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Scanning Electron Microscopy and Crystallinity of starches granules from cowpea, black and carioca beans in raw and cooked forms

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Abstract

Beans are a vital food for the population around the world because it contains a relatively high amount of protein, vitamins, fiber, and minerals, being an important vegetable source of iron, particularly when combined with ascorbic acid and cysteine. The starch content in the seed is between 45 and 60%. This paper evaluated the granular structure of starches from common beans (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) in raw and cooked forms by Scanning Electron Microscopy (SEM), and their crystallinity by X-ray diffraction (DR-X). By SEM, was observed the occurrence of gelatinization phenomenon in cowpea and carioca beans, and the resistance to this event in black beans. By DR-X, the diffraction angles found in this work are more consistent with the classification of the standard polyphorm A. The Relative Crystallinity (RC) of black bean cowpea and carioca beans, observed by X-ray diffraction, varied significantly.

Keyword: starch; bean; cowpea.

Practical Application: Physical chemical study of bean and cowpea starches for application in food industries, biocomponds encapsulation, bioplastic and others.

1 Introduction

Common beans (*Phaseolus vulgaris*) is one of the most consumed foods in many countries, containing a relatively high amount of proteins (Los et al., 2018; Rezende et al., 2018). Cowpea beans (*Vigna unguiculata*) is widely consumed in the North and Northeast of Brazil, as well as some African countries like Nigeria and Burkina Faso, featuring the typical cuisine of these regions (Freire-Filho et al., 2012). As in another species of leguminous plants, the starch content in dry basis in different cultivars of beans is between 45 and 60%, and therefore, the cooking quality of the beans is associated with postharvest handling and conditions for storage (Ferreira et al., 2017).

The legume starches are digested slowly and have a low glycemic index and are fermented in the large intestine to produce short-chain fatty acids (Kalpanadevi & Mohan, 2013). Besides the application in the food industry, the starches are industrially applied to the production of biodegradable plastics and nanofilms (Schmidt et al., 2013). As in the other species of starchy legumes, the starch content in the various bean species is between 45 and 60%. The granules shapes are ellipsoidal or spherical, with varying sizes, and contain high amounts of amylose (24 to 65%) (Shiga & Lajolo, 2005). The starch granule is a mixture of amylose and amylopectin polysaccharides, formed by dehydration synthesis.

When viewed microscopically under polarized light shows a typical model of the "Maltese Cross", resulting from birefringence in crystalline regions, which characterizes the radial orientation of the macromolecules. The center of the cross, the *hilum*, is considered the point of granule growth, and during the bean cooking, it swells irreversibly, losing the structural organization due to the gelatinization. Also, with a long storage time of grains, the gelatinized starches molecules lose the energy and the chains begin to reassociate in a more orderly state, this is called retrogradation phenomenon (Denardin & Silva. 2009). In legumes, the granules are ellipsoidal or spherical shapes and contain high amounts of amylose (24 to 65%). They are characterized as compounds, because many granules are in each amyloplast. By Scanning Electron Microscopy (SEM) is possible to observe the presence of some fissure and cracks. The surface of the granules is relatively impermeable to large molecules, such as amylases, due to the severe packaging of amylopectin chain. However, the presence of pores or channels allows the entry of hydrolyzers enzymes and other molecules into the interior. Cracks are related to the low integrity of starches, due to the weak interaction between the amylopectin chains radially arranged (Shiga & Lajolo, 2005; Martínez-Preciado et al., 2012). The shape and size of granule influence on their functional properties, such as paste viscosity. A high viscosity is desirable for industrial uses, in which the function is the thickener

