

## Research Article

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# Morphology and structure of acorn starches isolated by enzymatic and alkaline methods

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**Abstract:** Morphology and structure of starch from fruits of two acorns species, *Quercus rotundifolia* Lam. (QR) and *Quercus suber* Lam. (QS), isolated by enzymatic (ENZ) and alkaline (A3S) methods were studied. Acorn starches granules presented a round and oval shape, consisting of medium/small granules, with a mean granule size ranging between 9 and 13  $\mu\text{m}$ . Isolated acorn starches appear as light grayish-brown in naked eye, with high values of  $L^*$  for starches isolated by the ENZ method, and QR starches were duller than QS. No differences were observed for all the samples in FTIR spectra results. Acorn starches showed a C-type pattern, with a relative crystallinity between 43.1 and 46.6%. The  $^{13}\text{C}$  CP/MAS NMR spectra are different for the used isolation methods but are similar for both acorn species. However, acorn-isolated starches presented a predominant A-type allomorph packing type, and the A3S starches showed a higher degree of crystalline material. Those differences in the structure of acorn starches would be helpful to better understand the relationships among structure and functional properties for a possible potential industrial application of chestnut starches.

**Keywords:** acorn starch, morphology, X-ray diffraction, FTIR, NMR

## 1 Introduction

Acorn is the general name for the seed of Fagaceae family. Acorns contain abundant nutritional ingredients: 50–60% crude starch, 2–8% soluble sugar, 3–6% protein, 3–5% crude fiber, 1–5% crude fat, and 5–12% tannin (Lu et al. 2010). *Quercus suber* L. and *Quercus rotundifolia* Lam. forests are particularly abundant in Spain and Portugal, but they are also found in Greece, Italy, and France (Grove and Rackham 2001). Most of the fruit production goes to animal feeding, mainly Iberic pig. These fruits are also consumed by humans, and there are many different kinds of commercially available processed acorn products, including breads cakes, soups, snacks, noodles, wine, cake and tofu, jelly, and serving as fodder, which comprised principally of acorn flours (Rakic et al. 2006; Kim and Yoo 2009; Molavi et al. 2018; Zhang et al. 2019). The use of acorn flour for human nutrition is also traditional in the Iberian Peninsula. Ribeiro (1992) mentions that primitive Lusitanian people (III-I b.C. centuries) feeding was based on oats porridge, dark bread, and acorn flour. Nowadays, in Portugal, there are some uses of acorn flours in traditional recipes. However, the valorization of underexploited resources is now a major trend to improve sustainability of the agri-food chain.

Starch is an important material in both food and non-food industries. In these latest years, research has been performed on new starch sources, which achieved feasible requirements. But limited studies have been completed on isolation, characterization, and application of starches from nonconventional sources, which could help in improving the food and nonfood applications (Deng et al. 2020). Starch is the main component of QR and QS acorns, i.e., 48 and 49%, respectively (Correia et al. 2009), providing a potential alternative to traditional starchy raw materials. However, till now, only few studies have been performed on their structural and functional properties. Thus, to enhance starches uses, it is important to find new potentialities of these materials, and so further studies are needed. As mentioned by León-Camacho et al. (2004), the extraction of starch from acorns has some advantages: the introduction

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of a new starch source with new properties and possibility of new applications, contribution to the preservation of *Quercus* forests, and obtaining some valuable by-products such as oil.

Starches are semi-crystalline polymers of amylose and amylopectin, widely used as a gelling, stabilizing, thickening, and coating agent for numerous food and nonfood products (Liu and Liu 2020). The starch polymers are packed in granules containing crystalline and amorphous regions. The starch polymers are packed in granules, which have been shown by several methodologies, including electron microscopy. Starch granules can vary greatly with regard to fraction relationships, structure and organization of the amylopectin and amylose molecules, branching architecture of amylopectin, and also degree of crystallinity (Lindeboom *et al.* 2004). Generally, it is accepted that amylopectin structure is constituted by short branches, forming double helices, which in a great extent are organized into crystallites (Manners 1989). It is also believed that amylose forms part of the amorphous regions. The form and crystallinity, and the structure of amylose and amylopectin of starch granules, have been extensively studied using many complementary approaches. One of them is the diffraction technique that is indicative of crystallinity of the material, and other is the small-angle scattering technique that measures differences in electron density distribution (Copeland *et al.* 2009). Moreover, regarding the crystallites, it has been shown by X-ray diffraction (XRD) that they can assume in native starch granules two main types, A and B types, differing in their single-cell unit parameters and in the amount of structural water (Imberty *et al.* 1991). Spectroscopic methods such as nuclear magnetic resonance and Fourier transformed infra-red spectroscopy are other approaches used to obtain structural information on starch. These tools provide explicit information on molecular constituents and their organization in starch granules.

The main objective of this study is to contribute a deeper knowledge about morphology and structure of acorn starch from *Q. suber* and *Q. rotundifolia* and to better understand the related functional properties. This information would be very useful to a proper industrial application of these nonconventional and underexploited starch sources.

## 2 Material and methods

### 2.1 Materials

Acorns from *Quercus rotundifolia* (QR) and *Quercus suber* (QS) were collected in “montados” located in Idanha-a-Nova.

Three sets of 1 kg of each acorn fruits were randomly harvested at the maturity stage, dried, and milled as previously reported by Correia, Leitão, and Beirão-da-Costa (2009).

### 2.2 Starch extraction methods

Acorn starches were isolated according to the following methods (Correia and Beirão-da-Costa 2012).

#### 2.2.1 Alkaline pH using successively three sieves (A3S)

Acorn flours (120 g) were soaked in 250 mL of 0.25% NaOH at 5°C during 24 h. Suspensions were homogenized and screened through a 180  $\mu\text{m}$  sieve. This step was then repeated twice. The precipitate was screened in a 75  $\mu\text{m}$  sieve, followed by other with 53  $\mu\text{m}$  sieve. The mixture was centrifuged in a Universal 16 centrifuge (Hettich Zentrifugen Company, Germany) at  $800 \times g/15$  min, the mucilaginous layer was removed, and the precipitate was suspended in water. This procedure was repeated twice. Isolated starches were dried for 2 days at 40°C in a FD 115 Binder ventilated drying chamber (with an air flow of 300  $\text{m}^3/\text{h}$ ).

