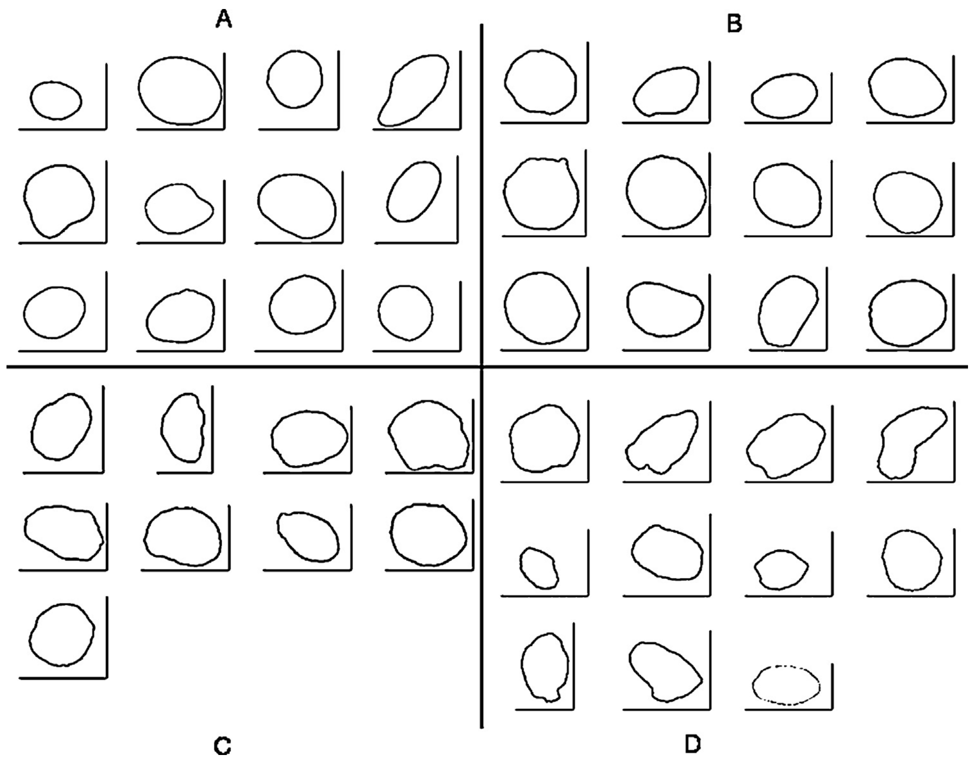

Fig. 4. Outline of contour of starch granular surface from scanning electron micrographs. (A) Native starch, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS.

