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The experimental identification of nixtamalized maize through starch spherulites

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ABSTRACT

Nixtamalization is a cooking technique that has played a significant role for thousands of years in the foodways of indigenous communities throughout the Americas. By cooking maize in an alkaline solution, often made from slaked lime, the process of nixtamalization increases the nutritional value of maize and helps to prevent severe malnutrition in populations dependent on maize as a staple food source. Due to the preservation bias against macrobotanical remains in tropical soils, microbotanical analyses of pottery residues are increasingly used to identify ancient plant use and preparation. However, to date no method has been developed to directly identify nixtamalization in the archaeological record via residue analysis. Through experimental replication of the nixtamalization process we have identified a unique product of the lime-based alkaline cooking process: residues that we conclude are starch spherulites. Here, we detail the range of diagnostic morphologies characteristic of starch spherulites and propose that the presence of starch spherulites found on cooking vessels and grinding stones, or within archaeological sediments, can act as a proxy for the use of the nixtamalization process. Through applications of polarized light microscopy, scanning electron microscopy, and SEM-EDS, this research lays the groundwork for the direct identification of nixtamalization in archaeological contexts, offering for the first time a direct mechanism with which to assess the inception and expansion of nixtamalization throughout the Americas.

1. Introduction

Practiced throughout the Americas, nixtamalization is a cooking method that transforms nutrient-deficient maize into a food source that provides vital niacin and calcium to large populations dependent upon maize as a staple food crop (Briggs, 2015). A form of cooking unique to this region, nixtamalization has become not only a critical way to ensure adequate nutrition in maize-dominated diets, but a cultural element in its own right. Numerous ethnohistorical records have highlighted the importance and endurance of the process of nixtamalization in the Americas (Bartram, 1853; Briggs, 2015; de la Vega, 1993; Wright, 1958). Due to the high carbohydrate content of maize kernels and the poor preservation of macrobotanical remains in most tropical settings within the Americas, microbotanical residue analysis provides a promising method through which to identify directly nixtamalization in the archaeological record.

Archaeological starch research is active in the Americas, but has largely focused on transitions to agriculture and timing the spread of maize cultivation in relation to the use of indigenous cultivars and wild plant use (Boyd and Surette, 2010; Boyd et al., 2008; Haas et al., 2013; Pearsall, 2015:246; Piperno et al., 2000; Zarrillo et al., 2008). While work has been done in the food science industry focusing on the effect of nixtamalization on starch granules (e.g., Gomez et al., 1989; Campus--Baypoli et al., 1999; Ratnayake et al., 2007), these findings have not been applied to the needs of archaeological research (Barton and Torrence, 2015). To date, there exist no archaeological studies that specifically analyze the impact of the nixtamalization process on microscopic starch granule morphology, or the ways in which starch affected by this process may be differentiated from that affected by other preparation or cooking methods (Henry, 2014:45). The lack of descriptive information surrounding nixtamalized maize starch morphology and the subsequent lack of comparative material means that there is currently no method for directly identifying this culturally and technologically significant process in the archaeological record, although detection of calcium deposits in cooking areas (Barba and Ortiz, 1992) and changes to carbonized seed morphology (Dezendorf, 2013) have been proposed as possible

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alternative approaches. Additionally, a dearth of photomicrographs clearly detailing the difference between nixtamalized maize starch and maize starch morphologies produced by different processing methods further complicates the issue of archaeological identification. A lack of reference collections has been identified as one of the main obstacles to more ubiquitous utilization of starch research (Barton and Torrence, 2015). This article attempts to bridge the gap between starch nixtamalization research conducted in food science and the specific descriptive needs required by archaeologists to identify ancient samples of starch.