Isolated starches presented a purity of 98.1 and 98.0% for *Q. suber* and *Q. rotundifolia*, respectively.

#### 2.2.2 Enzymatic method (ENZ)

Protease from *Aspergillus oryzae* was purchased from Sigma Chemical Co. One unit of protease was defined as the amount of enzyme that liberated 1.0  $\mu\text{mol}$  of tyrosine per minute from casein at pH 7.5 at 37°C. Protease was added (900 units) to 120 g of acorn flour. Water was added (360 mL), and the slurry was adjusted to pH 7.5 (with 0.1 M NaOH or 0.1 M HCl) and incubated at 37°C for a period of 2 h. Then, the slurry was centrifuged at the same conditions mentioned in the alkaline isolation method. Starch was suspended, washed with water (200 mL), and filtered through a 53  $\mu\text{m}$  sieve. The filtrate was centrifuged, and the supernatant and tailings were discarded and starch was dried as reported earlier.

The starch purity was evaluated by determining the starch content in the extract by the Ewers polarimetric method (ISO/DIS 10520, 1997). The purity of isolated starches was 97.6 and 97.8% for *Q. suber* and *Q. rotundifolia*, respectively.

### 2.3 Scanning electron microscopy

A scanning ISI-D 130 electron microscope (International Scientific Instrument) was used to observe the acorn starch samples. Samples were applied on an aluminum stub using double-sided adhesive tape, and the starch was coated with gold–palladium (80:20). An accelerating voltage of 10 kV was used.

### 2.4 Particle size and distribution analysis

To determine the size of acorn starch granules, a laser particle size analyzer Mastersizer X (Malvern Instruments Ltd, Malvern, United Kingdom) was used. A polydisperse mode of analysis and a 300 mm lens were used. Starch samples were dispersed in isopropyl alcohol to obtain an obscuration of 15–20%, using an equipment circulation unit, with mechanical shear and ultrasound for 5 min to disperse the starch clots. Measurements were taken at 2 min intervals. Size distributions were determined at three replications of duplicate samples ( $n = 6$ ). Results were expressed in terms of volume (%) occupied by starch granules. The classes defined based on Lindeboom et al. (2004) scale was used to classify starches: large ( $>25 \mu\text{m}$ ), medium ( $10\text{--}25 \mu\text{m}$ ), small ( $5\text{--}10 \mu\text{m}$ ), and very small ( $<5 \mu\text{m}$ ).

### 2.5 Color evaluation

A Chroma Meter CR-300 Minolta (Osaka, Japan) colorimeter and the classification by CIELAB (1986) system were used to analyze color. From  $L^*$   $a^*$   $b^*$ , chroma ( $c^*$ ) and hue angle ( $h^\circ$ ) were determined. Color lightness (value),  $L^*$  (100: white to 0: black), measures how light/dark is the color of the object; chroma or saturation,  $c^*$  (0–60), measures how dull/vivid is the object color; hue angle,  $h^\circ$  (0–360°), expresses the characteristic/dominant color (0 – red/purple; 90 – yellow; 180 – bluish/green). Color difference ( $\Delta E$ ) was determined by comparison to a white standard tile ( $L^* = 97.46$ ;  $a^* = -0.02$ ;  $b^* = 1.72$ ) and using equation (1):

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

A commercial maize (Maizena<sup>®</sup>, Copan, Portugal) starch was used as a reference.

Measurements were performed 25 individual times for each sample.

### 2.6 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of acorn starches from the *Q. rotundifolia* and *Q. suber* were obtained on a Mattson 7000 spectrophotometer. Starch sample was deeply mixed with dried potassium bromide powder (KBr) (1:100 in weight) and pressed in a die at 10,000 psi to yield a disc. The spectra recorded with a resolution of  $4.0 \text{ cm}^{-1}$  and 64 scans were registered in the medium-infrared area, which extends from 4,000 to  $400 \text{ cm}^{-1}$ . The calibration was carried out using KBr as a blank.

### 2.7 X-ray diffraction

The crystalline structure of the starches was carried out in a Philips diffractometer (X'Pert MPD, Almelo, Netherlands), using  $\text{CuK}\alpha$  radiation ( $\lambda = 0.154 \text{ nm}$ ) generated at 40 kV and 20 mA, at a moisture content between 8 and 10%. The sample was scanned through the  $2\theta$  (diffraction angle) from 3 to 50° at a speed of 8°/min. The degree of relative crystallinity was determined following the method previously reported by Huang et al. (2007) using equation (2):

$$\text{Crystallinity (\%)} = I_c / (I_a + I_c) \times 100 \quad (2)$$

where  $I_a$  is the amorphous area on the X-ray diffractogram and  $I_c$  is the crystallized area on the diffractogram.

### 2.8 Solid-state NMR

*Q. rotundifolia* and *Q. suber* starches were characterized by  $^{13}\text{C}$  NMR using cross polarization and magic angle spinning (CP/MAS), using a Bruker 500 spectrometer (Bruker, Germany). Before NMR measurements, samples were kept at 100% relative humidity for 48 h. The spectra were obtained with static magnetic field of the 11.7 T. The sample was placed in a zirconium rotor sealed of 4 mm with Kel-FTM caps and rotated at 9 kHz. The acquisition parameters used were as follows: proton pulse of 4  $\mu\text{s}$ , contact time of 2 ms, delay of 4 s, and 7,000 scans.

### 2.9 Statistical analysis

Experimental data were subjected to a one-way analysis of variance (ANOVA) using the Statistic<sup>®</sup> vs 6 software.

The significant difference or separation of means comparison of all parameters was tested by Tukey's HSD test. The level of significance used for all the statistical tests was 95%.