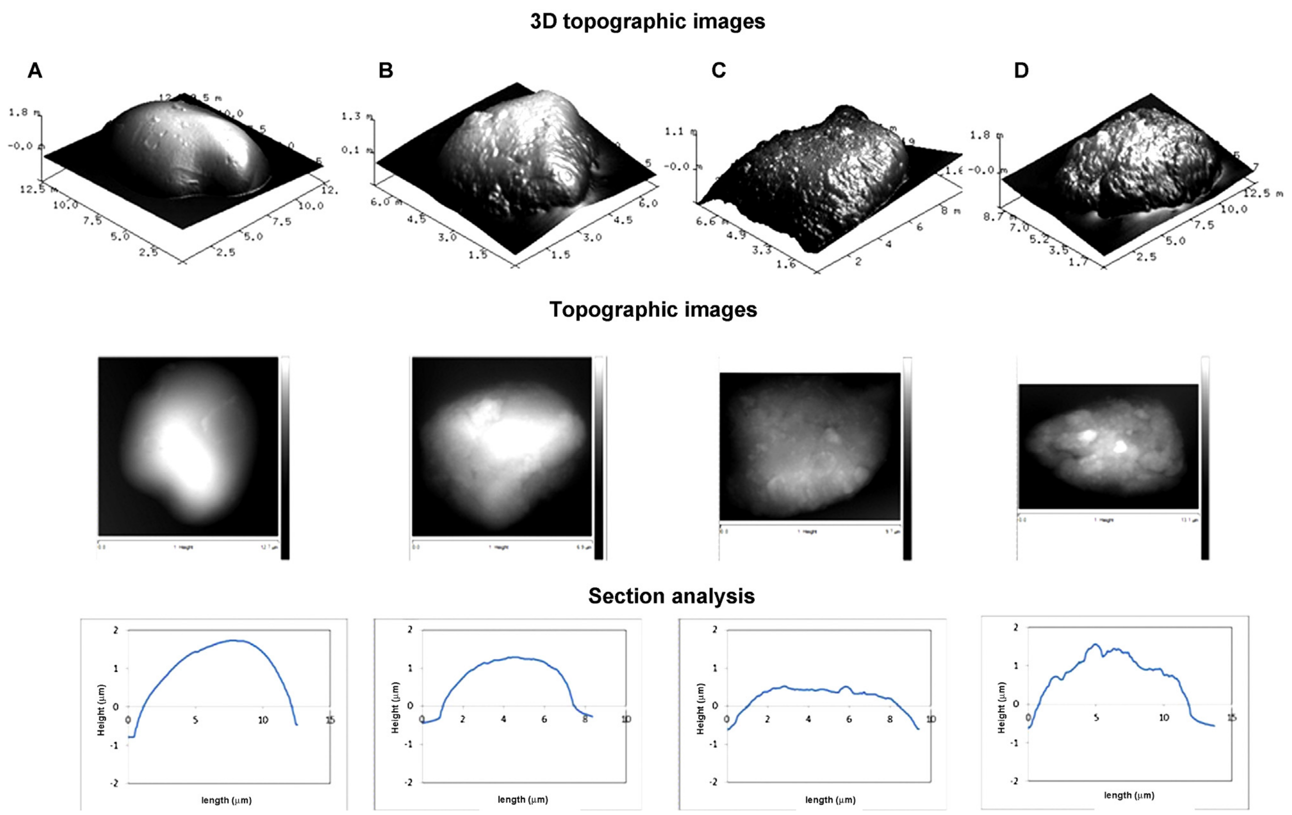

Fig. 5. 3D and 2D topographic atomic force microscopy (AFM) images and section analysis of starch granules. (A) Native, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS.