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(Ratnayake & Jackson, 2008). The diffraction X-ray is used to reveal the presence and characteristic of the crystalline structure of starch. Models of diffraction X-rays are demonstrating that the native starch granules contain between 15 and 45% crystalline material, which correspond to two polyphorm (A or B) and an intermediate form (C), the classification is based on the content of water and in the double helices of amylopectin packaging configuration (Singh et al., 2003). These patterns of crystallinity depend in part on the length of the amylopectin chains, the packing density within the granules, as well as the presence of water. Starches with type A crystallinity exhibit intensity peaks at 2θ diffraction angles at approximately 15.3°, 17.1°, 18.2° and 23.5°, Type B at about 5.6°, 14.4°, 17.2°, 22.2° and 24°, Type C at about 5.6°, 15.3°, 17.3° and 23.5°. There is also a fourth type of crystallinity, type V, formed by the crystallization of amylose with lipids, which shows peaks of intensity at the 2θ diffraction angles at approximately 12.6°, 13.2°, 19.4° and 20.6° (Lima et al., 2012). Type A crystallinity is described as a highly condensed and crystalline monocyclic cell unit, wherein 12 glucose residues from two-chain in the counter clock wise harbor four molecules of water between the helices. The A-type structure has a chain of amylopectin with lengths of 23 to 29 glucose units. The hydrogen bonding between the hydroxyl groups of the amylopectin molecule is responsible for the formation of the outer helical structure, among which linear chains of amylose moieties are packed through hydrogen bonds with outer chains of amylopectin. This polyphorm occurs in most cereals such as corn, rice, wheat, and oats. The type B pattern structure is more clearly defined, being composed of a basic unit of chains that are packaged in a hexagonal array, where the cellular unit has two double helices in the counter clock wise direction, aligned and arranged in parallel. Type B crystallinity has amylopectin chain lengths of 30 to 44 glucose molecules, containing 36 molecules of water for each 12 glucose residues, being half of that water tightly bound to the double helices, and the other half being concentrated in a screw shaft. In addition to being considered richer in amylose, these types of starch have similar shapes and sizes and are resistant to hydrolysis, both enzymatic and acidic. The C-type structure is an amylopectin intermediate structure, with amylopectin chain lengths between 26 to 29 glucose units, being common in some roots and legumes (Singh et al., 2017; Santiago-Ramos et al., 2018). Starches type A crystallinity are more susceptible to hydrolysis due to the presence of pores on the surface, permeable to certain enzymes, while type B have protective shells, called crystalline blocks. However, type B crystals have a lower melting temperature (77 °C) when compared to crystals type A, 90 °C. (He & Wei, 2017). This work aimed to evaluate, by SEM, the starch granules present in cowpea beans and common beans in the black and carioca varieties, in raw and cooked forms. Comparing the relative crystallinity of the three varieties of beans through X-ray diffraction.

2 Materials and methods

2.1 SEM

The samples were raw or cooked in a pressure cooker at 250 °C for 25 minutes. Before this procedure, the cooked beans were dehydrated at 40 °C for 18 hours and then stored in a freezer at -10 °C. After that, it was posted on sheet metal and coated with 30-35 nm of gold at 6.10-2 atm in Balzers sputter Gold Sputter FL9496 Balzers (Liechtenstein). The observations and documentation of the material were carried out at the National Center of Bioimaging of Federal University of Rio de Janeiro (CENABIO – UFRJ) using microscope Zeiss brand EVO MA10 (Germany) tungsten filament, 10 mm working distance, voltage -15 kV. The images were achieve with Secondary Electron Detector. Starch Isolation The starch extraction was based on the method described by Wang & Wang (2004) with some modifications. The samples were ground in a laboratory mill (Perten, 3100) soaked in 0.1% NaOH solution at a ratio of 1:5 and to standby for 20 hours. After, the dispersion was subjected to vigorous stirring in a blender for two minutes. The resulting material was passed through a 63 µm bolter and centrifuged (Sorvall® RC 6 Plus centrifuge, Thermo Fisher Scientific, Canadian) at 1200 rpm for five minutes at room temperature (25 ± 2 °C). The supernatant was discarded, and the pellet was resuspended in 0.1% NaOH solution then centrifuged again, the operation being performed twice. The extracted starch was dispersed in distilled water and neutralized with HCl 1 $mol.L^{-1}$ to pH 6.5 and centrifuged. The pelleted material was resuspended in distilled water and centrifuged, the operation being performed twice. The resulting starch was dried in an oven with air circulation at 40 °C to 11 ± 0.5% moisture. The starch extraction yield was calculated based on the difference between the dry pasta flour before and after the isolation of starch.