**Ethical approval:** The conducted research is not related to either human or animal use.

## 3 Results and discussion

### 3.1 Morphology of acorn starches

Isolated acorn starches appeared to the naked eye as a powder having a light greyish-brown color. Starches isolated by the ENZ method presented higher values of  $L^*$ , meaning that they were whitest (Table 1). Starches presented a yellow predominant color ( $h^\circ$  values near  $90^\circ$ ). QR produces starches duller than QS, and the A3S methods presented the higher values. Furthermore, some of these aspects are probably related to color characteristics of the origin flours since this tendency was also observed for QR and QS flours (Correia *et al.* 2009). The  $\Delta E$  calculation clearly puts in evidence major differences between samples. According to Drlange (1994), acorns starches presented values that are classified as great (between 6.0 and 12.0) to very great color difference (more than 12). Based on these results, acorn starches showed not to be recommended for use in products requiring light uniform color, but they could find applications in baked goods (e.g., cookies, donuts) that are not affected by the starch colour. The isolation method seems to affect the colour parameters. Starches produced by the A3S method presented higher values of total color difference parameter and lower  $L^*$  values, probably due to the lower efficiency to extract and eliminate some compounds formed during the drying process of the respective fruits, such as those resulting from the Maillard reaction.

Granule size distribution and SEM images of acorn starches produced by alkaline and enzymatic isolation methods are shown in Figure 1. Granules are round and oval in shape, showing some fractures. Images analysis does not establish differences on the isolation method effects. Acorn starches consist of medium/small granules, with starch granules size lower than  $18\ \mu\text{m}$ , representing about 80% of the total volume of particles, with about 25% of volume granules lower than  $5\ \mu\text{m}$ . The mean granule size ranged between 9 and  $13\ \mu\text{m}$ . These were also found for other acorn starches (Cho and Kim 2000; Stevenson *et al.* 2006; Molavi *et al.* 2018). Wilson *et al.* (2006) used a size scale where starch granules are classified into three size ranges: A-type granules ( $>15\ \mu\text{m}$ ), B-type granules ( $5\text{--}15\ \mu\text{m}$ ), and C-type granules ( $<5\ \mu\text{m}$ ). Based on this classification, acorn starch granules should be classified predominantly as B-type granules, and thus, some equipment used for extraction starch from wheat can be used for acorns. However, QS starch showed a higher range of sizes, with some granules larger than  $60.0\ \mu\text{m}$  in diameter. The ENZ method had removed/destroyed larger granules when compared to granule size distribution showed by the A3S method. This effect was also reported by other authors in their studies (Daiuto *et al.* 2005). From the results, it is evident that the granule diameter of acorn starch varies greatly depending on species and starch isolation methods.

### 3.2 Fourier transform infrared spectroscopy

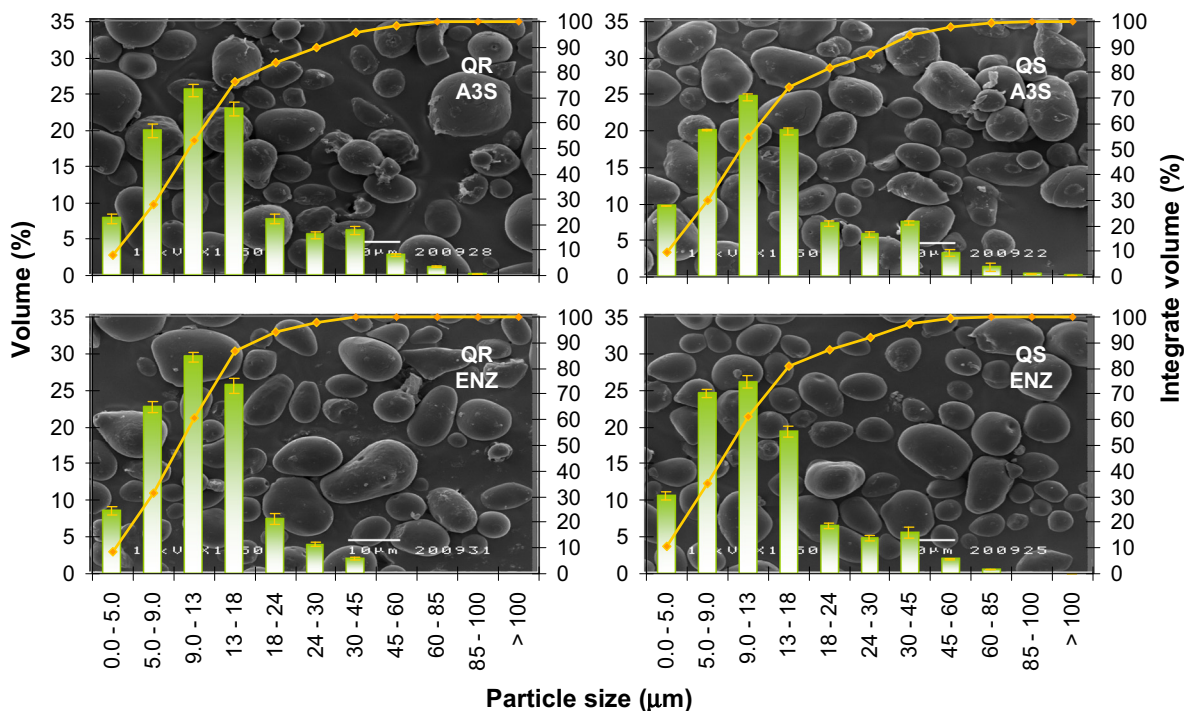
Fourier transform infrared (FTIR) analysis is a powerful tool to analyze, in a qualitative way, the interactions between starch molecules. It analyzes the presence of functional groups in the starch granules to confirm the structure of starches, which are detected through peaks, presented in a spectra, originated by vibrational modes of starch components. Usually, the spectra of sample show peaks that confirm the polysaccharide nature of

**Table 1:** Colour parameters of acorn starches

Variety	Isolation method	$L^*$	$c^*$	$h^\circ$	$\Delta E$
QR	A3S	$85.8 \pm 0.3^c$	$14.0 \pm 0.2^b$	$93.1 \pm 0.2^a$	$16.9 \pm 0.3^b$
	ENZ	$90.7 \pm 0.3^a$	$9.4 \pm 0.2^d$	$92.3 \pm 0.2^b$	$10.2 \pm 0.2^d$
QS	A3S	$83.2 \pm 0.2^d$	$15.0 \pm 0.2^a$	$93.0 \pm 0.2^a$	$19.5 \pm 0.1^a$
	ENZ	$87.8 \pm 0.2^b$	$13.3 \pm 0.1^c$	$92.2 \pm 0.1^b$	$15.1 \pm 0.2^c$

Values are mean  $\pm$  standard error of mean.