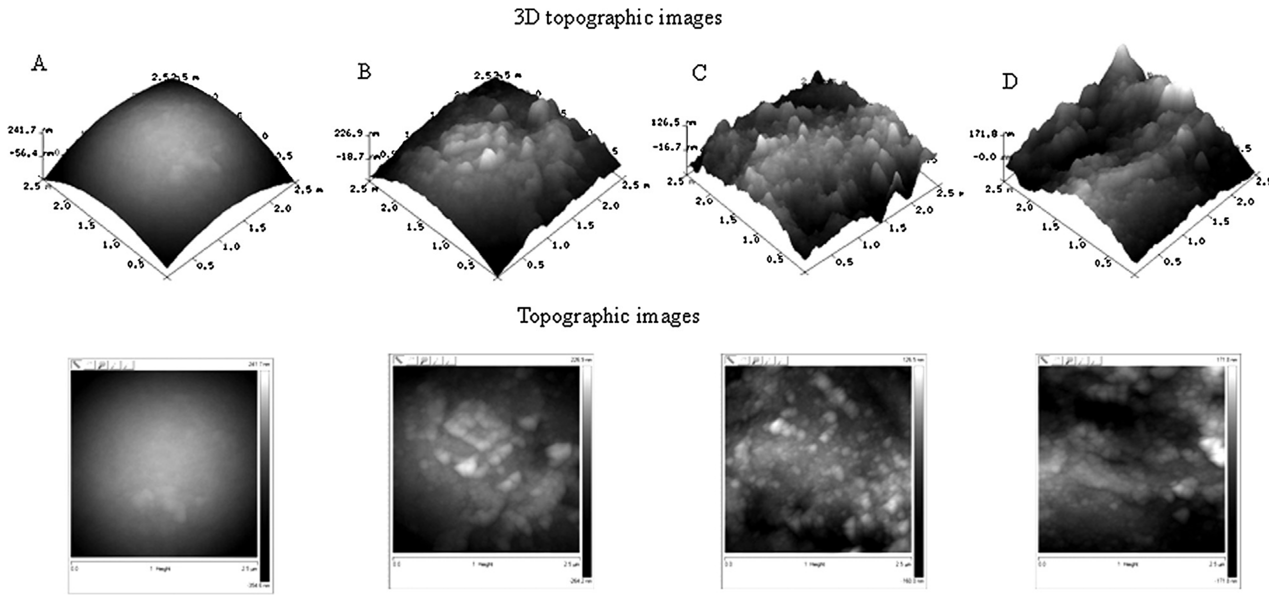

Fig. 6. Surface profile of native and damaged starch granules. 3D and 2D topographic atomic force microscopy (AFM) images (2.5 × 2.5 μm). (A) Native, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS.

R_a and R_q values of 14.2 ± 2.7 nm and 11.3 ± 2.3 nm, respectively, both values described smooth surfaces. R_a and R_q values increased to 58.2 ± 8.0 nm and 46.5 ± 7.5 nm with the highest damaged starch level. No roughness average (R_a) and root mean square roughness (R_q) values of cereal starch were found in the bibliography.

The homogeneity (H), entropy (E) and fractal dimension of the surface (FDs) values of the surface granules are summarized in Table 1. Homogeneity (H) can be associated to monotonous or homogenous images, while the entropy (E) value measures the disorder or randomness of images (Haralick et al., 1973; Mendoza et al., 2007). Damaged granules exhibited no significant differences of energy and contrast parameters ($p \leq 0.05$) compared to native granules (results not shown). Nonetheless, damaged granules showed higher entropy values than the native granules and the opposite effect was observed on surface homogeneity.

The fractal dimension of the surface (FDs) of starch granules (Table 1) ranged between 2.047 and 2.137 in the native sample and in the 72.9 DS sample, respectively. FDs of granules is a measure directly related to the degree of surface roughness, since an image with a smooth or uniform texture will present little or no variation in brightness (e.g., a smooth white image), while an image presenting a rough or heterogeneous texture will show a high variation in the gray-scale. For 2-D gray-scale images, values from 2 to 3 can be obtained; FDs values close to 2 indicate smoother surfaces; and values close to 3 indicate a high degree of roughness (Quevedo et al., 2009; Arzate-Vázquez et al., 2012). Damaged granules showed higher FDs values than native granules. Textural features values can be explained by the increase of the structural complexity of the granule surfaces as a consequence of the mechanical damage.

The damaged starch samples showed qualitative and quantitative rougher surfaces at all magnifications levels (results not shown), as compared to native starch samples which presented a smoother surface. The microstructure observed in the damaged starch samples could be explained by the erosion process by effect of the disk milling.

To elucidate details of the effects of mechanical damage, different areas (1.0 μm × 1.0 μm, 500 nm × 500 nm and 250 nm × 250 nm) were obtained by non-contact atomic force microscopy (AFM). Fig. 7 shows AFM images of the surface of starch granules at a higher magnification level (scan sizes 500 nm × 500 nm), said images were obtained simultaneously by the topographical and amplitude error image modes.

High-resolution topographical images of native wheat starch granule surface (Fig. 7A) had a textural appearance with semi-spheroidal objects. Numerous raised nodules typically 40–100 nm in size (evident as small bright areas) were observed.

The amplitude error image is a type of AFM image that emphasizes the edge of the samples. High-resolution amplitude error images showed an undulating, regular appearance and grain-like structures with numerous raised nodules. These images were typical of the native samples examined and they were comparable with those reported in the literature (Baldwin, Adler, Davies, & Melia, 1998; Ohtani, Yoshino, Hagiwara, & Maekawa, 2000).