In this article we demonstrate that maize nixtamalized following traditional Mesoamerican practices produces a distinctive radial structure formed of starch molecules dissociated from their original configuration within a naturally occurring native starch granule; this structure is termed a starch spherulite (Nordmark and Ziegler, 2002a). These starch spherulites exhibit a consistent and characteristic morphology distinct from those of native starch granules, as described in previous research conducted in the food science industry (Davies et al., 1980; Gomez et al., 1989; Nordmark and Ziegler, 2002b), and distinct from morphologies produced by other cooking and preparation techniques (e.g., Henry et al., 2009). We identify distinct morphologies of starch spherulites that are markedly different in optical properties and both elemental and morphological structure from starch granules not exposed to an alkaline solution, which is a requisite component of the nixtamalization process. Thick concretions of these nixtamalized morphologies that accumulate on the interior of cooking vessels suggests that both cooking vessel residues and archaeological sediments should be tested to identify nixtamalization in the archaeological record.

2. Nixtamalization in the Americas

The term nixtamalization is derived from the Aztec language Nahuatl, where the word for the product of this process is *nixtamalli* or *nextamalli* (Briggs, 2015). The practice was first recorded historically by Bernardino de Sahagun—a Spanish missionary priest and ethnographer—in his account *Historia General de las Cosas de Nueva España* (also known as the Florentine Codex). Another post-Columbian account of Aztec life, the Codex Mendoza, contains pictorial illustrations of Aztec daily life, including depictions of a woman teaching her daughter how to properly grind nixtamalized maize for tortillas (Berdan and Anawalt, 1997). The illustrations show tortillas cooking on a *comal* (a type of ceramic griddle), as well as a type of ceramic vessel labeled an *olla* that is believed to contain nixtamalized maize (Berdan and Anawalt, 1997). De Sahagun noted in his text that tortillas were popular in both domestic and economic settings (World Digital Library, 2016).

Regional differences do exist in the alkalizing agent used for nixtamalization and in how maize was prepared. In Mesoamerica, the widespread availability of limestone made slaked lime the predominant choice for the alkalizing agent, and nixtamalized maize was commonly ground into *masa*, the dough used to make tortillas and tamales (Barba Pingarrón 2013). Milling stone slabs discovered archaeologically and inferred to have been used to grind cooked maize have been adopted as a proxy for the onset of alkaline cooking processes. Evidence of this processing equipment dates as early as 1500–1200 BCE (Staller and Carrasco, 2009). In the American Southwest and Eastern Woodlands, however, lye strained from wood ash and other burned plant materials was most common (Beck, 2001; Briggs, 2015; Katz et al., 1974). In contrast to Mesoamerican masa-based foodways, in North America hominy corn stew and porridge dominated (Briggs, 2015; Hudson, 1976). Dent maize is preferred in all regions for nixtamalization over flint, flour, and popcorn varieties due to its high proportion of soft to hard endosperm.

Nixtamalization was practiced not only for the desired culinary changes it produced in maize kernels (loosened pericarp, improved plasticity, and distinctive taste), but for biochemical improvements as well. In maize, the bioavailability of niacin (vitamin B_3)— an essential nutrient—is low because it is bound in indigestible forms as niacinogen and niacytin (Rebor, 2001; Villines et al., 2012; Watson and Ramstad, 1987), but when cooked in an alkaline solution, the bound niacin is freed and made available to the human body for absorption (Ellwood et al., 2013; Gutierrez-Dorado et al., 2008). This transformation prevents pellagra, a potentially fatal disease caused by chronic niacin deficiency (Hegyi et al., 2004). Additionally, cooking maize in an alkaline solution improves the quality of protein available for the human body to digest by balancing the ratio of essential amino acids (Bressani et al., 1990; Katz et al., 1974; Villines et al., 2012), and promotes the absorption of calcium, providing up to 67% of daily calcium needs (Biskowski, 2017). These nutritional changes are especially important for healthy growth and development of children with maize-based diets.