Means sharing the same letters in columns are not significantly different from each other (Tukey's HSD test,  $p < 0.05$ ).



**Figure 1:** SEM ( $\times 1,500$ ) and granule size distribution of acorn starch: QR – *Q. rotundifolia*; QS – *Q. suber*; A3S – alkaline pH and using successively three sieves method; ENZ – enzymatic method.

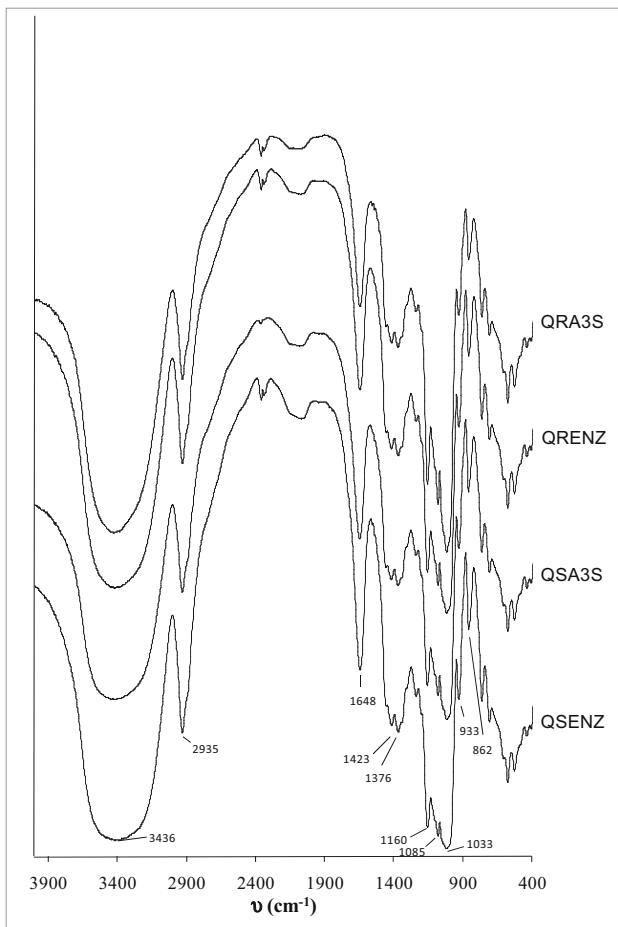
the starches at approximately 3,320, 2,920, 1,645, 1,426, 1,331, 1,156, 1,079, 1,020, 923, and 915–757  $\text{cm}^{-1}$  (Deng et al. 2020).

Figure 2 shows a comparison between the FTIR spectra obtained for the acorns starch granules isolated by A3S and ENZ methods. No differences can be observed in those spectra. The characteristic peaks of starch molecules interactions are presented in Table 2, and as an example, the spectra of the sample *Q. suber* isolated by the ENZ are shown in Figure 2. The peaks at 2,935 and 3,436  $\text{cm}^{-1}$  could be due to the complex vibration stretches associated with –CH stretches attributed to the ring methine hydrogen atoms and free intramolecular and intermolecular hydroxyl groups, respectively (Reddy et al. 2017; Xu et al. 2019). The absorptions near 1,376 and 1,648  $\text{cm}^{-1}$  may be attributed to water bending vibration (Zhang et al. 2007; Luo et al. 2009; Reddy et al. 2017). Between 933 and 1,160  $\text{cm}^{-1}$  appeared three characteristic peaks, which are associated with C–O bond stretching. Furthermore, the anhydro-glucose ring C–O stretch peaks are observed at 1,085 and 1,033  $\text{cm}^{-1}$  (Fang et al. 2002; Wu, Geng et al. 2009), and the peak at 862  $\text{cm}^{-1}$  is associated with the C–H of residual carbons of *b*-galactose (Freile-Pelegrín et al. 2007). Thus, the 800–1,200  $\text{cm}^{-1}$  region in these spectra reflects C–C, C–OH, and C–H stretching vibrations, starch polymer conformation, and

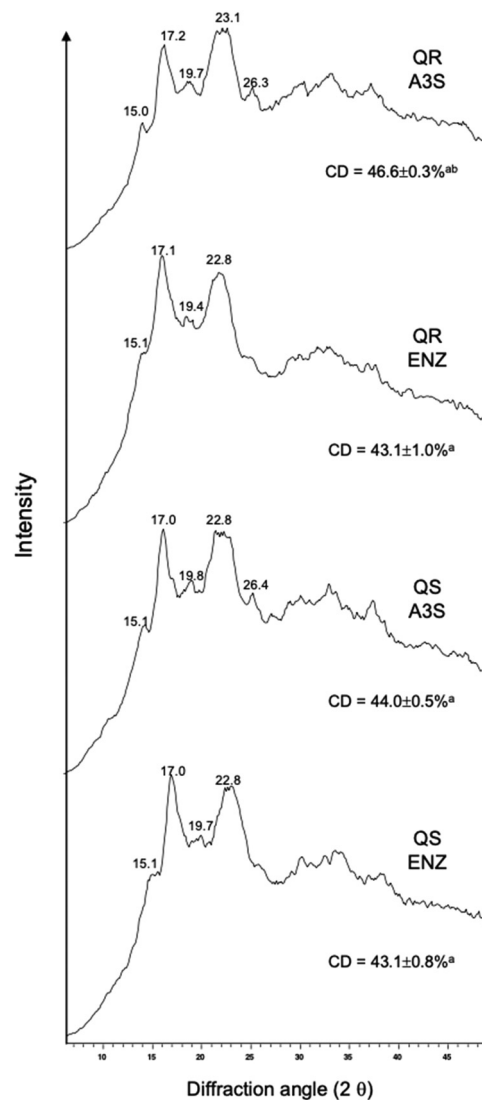
hydration process (Li et al. 2020). Furthermore, the absorbance ratio of 1,045/1,022  $\text{cm}^{-1}$  show the ordered degree of starch, being the absorbance at 1,045  $\text{cm}^{-1}$  relative to the ordered/crystalline region of starch, and the one at 1,022/995  $\text{cm}^{-1}$  reflects the proportion of amorphous to the ordered carbohydrate structure in starch, indicating the internal changes in the double-helical structure (Li et al. 2020; Sevenou et al. 2002). These encountered results are closed to those obtained for native potato starch (Luo et al. 2009; Wu et al. 2009).