According to Baldwin et al. (1998) wheat starch had protrusions made up of 10–50 nm structures and some larger structures of 50–300 nm in diameter. Later, Juszczak, Fortuna, and Krok (2003) examined the surface of starch granules of selected cereals by non-contact atomic force microscopy and reported elongated granules with a rough surface, and protruding surface structures of sizes below 200 nm.

These structures are of carbohydrate origin, and consist of end fragments of amylopectin clusters. The structure of starch, composed of spherical "blocklets" (raised nodules) ranging from 20 to 500 nm in diameter (depending on the starch botanical type and their location in the granule), was described by Gallant, Bouchet, & Baldwin (1997). In A-type crystalline starches (e.g. wheat starch) the "blocklets" ranged from 25 to 100 nm in diameter. The "blocklets" ranging from 50 to 500 nm in diameter would, therefore, contain between 5 and 50 amylopectin side-chain clusters (Pérez et al., 2009)

The appearance of the raised nodules changed significantly as a consequence of the mechanical damage. The AFM images of damaged granules had a textural appearance with semi-spheroidal but elongated objects, which became more evident with higher mechanical damage (Fig. 7D). The amplitude error images clearly showed that the surface of the damaged starch was composed of larger elongated "blocklets". The long axis of the elongated raised nodules ranged between 82–186 nm, 129–196 nm, and 113–212 nm to 13.3 DS, 34.1 DS and 72.9 DS samples, respectively.