2.2 X-ray diffraction

Diffraction of X-rays crystallinity tests were performed in the multiuser laboratory of the Chemistry Institute of the Federal University of Uberlândia, using a diffractometer X-ray (XRD-6000, Shimadzu, Brazil), where the scan region of diffraction ranged from 5-30°, with a target voltage of 30 kV, current of 30 mA and the scan speed 1 min^{-1}. The relative crystallinity (RC) of the starch granules was calculated by XRD-6000 software. V. 5.2. The CR values of all bean samples were evaluated by Graphpad PRISM® software, using analysis of variance (ANOVA).

3 Results and discussion

3.1 SEM

In Figure 1 is possible to visualize the image obtained by SEM of cowpea endosperm. In A, the raw sample with kidney-shaped starches (magnification 5.52 KX), in B, it is observed irregularities in the starch surface and the presence of cracks (magnification 30.36 KX), in C, the cooked sample, it is observed that the starch granules are grouped, due to gelatinization phenomenon (magnification 400X), confirmed in D (magnification 19,14 KX).

Salgado et al. (2005a, b) described that in the conditions which are conducted their experiments, the morphology of the starch granules were not influenced by the grain maturation stage. It has kidney-shaped, and the size varied between 11.8 µm and 26.7 µm with smooth surface, while the pattern of crystallinity was higher in mature than in the green beans. According to the authors, the phenomenon of gelatinization followed by retrogradation can be considered beneficial from