### 3.3 X-ray diffraction pattern and crystallinity

The X-ray diffraction patterns of acorn starches are shown in Figure 3, which also presents the corresponding X-ray diffraction parameters and crystallinity degree. Starch samples exhibit a X-ray pattern, where the polymorphs were present in varying proportions, with a strongest diffraction peaks at  $2\theta$  of about 17° and 23° and a few small peaks at around 15°, 20°, and 26°. These patterns may be classified as a C-type X-ray pattern (a mixture of “A” and “B” unit cells) (Chung et al. 2010). B-type starch has one strongest diffraction peak at around 17°  $2\theta$ , a few small peaks at around 15°, 22, and 24°, and also a characteristic peak at about 5.6°  $2\theta$ , like potato



**Figure 2:** The FTIR spectra of acorn starch: QR – *Q. rotundifolia*; QS – *Q. suber*; A3S – alkaline pH and using successively three sieves method; ENZ – enzymatic method.



**Figure 3:** X-ray diffraction patterns of acorn starches: QR – *Q. rotundifolia*; QS – *Q. suber*; A3S – alkaline pH and using successively three sieves method; ENZ – enzymatic method; DC – crystallinity degree (%). Data with the same superscript letter are not significantly different ( $p < 0.05$ ).

starches and some legumes starches (Jayakody et al. 2007; Singh et al. 2008; Guo et al. 2017). Conversely, pure A-type starches such as wheat and maize exhibit a shoulder/doublet at around  $17^\circ$  and  $18^\circ$   $2\theta$ , which do not show the  $2\theta$  peak at  $5.6^\circ$ , but show a unique and strong peak at around  $15$  and  $23^\circ$   $2\theta$ , instead of the doublet  $22$ – $24^\circ$   $2\theta$  (Jayakody et al. 2007; He and Wei 2017), and another peak at  $27^\circ$  (Yu and Wang 2008). A- and B-types

starches presented the same double helical conformation but different packing arrangement, being the A-type

**Table 2:** The wavenumbers and the corresponding groups of starch in the FTIR spectrum of QS ENZ

Wavenumbers ( $\text{cm}^{-1}$ )	Bonds	Groups in starch by FTIR spectrum
3,436	Str. OH	Intermolecular and intramolecular
2,935	Str CH	Of the anhydroglucose ring
1,648, 1,376	$\text{H}_2\text{O}$	In the starch and strictly bonded
1,160, 1,085	Str Co of COH	OH group of starch takes part in the hydrogen bond formation
1,033, 933	Str CO of COC	Group in the anhydroglucose ring
862	C–H	Residual carbons of $\beta$ -galactose

denser and with less intracrystalline water content (Wang et al. 2009). Furthermore, the structure of the C-type starch granules is distinct, being an aggregation of both A- and B-type polymorphs, giving to C-type starches different processing suitability (Guo et al. 2017), more complex than the other two types due to the different contents and distribution patterns of A- and B-type crystals in them (Wang et al. 2009).

The C-type spectrum are classified into C<sub>A</sub>-type, C<sub>B</sub>-type, and C<sub>C</sub>-type (Cai et al. 2014; He and Wei 2017) based on their similarity to A and B-type polymorphs. According to He and Wei (2017), the X-ray pattern of C<sub>C</sub>-type starch shows a single peak at about 17° and 23° 2θ, and a few small peaks at around 5.6° and 15° 2θ, and that of C<sub>A</sub>- and C<sub>B</sub>-type starches are similar to that of the C<sub>C</sub>-type, but they presented a shoulder peak at around 18° 2θ and a strong singlet at 23° 2θ for C<sub>A</sub>-type starch, and two shoulder peaks at about 22° and 24° 2θ for the C<sub>B</sub>-type starch. In this sense, because of the peaks revealed by X-ray, the *Q. rotundifolia* and *Q. suber* acorn starches could be classified as a C<sub>A</sub>-type. It should also be noted that the presence of a peak near 20° indicates the occurrence of crystalline amylose–lipid complexes (Yang et al. 2010). The complex amylose–lipid complexes are more evident on starches isolated by the A3S method, which means that this method is less effective in destroying these complexes. Furthermore, the type of X-ray diffraction patterns of acorn starches seems to be dependent of acorn species because Stevenson et al. (2006) observed a A-type pattern for pin oak acorn starches. Kim and Lee (1976) reported acorn starch has B-type, but Molavi et al. (2018) found that *Q. brantii* a C-type pattern.

Concerning crystallinity, no significant differences are presented between acorn starches, showing a high percentage of this parameter, when compared, for example, with pin oak acorn starch (Stevenson et al. 2006) but similar to Persian starch (Molavi et al. 2018). QR starches showed higher crystallinity values, and the A3S method produced also starches with higher degree of crystallinity, which could mean that its original structure is less disturbed, mainly the amylopectin crystallites.

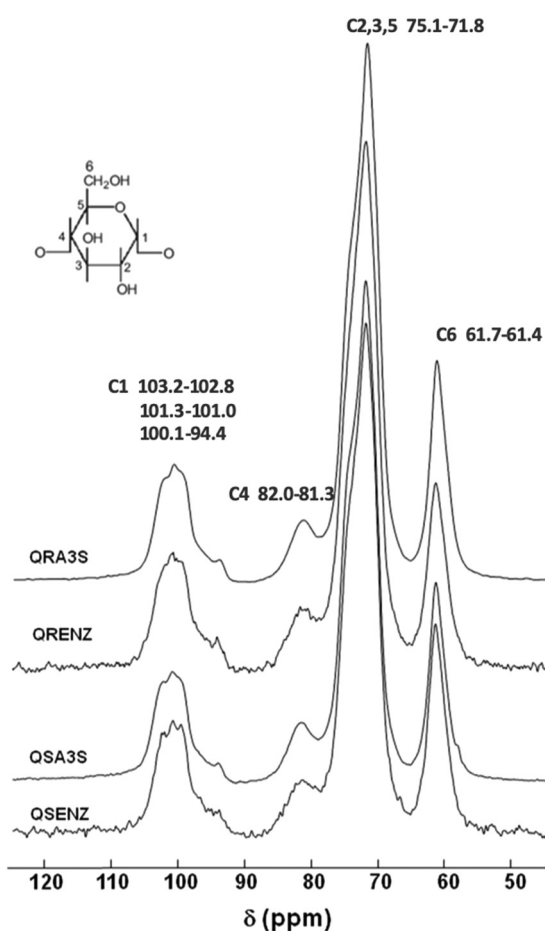
### 3.4 Solid-state NMR

Molecular order in a starch granule is composed of two types of helices from amylopectin side chains: (1) packed helices in regular arrays, which form crystallinity, measured by <sup>13</sup>C CP/MAS solid-state NMR and X-ray