3. Role of starch in archaeobotanical analysis

In the archaeological record, starch is typically preserved and recovered in residues found on cooking wares and food processing tools, as well as in dental calculus (fossilized plaque) that forms on human teeth (Henry and Piperno, 2008; Loy et al., 1992; Pearsall, 2015:341; Piperno and Holst, 1998). Starch granules can be identified to the genus and even species level by looking at a number of diagnostic elements: the size and shape of the starch granule, the position of the hilum and extinction cross, pattern of lamellae, and distinctive fissures (Henry, 2014; Henry et al., 2009; Pearsall, 2015).

Archaeological starch research has focused on understanding how specific cooking and preparation processes affect starch granules (del Pilar Babot, 2003, 2006; del Pilar Babot et al., 2014; Cortella and Pochettino, 1994; Henry et al., 2009; Messner and Schindler, 2010; Raviele, 2011). In their landmark study, Henry et al. (2009) subjected ten domesticated plant species to experimental grinding, soaking, baking, parching, and popping. Their results indicate that while specific cooking methods can be difficult to distinguish, and that in some cases multiple cooking methods produced identical products, all samples were still identifiable as starch, and the presence of damaged starch can be used to identify plant processing. Finally, they note that cooking often affects the microscopic morphology of starch granules and that "a full range of cooking experiments" is necessary to improve understanding of starch survival and morphology in the archaeological record (Henry et al., 2009:921). Experimental studies conducted at the University of Missouri have explored the effects of baking, fermentation, toasting, pounding, and cutting on starch granules. These are accompanied by clear photomicrographs that demonstrate the corresponding morphologies (Pearsall, 2015:342, 345).

Studies concentrating on differential starch survival and the pathways through which starch is deposited in the archaeological record have allowed researchers to interpret their finds more accurately (Barton, 2009; Barton and Matthews, 2006; Collins and Copeland, 2011). A recent study extends this line of inquiry to include close examination of the methods that archaeologists use to recover and prepare ancient samples for microscopic analysis (Cuthrell and Murch, 2016). By analyzing the morphologies of unaltered starch, the effects of cooking processes, and the additional ways in which starch granules may be modified following deposition, archaeologists can understand better the processes that affect the morphology of the starch they analyze.

4. Previous experimental work

4.1. Food science studies and nixtamalized starch granules

With a long history of research focusing on starch, food science has explored questions regarding starch classification and experimentation long before archaeologists became interested in similar questions (Schwartz and Whistler, 2009). In fact, much of the early understanding of starch formation, destruction, and damage that is used in archaeological research comes from these early studies (e.g., Campus-Baypoli et al., 1999; Gomez et al., 1989, 1990; Radley, 1976). Significantly,

Trejo-González et al. (1982) and Gomez et al. (1989) provide some of the earliest descriptions of the effects of nixtamalization on starch granules, noting that the process causes starch sampled from cereal grains to swell and increases the amount of agglomeration of starch as viewed on microscope slides (Gomez et al., 1989:332; Trejo-González et al., 1982:256). However, the published figures of Gomez et al. (1989) include only cross-polarized images of nixtamalized sorghum starch, and those of Trejo-González et al. (1982) include only scanning electron, but not polarized light, imagery. Similarly, Ratnayake et al. (2007) focus on the structural changes that occur in starch granules after undergoing nixtamalization while specifically focusing on isolating starch granules. Although such articles have distinct aims of varying relevance to archaeologists, they offer valuable insight into the changes that nixtamalization produces in maize and provide archaeologists interested in experimental analyses with a template for doing so. While many of these studies include additional processing steps beyond nixtamalization that may be common in the food industry (such as milling, drying, and freezing the product), such studies may be easily adapted to suit the needs of experimental projects designed with archaeological applications in mind.