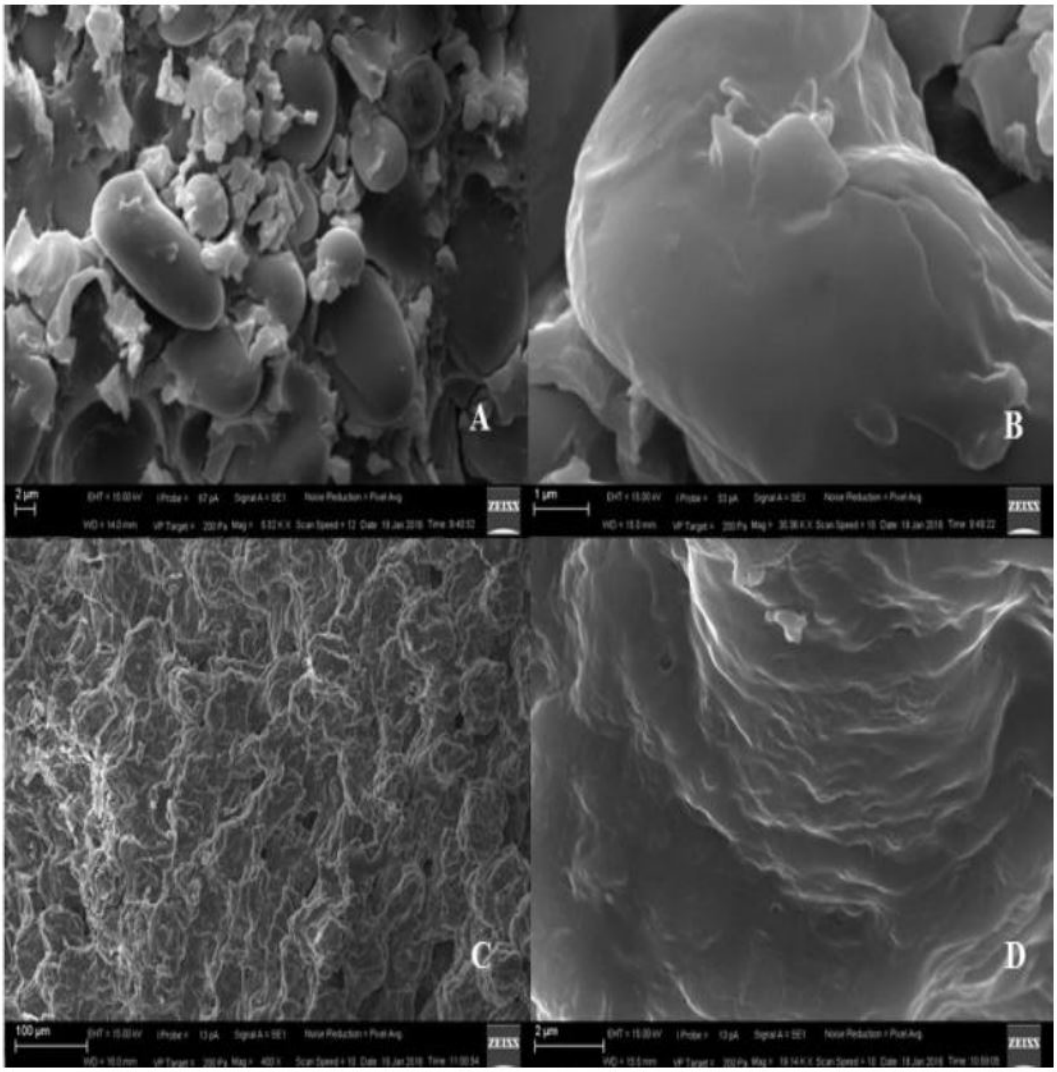

Figure 1. (A) SEM raw cowpea endosperm, magnification 5.52 KX; (B) SEM raw cowpea endosperm, magnification 30.36 KX; (C) Cowpea cooked with gelatinized starches, magnification 400X; (D) Cowpea cooked cotyledon, magnification of 19.14 KX.

a nutritional point of view by increasing the fiber content. This fact proves a functional property attributed to food, especially considering the role of short-chain fatty acids, produced during the fermentation of resistant starch by bacteria present in the colon. Miranda et al. (2015) observes by Optical Microscopy with polarized light, the empty amyloplasts in cooked cowpea bean, suggesting the lost of birefringence of starches, due to the occurrence of gelatinization.

Figure 2 below shows the SEM of blackbean endosperm. In A, the ellipsoid shape of starches in raw samples (magnification 9.81 KX); in B cracks in the starch surface can be observed (mag. 19.89 KX); in C is observed cotyledon of cooked black bean (magnifications of 275 X); in D, is possible to observe that the gelatinization was not complete as in cooked cowpea (Figure 1D), indicating a resistance to this phenomenon, due to the hard-to-cook occurrence (magnification 29.01 KX)

Kaur & Singh (2007) described the oval shape of blackbean starches and a smooth surface without cracking, observed under normal conditions. Ambigaipalan et al. (2011), by SEM images, also did not find the presence of cracks in black bean starches. Martínez-Preciado et al. (2012) described the morphological structure of common beans starch granules by SEM, observing an irregular oval shape with sizes 10-40 µm in length and 10-25 µm in width, and small spherical starches of 10 µm. It was also observed that the starch granules were well-defined and not suffer any damage. Miranda et al. (2015) observed by optical microscopy with polarized light, amyloplasts filled with starches in cooked black bean samples, suggesting that is no gelatinization, due to the occurrence of hard-to-cook (HTC) phenomenon. SEM, however, allows to see that the gelatinization occurs, but partially. At microstructural level, the result of HTC seems to be associated with the inability of the middle lamella of cotyledons cells to dissolve and separate cells. Chigwedere et al. (2018) investigated the relative contributions of cotyledons and seed coats towards hardening of common beans were and the rate-limiting process which controls bean softening during cooking was determined. Fresh or aged whole beans and cotyledons were