diffraction, and (2) unpacked helices that are in a regular form or packed in short-range distance, which can be detected by <sup>13</sup>C CP/MAS solid-state NMR but cannot be found by X-ray diffraction (Cooke and Gidley 1992).

Figure 4 summarizes the <sup>13</sup>C CP/MAS NMR spectra of starch *Q. rotundifolia* and *Q. suber*, with reference to <sup>13</sup>C chemical shift values for all resolved signals. Assignments of resonances are consistent with the literature data (Gidley and Bociek, 1985; Veregin et al. 1986; Bogracheva et al. 2001; Atichokudomchai et al. 2004; Wang et al. 2009). RMN spectra present a different crystallite types. Signal of C1 resonance contains information both on the noncrystalline (but rigid) chains as well as the crystalline nature. A triplet characteristic resonance for A-type crystallites was observed for all the starch (Figure 4). The multiplicity of the C1 resonance is related to the type of packing of granules in the region 99–104 ppm intensity. Maltotriose is the repeat unit in an A-type starch, and the twofold axis generates the double helix causing three different environments for C1; thus, the C1 peak in A-type starch spectra is a triplet (~102, 101, and 100 ppm). Moreover, maltose is the repeat unit for B-type starch, and the threefold screw axis generates a double helix, which provides two different environments for C1. Thus, the C1 peak in B-type starch spectra is a doublet (~101 and 100 ppm) (Gidley and Bociek 1985; Veregin et al. 1986). Signal at 81–84 ppm is attributed to C4. The peaks in the C2, C3, and C5 region (70–79 ppm) were sharpened, and the signal near 76 ppm (B-type double helices from residues of free amylose) was pronounced for the nonwaxy starches (Atichokudomchai et al. 2004).

The results show that *Q. rotundifolia* and *Q. suber* have similar starch structures. Moreover, the isolation methods clearly affected both varieties. Since C-type starches have both A- and B-type crystallites, it can be suggested that the resonances in the spectrum mainly depend on the relative proportions of A- or B-allomorph in the sample (Bogracheva et al. 2001). Signals at 99–104 and 58–65 are attributed to C1 and C6 in hexapyranoses, respectively. As described earlier, the C1 resonances for the ENZ method, in both acorns, indicates that B-type allomorph is actually predominant in the acorn starch isolated by this method, whereas a typical A-type characteristic can be found in the A3S method. The two broad shoulders that appear at ~103 and ~82 ppm resonances could arise from the amorphous domains for C1 and C4 (Wang et al. 2009). The overlapping signal around 72 and 74 ppm is associated with C2, C3, and C5, and the resonance around 61.4 ppm is assigned to C6. Except the above peaks, the weak peak appearing at 94.5 ppm could be attributed to high-energy, twisted conformations remote



**Figure 4:**  $^{13}\text{C}$  CP/MAS NMR spectra of acorn starch: QR – *Q. rotundifolia*; QS – *Q. suber*; A3S – alkaline pH and using successively three sieves method; ENZ – enzymatic method.

from those characteristic of single helices (102–103 ppm) and double helices (99–101 ppm) (Wang et al. 2009).

Generally, one particular difference was observed in the  $^{13}\text{C}$  CP/MAS NMR patterns for acorn starch isolated by the A3S and ENZ methods. The intensity of C-1 and C-4 amorphous resonances seems to be smaller in A3S method than in the ENZ method. Amorphous compounds give broad resonances as the distribution of local molecular environment gives rise to a wide distribution of chemical shifts for each carbon. Some researchers mentioned that ordered materials show narrower resonances due to more regularity of the environment (Gidley and Bociak 1985; Veregin et al. 1986), which reflected the stricter polymer configurations in the ordered parts of the starch (Paris et al. 1999). Because of the decrease in the amount of amorphous phases in the starch granules, the resonances decreased, which leads to obtain more crystalline material in the compounds obtained by the A3S method. These results are also corroborated by the X-ray diffraction crystallinity results.

## 4 Conclusions

Starch isolated from the two studied acorns seems to have similar morphology. Acorn starch granules were found to be oval and round in shape, medium/small in size, being the size granules classified predominantly as B-type granules. Acorn starches have high relative crystallinity values, more than 43.1%, with similar interactions between starch molecules, and they could be classified as a  $C_b$  types. Morphology and structural properties of starches seems to be significantly affected by isolation methods. Starch isolated by the A3S method presented high range of granule sizes, more than 60  $\mu\text{m}$ . The interactions between molecules in starch granule were similar for both extraction methods, but the structures of the granules were significantly different. In general, the XRD display no significant differences on the crystallinity degree of acorn starch granules, but the  $^{13}\text{C}$  CP/MAS NMR patterns showed some differences for starches isolated by the A3S and ENZ methods.

The ENZ method exhibits smaller intensity of C-1 and C-4 amorphous resonances than in the A3S method. The C1 resonances indicate that A-type allomorph is predominant in the acorn starches, and starch isolated by the A3S method presented a high compact crystalline structure of the double helices.

These nonconventional and underexploited starch acorn sources presented some structural differences, and even higher considering the isolation method. So, it is possible to preview that the functional properties will be also different, leading to useful information, which would be very important to find proper industrial applications.