4.2. First recognition and description of starch spherulites

Spherulites are three-dimensional, radially symmetrical structures formed of polymer molecules that self-assemble under a variety of experimental and real-world conditions (Sharples, 1966). As noted by Nordmark and Ziegler (2002a), such structures form readily in synthetic polymers but rarely in biopolymers. Their distinct optical qualities include a "Maltese cross" pattern of birefringence under cross-polarized light, in which the arms of the extinction cross are broader at the edges of the spherulite and form a narrow point at the center. Spherulite structures formed of calcium carbonate are produced in the guts of ruminant animals and found in their dung, thus commonly termed "dung spherulites" or "fecal spherulites" (Canti, 1997, 1998; Shahack-Gross, 2011). In addition to their overall shape and optical properties, spherulites are also characterized by variable size (and, at times, morphology) based on specific environmental conditions under which they formed (Buléon et al., 2007; Fanta et al., 2002). The radial arrangement of polymer molecules characteristically results in a rough surface to the spherulite at high magnification and a radial, crystalline appearance when examined in section under scanning electron microscopy (SEM).

Spherulites of starch have been formed under experimental conditions from a variety of plant starches: rice (Fanta et al., 2002; Kiatponglarp et al., 2015), potato (Fanta et al., 2002), yam (i.e., *Dioscorea* sp.; Hongsprabhas et al., 2014), manioc (Israkarn and Hongsprabhas, 2017; Israkarn et al., 2014), mung bean (Israkarn and Hongsprabhas, 2017; Israkarn et al., 2014), and, especially, maize (Creek et al., 2006; Davies et al., 1980; Fanta et al., 2006; Nordmark and Ziegler, 2002a, 2002b; Peterson et al., 2005). In particular, the amylose fraction of the native starch grain is responsible for the formation of spherulites: this is due to the higher melting point of amylose crystals versus those of amylopectin, and that amylopectin as a branched molecule inhibits the formation of such crystals (Lay Ma et al., 2011; Ziegler et al., 2003). Notably, most often such experiments have formed spherulites under conditions impossible in pre-modern societies, by heating to 120–180 °C in an aqueous solution, which is only possible under pressure, and today is achieved in an autoclave or similar (Davies et al., 1980; Fanta et al., 2002). In some cases, however, spherulites have also been formed at below boiling temperatures through the addition of Ca^{2+} ions in an alkaline environment (e.g., via $CaCl_2$ and NaOH; Israkarn and Hongsprabhas, 2017). It is thought that these two methods (high heat and rapid cooling, and Ca^{2+} presence within an alkaline environment) produce similar effects in dissociating amylose molecules from their original structure (gelatinizing the starch) and permitting recrystallization (often termed "retrogradation") upon cooling (Bryant and Hamaker, 1997; Fanta et al., 2002; Galliard and Bowler, 1987; Gomez et al., 1990; Mondragón et al., 2004; Nordmark and Ziegler, 2002a; Oosten, 1982, Oosten, 1990). Although spherulites have been produced from yam starch under alkaline conditions (Hongsprabhas et al., 2014), it has not yet been determined whether similar processes can produce spherulites in maize in the presence of an alkaline, calcium-rich solution without heating >100 °C, as in the studies described above.

Some additional similarities, however, have been noted between superheating and alkaline experiments with maize starch. Both heating (Fanta et al., 2002) and nixtamalization (Mariscal-Moreno et al. 2017; Mondragón et al., 2004) produce a rearrangement of amylose into a V-type (helical) crystalline structure, a conformation not present in native starch, which is comprised entirely of A- and B-type crystals. There is also evidence that nixtamalization results in calcium joining the amylose complex (e.g., Trejo-González et al., 1982). Bryant and Hamaker (1997) suggest this is due to the ionization of hydroxyl groups within the amylose, due to the high pH, creating binding sites for calcium ions that cross-link molecules, which has been observed with nixtamalized yam starch (Hongsprabhas et al., 2014). Varying concentrations of lime, as well as boiling time, steeping time, and kernel damage affect calcium concentrations within maize starch (Fernández-Muñoz et al., 2002; Lobato-Calleros et al., 2015; Trejo-González et al., 1982:255). The calcium-starch complex inhibits starches from complexing with iodine (Mondragón et al., 2004:417), rendering nixtamalized maize starch unaffected by the typical color change introduced by iodine-based starch tests, such as Lugol's solution (Barton, 2007; Flint, 1994). As the interaction of starch molecules with other dyes, such as Congo Red and Trypan Blue, is contingent on hydrogen bonds and water absorption (which is why these are useful tests for damaged or gelatinized starches; Barton, 2007; Flint, 1994; Lamb and Loy, 2005; Loy, 1994), we anticipate that calcium-amylose complexes should exclude these stains as well.