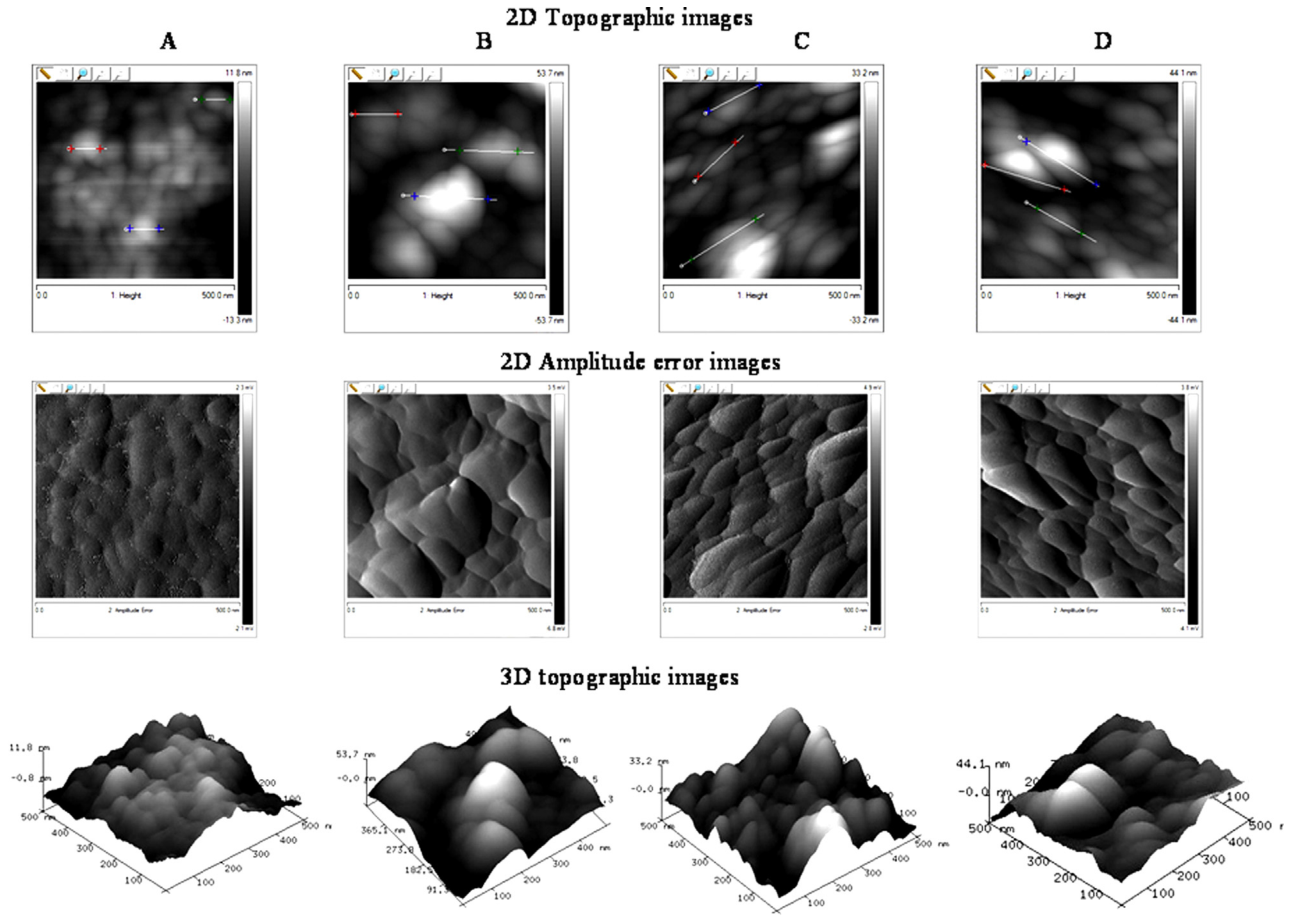

Fig. 7. 2D and 3D topographic and amplitude atomic force microscopy (AFM) images of starch granules (500 × 500 nm). (A) Native, (B) 13.3 DS, (C) 34.1 DS and (D) 72.9 DS.

Atomic force microscopy 3D images (Fig. 7) of damaged granules showed similar information; however, it is worth mentioning that the raised nodules of damaged granules showed higher height than native granules.

The analysis of atomic force microscopy images indicated that the mechanical process produced structural modifications at the "blocklet" level of structure. It seemed that the shearing on the granules produced the deformation of the objects which generated the presence of larger, elongated "blocklets" (and not spherical ones as observed in native granules).

As a result of many researches (Morris, Ridout, & Parker, 2005; Ridout, Gunning, Wilson, Parker, & Morris, 2002; Ridout, Parker, Hedley, Bogracheva, & Morris, 2003; Ridout, Parker, Hedley, Bogracheva, & Morris, 2004; Ridout, Parker, Hedley, Bogracheva, & Morris, 2006) structural differences were characterized by means of atomic force microscopy, allowing a totally new interpretation of the internal structure of the starch granule. The general morphology of the blocklets was imaged using shaded topography and error signal mode images, and the matrix was characterized by means of the force modulation image. From these works, it was possible to demonstrate that the internal granule structure is heterogeneous. These studies have shown that in some regions, the starch granule is composed of continuous hard blocklets dispersed in a softer matrix material. Besides, they concluded that in others starch granule regions, the matrix in which the blocklets are embedded contained a fine hard network structure, which is caused by the presence of a crystalline amylose network. Based on these findings, it is hypothesized that the friction on the granules produced the erosion of outer regions, being this effect more pronounced on softer matrix material than on hard blocklets. This might cause the differences in the height of blocklets of damaged samples compared to native starch.

4. Conclusions

The physical damage caused to the granular surface by the milling process resulted in starch granules with irregular, rough and less uniform surfaces, and in a reduced granular crystallinity.

Micro and nanostructural characterization of starch granules by means of microscopy techniques and images analysis provided relevant qualitative and quantitative information, which can be useful for the study of the microstructure of cereal products and also for its processing and utilization. The images and fractal analysis were successful to evaluate the changes on the morphology and surfaces of the starch granules due to mechanical damage induced in the material. Fractal and textural parameters can be helpful to evaluate the quality of cereal products and the efficiency of the milling process. The results obtained in this research integrate qualitative and numerical information that could be useful for process engineering and for understanding the properties–structure relationships of starch granules. The results indicated that the mechanical process produced structural modifications at nanometric scale that can be important for quality control of this kind of foodstuff.

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