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## References

- [1] Atichokudomchai N, Varavinit S, Chinachoti P. A study of ordered structure in acid-modified tapioca starch by <sup>13</sup>C CP/MAS solid-state NMR. *Carbohydr Polym.* 2004;58:383–9. doi: 10.1016/j.carbpol.2004.07.017.
- [2] Bogracheva TY, Wang YL, Hedley CL. The effect of water content on the ordered/disordered structure in starches. *Biopolymers.* 2001;58:247–59. doi: 10.1002/1097-0282(200103)58:3<247:aid-bip1002>3.0.co;2-l.
- [3] Cai C, Cai J, Man J, Yang Y, Wang Z, Wei C. Allomorph distribution and granule structure of lotus rhizome C-type starch during gelatinization. *Food Chem.* 2014;142:408–15. doi: 10.1016/j.foodchem.2013.07.091.
- [4] CIELAB. Colorimetric. 2nd ed., Vienna: Publication C.I.E. No. 15, 2 Central Bureau of Commission International de L'Éclairage; 1986.
- [5] Cho SA, Kim SK. Particle size distribution, pasting pattern and texture of gel of acorn, mungbean, and buckwheat starches. *Korean J Food Sci Technol.* 2000;32:1291–7.
- [6] Chung HJ, Liu Q, Hoover R. Effect of single and dual hydro-thermal treatments on the crystalline structure, thermal properties, and nutritional fractions of pea, lentil, and navy bean starches. *Food Res Int.* 2010;43:501–8. doi: 10.1016/j.foodres.2009.07.030.
- [7] Cooke D, Gidley MJ. Loss of crystallinity and molecular order during starch gelatinization: origin of the enthalpic transition. *Carbohydr Res.* 1992;227:103–12. doi: 10.1016/0008-6215(92)85063-6.
- [8] Copeland L, Blazek J, Salman H, Tang MC. Form and functionality of starch. *Food Hydrocoll.* 2009;23:1527–34. doi: 10.1016/j.foodhyd.2008.09.016.
- [9] Correia PR, Beirão-da-Costa ML. Starch isolation from chestnut and acorn flours through alkaline and enzymatic methods. *Food Bioprod Process.* 2012;90(2):309–16. doi: 10.1016/j.fbp.2011.06.005.
- [10] Correia P, Leitão A, Beirão-da-Costa ML. Effect of drying temperatures on chemical and morphological properties of dried acorns flours. *Int J Food Sci Technol.* 2009;44:1729–36. doi: 10.1111/j.1365-2621.2009.01988.x.
- [11] Daiuto E, Cereda M, Sarmento S, Vilpoux O. Effects of extraction methods on yam (*Dioscorea alata*) starch characteristics. *Starch/Särke.* 2005;57:153–60. doi: 10.1002/star.200400324.
- [12] Deng M, Reddy CK, Xu B. Morphological, physico-chemical and functional properties of underutilized starches in China. *Int J Biol Macromolecules.* 2020;158:648–55. doi: 10.1016/j.ijbiomac.2020.05.031.
- [13] Drlange. Colour Review. Drlange Application Report No. 8.0 e. USA: Drlange; 1994.
- [14] He W, Wei C. Progress in C-type starches from different plant sources. *Food Hydrocoll.* 2017;73:162–75. doi: 10.1016/j.foodhyd.2017.07.003.
- [15] Fang JM, Fowler PA, Tomkinson J, Hill CAS. The preparation and characterisation of a series of chemically modified potato starches. *Carbohydr Polym.* 2002;47:245–52. doi: 10.1016/S0144-8617(01)00187-4.
- [16] Freile-Pelegrín Y, Madera-Santana T, Robledo D, Veleza L, Quintana P, Azamar JA. Degradation of agar films in a humid tropical climate: Thermal, mechanical morphological and structural changes. *Polym Degrad Stab.* 2007;92:244–52. doi: 10.1016/j.polymdegradstab.2006.11.005.
- [17] Gidley MJ, Bociek SM. Molecular organization in starches: A <sup>13</sup>C CP/MAS NMR study. *J Am Chem Soc.* 1985;107:7040–4. doi: 10.1021/ja00310a047.
- [18] Grove AT, Rackham O. *The Nature of Mediterranean Europe: An Ecological History.* London: Yale University Press; 2001. p. 384.
- [19] Guo Z, Jia X, Zhao B, Zeng S, Xiao J, Zheng B. C-type starches and their derivatives: structure and function. *Ann NY Acad Sci.* 2017;1398:41–61. doi: 10.1111/nyas.13351.
- [20] Huang J, Schols HA, Soest JGG, Jin Z, Sulmann E, Voragen AGJ. Physicochemical properties and amylopectin chain profiles of cowpea, chickpea and yellow pea starches. *Food Chem.* 2007;101:1355–62. doi: 10.1016/j.foodchem.2006.03.039.
- [21] Imberty A, Buléon P, Tran V, Perez S. Recent advances in knowledge of starch structure. *Starch/Stärke.* 1991;43:375–84. doi: 10.1002/star.19910431002.
- [22] ISO/DIS 10520. Native starches – Determination of starch content – Ewers polarimetric method. Geneva, Switzerland: International Organization for Standardization; 1997.
- [23] Jayakody L, Lan H, Hoover R, Chang P, Liu Q, Donner E. Composition, molecular structure, and physicochemical properties of starches from two grass pea (*Lathyrus sativus* L.) cultivars grown in Canada. *Food Chem.* 2007;105:116–25. doi: 10.1016/j.foodchem.2007.03.057.
- [24] Kim JO, Lee M-J. Studies on some physicochemical properties of the acorn starch. *Korean J Food Sci Technol.* 1976;8:230–5.