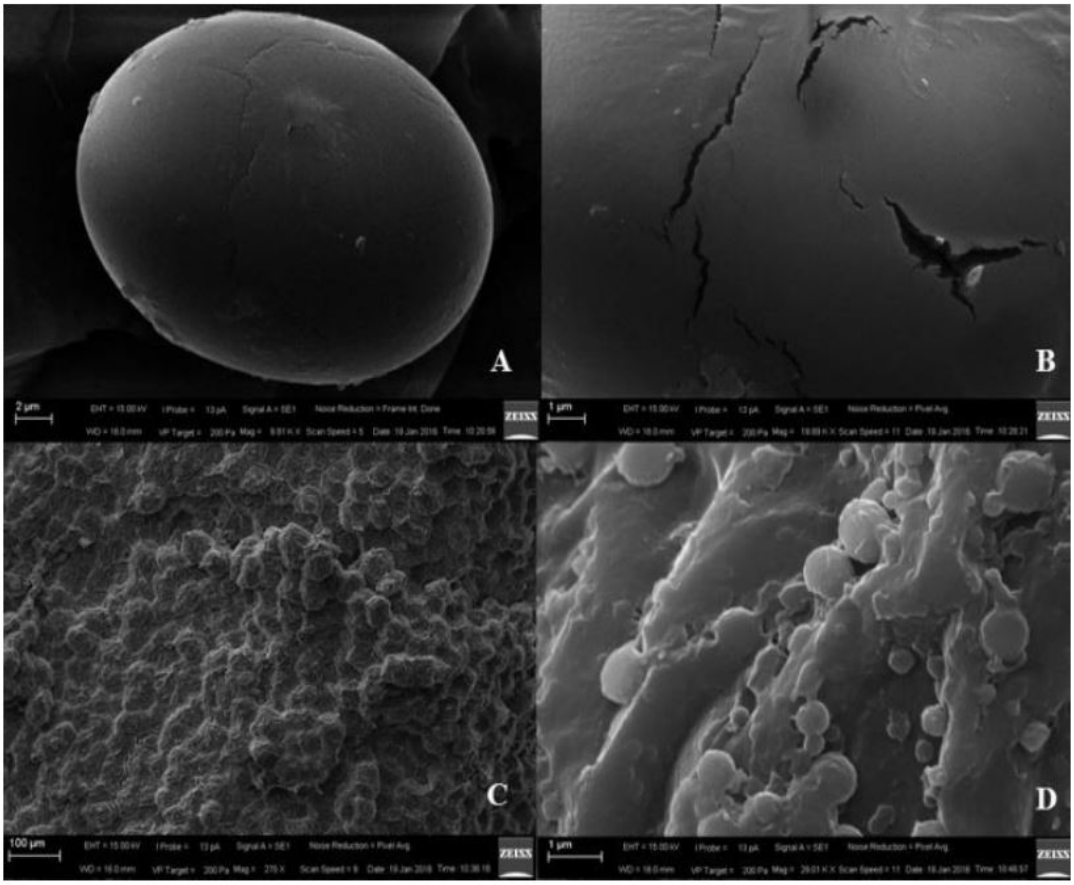

Figure 2. (A) SEM raw blackbean endosperm, magnification 9.81 KX; (B) Raw blackbeans endosperm, magnification 19.89 KX; (C) Cooked black bean cotyledon, magnification 275 X; (D) Cooked black beans without complete gelatinization of starches (magnification 29.01 KX).

soaked and cooked in demineralised water or 0.1 M $NaHCO_3$ solution, and texture evolution, microstructure changes and thermal properties were studied. The authors suggested that the rate-determining process in bean softening relates to cell wall/middle lamella changes influencing pectin solubilization.

In Figure 3 below, is observed the SEM of carioca bean endosperm. In A, the ellipsoid shape of starches in raw samples, similar to the black bean (magnification 6.00 KX); in B (Figure 2A). In B, it is possible to observe irregularities in the starch surface (magnification 15,61 KX). In C is observed cotyledon of cooked carioca bean (magnifications 370 X). In D, is observed the gelatinization and no intact chains (magnification 15.61 KX).

In the work of Ambigaipalan et al. (2011) was not found the presence of cracks in carioca beans starches, as well as in black bean starches, both varieties evaluated by SEM. Wang & Ratnayake (2014) was observed, by SEM, that has no damage on the starch surface from different Great-Northern bean cultivars. However, some samples had the presence of a strange material. All cultivars showed spherical, oval or elliptical starches shapes. According to the authors, bean starch granules usually have similar morphologies among the varieties of *P. vulgaris*, but unlike other starches such as tapioca and banana. Rupollo et al. (2011) examined SEM starch granules isolated from common bean, stored for 360 days, under three conditions: in hermetically sealed containers at 5 °C, in modified atmosphere with nitrogen at 15 °C and standard atmosphere at 25 °C. The author observed a great similarity between the granules, even at different storage conditions of seed. However, the starch granules stored in a conventional atmosphere at 25 °C appeared to be more aggregated than others. The samples stored in modified atmosphere with nitrogen at 15 °C did not differ in solubility and gel properties over the beans stored in a conventional atmosphere at 25 °C. However, the gel properties of this both conditions, differs from the hermetic samples at 5 °C, which had lower crystallinity, as well as swelling power and gelatinization heat. The starch grains stored in modified atmosphere with nitrogen at 15 °C, in turn, showed lower crystallinity, swelling power, and gelatinization heat than samples stored in a conventional atmosphere at 25 °C. Studies about the effects of storage conditions on bean starch properties are important to understanding the hardening phenomenon (hard-to-cook) and, moreover, make possible a storage standardization for industrial use. Vanier et al. (2014) evaluate the effects of nitrogen-modified atmosphere storage on coat color preservation, and the development of the hard-to-cook