- [25] Kim W-W, Yoo B. Rheological behaviour of acorn starch dispersions: effects of concentration and temperature. *Int J Food Sci Technol.* 2009;44:503–9. doi: 10.1111/j.1365-2621.2008.01760.x.
- [26] León-Camacho M, Viera-Alcaide I, Vicario IM. Acorn (*Quercus* spp.) fruit lipids: Saponifiable and unsaponifiable fractions: A detailed study. *J Am Oil Chem Soc.* 2004;81:447–53. doi: 10.1007/s11746-004-0921-8.
- [27] Li X, Chen W, Chang Q, Zhang Y, Zheng B, Zeng H. Structural and physicochemical properties of ginger (*Rhizoma curcumae longae*) starch and resistant starch: A comparative study. *Int J Biol Macromolecules.* 2020;144:67–75. doi: 10.1016/j.ijbiomac.2019.12.047.
- [28] Lindeboom N, Chang PR, Tyler RT. Analytical, biochemical and physicochemical aspects of starch granule sizes, with emphasis on small granule starches: a review. *Starch/Stärke.* 2004;56:89–99. doi: 10.1002/star.200300218.
- [29] Liu K, Liu Q. Enzymatic determination of total starch and degree of starch gelatinization in various products. *Food Hydrocoll.* 2020;103:105–639. doi: 10.1016/j.foodhyd.2019.105639.
- [30] Lu ZD, Feng H, Yue S, Lu MB, Yu LJ. Fermentative production of L (+)-lactic acid using hydrolyzed acorn starch, persimmon juice and wheat bran hydrolysate as nutrients. *Bioresour Technol.* 2010;101(10):3642–8. doi: 10.1016/j.biortech.2009.12.119.
- [31] Luo F, Huang Q, Fu X, Zhang L, Yu S. Preparation and characterisation of crosslinked waxy potato starch. *Food Chem.* 2009;115:563–8. doi: 10.1016/j.foodchem.2008.12.052.
- [32] Manners DJ. Recent development in our understanding of amylopectin structure. *Carbohydr Polym.* 1989;11:87–112. doi: 10.1016/0144-8617(89)90018-0.
- [33] Molavi H, Razavi SMA, Farhoosh R. Impact of hydrothermal modifications on the physicochemical, morphology, crystallinity, pasting and thermal properties of acorn starch. *Food Chem.* 2018;245:385–93. doi: 10.1016/j.foodchem.2017.10.117.
- [34] Paris M, Bizot H, Mery J, Bizarre JY, Buléon A. Crystallinity and structuring role of water in native and recrystallized starches by <sup>13</sup>C CP/MAS NMR spectroscopy I: spectral decomposition. *Carbohydr Polym.* 1999;39:327–39.
- [35] Rakic S, Povrenovic D, Tesevic V, Simic M, Maletic R. Oak acorn, polyphenols and antioxidant activity in functional food. *J Food Eng.* 2006;74:416–23. doi: 10.1016/j.jfoodeng.2005.03.057.
- [36] Reddy CK, Luan F, Xu B. Morphology, crystallinity, pasting, thermal and quality characteristics of starches from adzuki bean (*Vigna angularis* L.) and edible kudzu (*Pueraria thomsonii* Benth). *Int J Biol Macromolecules.* 2017;105:354–62. doi: 10.1016/j.ijbiomac.2017.07.052.
- [37] Ribeiro O. *Formação de Portugal*. Ed. Lisbon, Portugal: Instituto de Cultura e Língua Portuguesa (ICALP); 1992. p. 139.
- [38] Sevenou O, Hill SE, Farhat IA, Mitchell JR. Organisation of the external region of the starch granule as determined by infrared spectroscopy. *Int J Biol Macromolecules.* 2002;31:79–85. doi: 10.1016/s0141-8130(02)00067-3.
- [39] Singh N, Nakaura Y, Inouchi N, Nishinari K. Structure and viscoelastic properties of starches separated from different legumes. *Starch/Stärke.* 2008;60:349–57. doi: 10.1002/star.200800689.
- [40] Stevenson DG, Jane J-L, Inglett G. Physicochemical properties of pin oak (*Quercus palustris* Munchh.) acorn starch. *Starch/Stärke.* 2006;58:553–60. doi: 10.1002/star.200600533.
- [41] Veregin RP, Fyfe CA, Marchessault RH, Taylor MG. Characterization of the crystalline A and B starch polymorphs and investigation of starch crystallization by high-resolution <sup>13</sup>C CP/MAS NMR. *Macromolecules.* 1986;19:1030–4. doi: 10.1021/ma00158a016.
- [42] Xu J, Ma Z, Ren N, Li X, Liu L, Hu X. Understanding the multi-scale structural changes in starch and its physicochemical properties during the processing of chick-pea, navy bean, and yellow field pea seeds. *Food Chem.* 2019;289:582–90. doi: 10.1016/j.foodchem.2019.03.093.
- [43] Wang S, Yu J, Zhu Q, Yu J, Jin F. Granular structure and allomorph position in C-type Chinese yam starch granule revealed by SEM, <sup>13</sup>C CP/MAS NMR and XRD. *Food Hydrocoll.* 2009;23:426–33. doi: 10.1016/j.foodhyd.2008.02.012.
- [44] Wilson JD, Bechtel B, Todd TC, Seib PA. Measurement of wheat starch granule size distribution using image analysis and laser diffraction technology. *Cereal Chem.* 2006;83:259–8. doi: 10.1094/CC-83-0259.
- [45] Wu Y, Geng F, Chang PR, Yu J, Ma X. Effect of agar on the microstructure and performance of potato starch film. *Carbohydr Polym.* 2009;76:299–304. doi: 10.1016/j.carbpol.2008.10.031.
- [46] Yang B, Jiang G, Prasad KN, Gu C, Jiang Y. Crystalline, thermal and textural characteristics of starches isolated from chestnut (*Castanea mollissima* Bl.) seeds at different degrees of hardness. *Food Chem.* 2010;119:995–9. doi: 10.1016/j.foodchem.2009.08.002.
- [47] Yu J, Wang S. Morphological and crystalline properties of starches from new sources-traditional Chinese medicines (TCMs). *Starch/Stärke.* 2008;60:110–4. doi: 10.1002/star.200700661.
- [48] Zhang N, Jiang J-C, Yang J, Wei M, Zhao J, Xu H, et al. Citric acid production from acorn starch by tannin tolerance mutant *Aspergillus niger* AA120. *Appl Biochem Biotechnol.* 2019;188:1–11. doi: 10.1007/s12010-018-2902-4.
- [49] Zhang SD, Zhang YR, Zhu J, Wang XL, Yang KK, Wang YZ. Modified corn starches with improved comprehensive properties for preparing thermoplastics. *Starch/Stärke.* 2007;59:258–68.