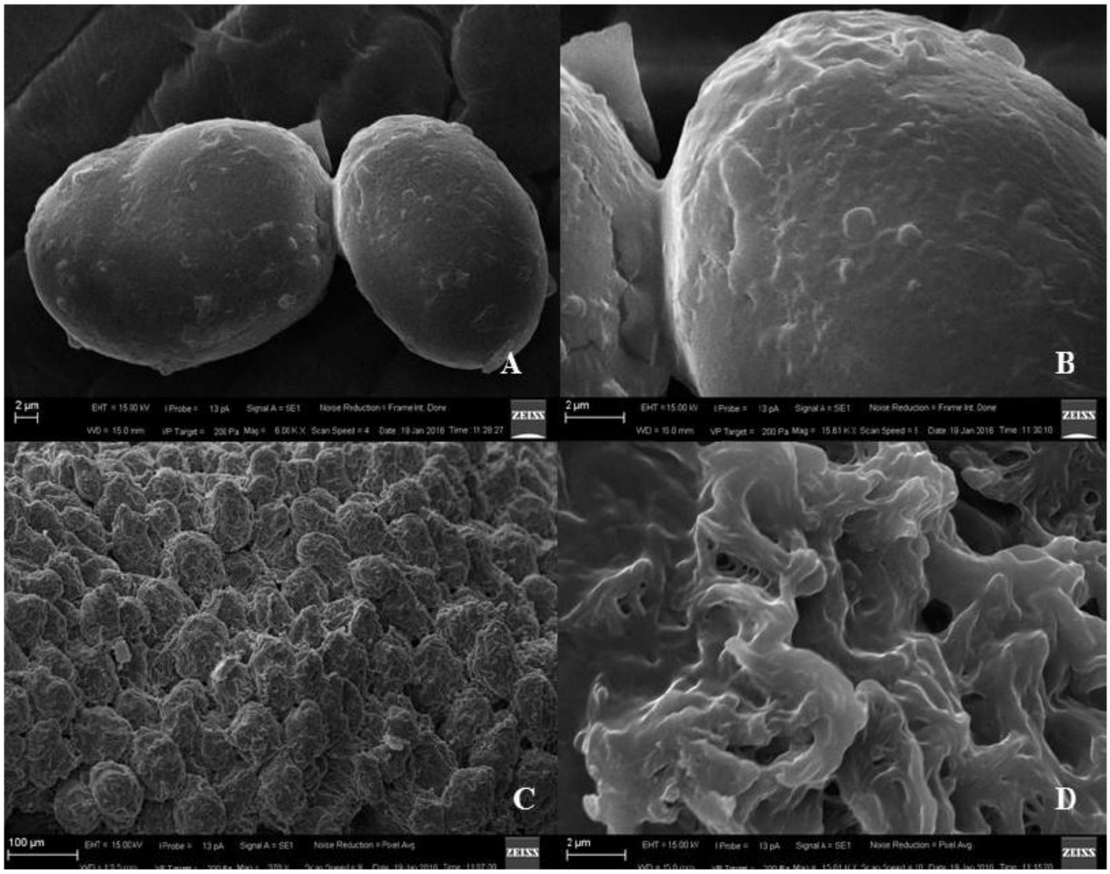

Figure 3. (A) SEM raw carioca bean endosperm, magnification of 6.00 KX; (B) Raw carioca bean endosperm magnification 15.61 KX; (C) Cooked carioca beans, magnification 370 X; (D) Cooked carioca cotyledon, magnification 15.61 KX.

defect in Carioca beans stored for 360 days. They observed that this condition of storage preserves the hydration coefficient and the electrical conductivity of carioca bean and slows the development of the HTC defect, at least for 360 days storage. This storage system also reduces the cellular stresses caused by storage in the normal atmosphere at 25 °C. Vanier et al. (2018) characterized starches from four common bean genotypes to use in production of biodegradable films. Starches were characterized by their swelling power, solubility, amylose content, granule morphology, relative crystallinity, thermal and pasting properties, and susceptibility to α-amylase hydrolysis. Films were also characterized. The authors observed that depending on the common bean genotype, a great variation on starch properties was found, which, in turn, clearly impacted on the characteristics of the starch-based films.

3.2 X-ray diffraction

The properties of X-ray diffraction provides evidence of the ordered structure of the starch granule. According to Figure 4 below, the starches of the common beans and cowpea showed a characteristic of C pattern, as in general legumes, and peaks of highest intensity in the diffraction regions is in the spacing close at 15° and 18° (2Θ).

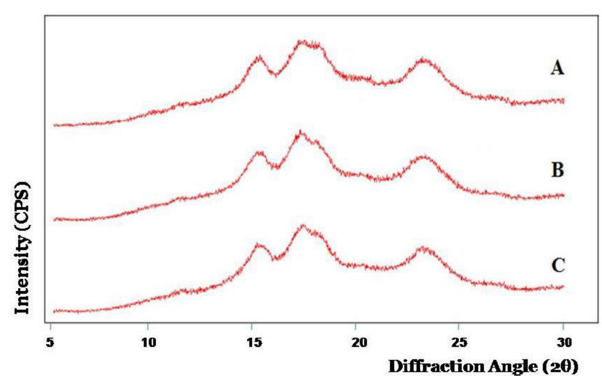

Figure 4. Intensity diffraction peaks of X-ray starch isolated from beans (A) cowpea (B) black, and (C) carioca. CPS: Cont Per Second.

The starch isolated from black beans shows the highest peak values of the three evaluated. The diffraction angles 15°, 17° 23° represent the largest of intensity peaks detected in X-ray diffraction, being higher in 17° for all starches analyzed in this study. Although the literature description generally classifies the crystalline structure of the legume starch in the standard polyform C, the diffraction angles encountered in this work are more consistent with the

Table 1. Starch yield, Intensity of the main peaks and Relative Crystallinity of starches isolated from cowpea, blackbeans and carioca.

Beans	Starch Yield (%)	Intensity (CPS*)			Relative Crystallinity (%)
		15°	17°	23°	
Cowpea	15.8 ± 0.3	1863	2214	1822	10.57 ± 0.2
Blackbean	17.1 ± 0.2	1938	2358	1834	10.62 ± 0.3
Carioca	16.1 ± 0.3	1922	2306	1806	10.53 ± 0.2

*CPS: Cont Per Second.

classification of the standard polyphorm A (Lima et al., 2012; Singh et al., 2017). The yield of isolated starch, the intensity of the main peaks and the relative crystallinity can be observed in Table 1, bellow.

The relative crystallinity following descending order: blackbeans (10.64%) > cowpea (10.57%) > carioca beans (10.50%). Being significantly varied, considering the analysis of variance. According to Hoover & Ratnayake (2002), differences in relative crystallinity of starches are affected by the size of the crystal. The number of crystalline regions is influenced by the content and length of the amylopectin chain, and the guidelines of the double helices in the crystalline domains, as well as the degree of interaction between double helices.

Pinto (2014) evaluated carioca bean starch subjected to different treatments, and noted the following sequence on the degree of relative crystallinity: enzymatic hydrolysis > native starch > heating > ultrasound > heat treatment of low humidity.

4 Conclusion

The SEM method allows observing the difference between the starches of varieties of beans, as well as their modification after beans treatment, like the loss of structural organization, which is characteristic by the gelatinization phenomenon, occurring when the matrix was subjected to temperatures greater than 50 °C. It was also possible to see the occurrence of hard-to-cook phenomenon in blackbean, which would be very important in the supervision of legumes offered for sale and consumption. Regarding the studies of the crystallinity of the starch granules by DR-X, the diffraction angles found in this work are more consistent with the classification of the standard polyphorm A, and the Relative Crystallinity of blackbeans, cowpea and carioca beans, observed by X-ray diffraction, varied significantly, considering a variance analysis.

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