5. Materials and methods

5.1. Samples and equipment

Maize used in this study was an organic, South Carolina-grown heirloom hominy corn free from genetic modification, obtained from a distributor that provides retail and wholesale heirloom grains (Anson Mills, 2016). This particular variety is a dent landrace of *Zea mays*. Following traditional methods (Briggs, 2015), the kernels were left to dry on the cob in the field prior to harvesting (Anson Mills, 2016). Powdered calcium hydroxide, $Ca(OH)_2$, also known as slaked or pickling lime, was used as the alkalizing agent. To approximate the ceramic cookware and slow cooking times typical of nixtamal preparation (Beck, 2001; Briggs, 2015), a commercial electric slow cooker with a glazed earthenware cooking insert (Crock-Pot brand) was used to provide slow heating for all cooked samples. An advantage of the glazed surface is that the insert could be completely cleaned, scrubbed with soap, rinsed, and then scrubbed with dilute acetic acid, and rinsed again, to prevent cross-contamination between trials.

5.2. Experimental design

The nixtamalization process employed in this study was adapted from a recipe on Anson Mills' website (Anson Mills, 2016), as well as numerous ethnographic accounts (Beck, 2001; Harrington, 1908; Katz et al., 1974; Waugh, 1916). To create the alkaline solution, 45 g of slaked lime was dissolved in 2 L of boiling water. This solution was left to dissolve for 5.5 h, at which point it was decanted into the ceramic insert of the slow cooker, using caution not to introduce remaining lime solids into the decanted solution. Dried maize (120 g) was then added to this solution and left to sit for 14 h. This pre-cooking soak begins to soften the kernel and allows for a shorter cooking time, following traditional methods used in Mesoamerica and the Eastern Woodlands, and

identified in ethnographic records as occurring throughout the 17th and 18th centuries (Briggs, 2015). Following this soaking period, the slow cooker was turned to the "low" heat setting and the maize was left to cook for 7 h. This setting maintains a temperature of approximately 85 °C for the duration of the cooking process, based on periodic direct measurement of the vessel contents. This experiment is termed Trial 1 (Table 1).

As a control, the above process was replicated without the addition of slaked lime to either the soaking or cooking liquid (Trial 2). Subsequently, heat, lime concentration, and time exposed to the alkaline solution were manipulated to determine their impacts on morphology and free-floating starch production. To assess the degree to which the concentration of slaked lime affected starch granules, a trial with half the amount of lime (22.5 g) was run (Trial 3). To address the degree to which the introduction of lime affects starch granules absent of heat, two trials (one with lime introduced [Trial 4] and one without [Trial 5]) were left to soak for a total of 144 h without heating. Finally, three boiling trials (Trials 6–8) prepared in a stainless steel pot heated on a stove were conducted in order to evaluate the effect of both increased heat and lime treatment on starch granule morphology. Boiling samples were maintained at 100 °C for the duration of the trials, and varied only in the presence/absence of lime and the presence/absence of a pre-soak period. For boiling trials, 500 mL of water was added every hour to maintain an adequate level of solution in the pot. While the evaporation and addition of water throughout this process is likely to have had an effect on the lime concentration, the resulting maize kernels were indistinguishable from nixtamalizing or control trials (Trials 1 and 2, respectively), indicating that the difference was not significant. A summary of all experimental processes is given in Table 1.

For all comparative processes, the same amount of maize and water was used as in the standard nixtamalization process (Trial 1). All trials for which there was a pre-soaking period were pre-soaked for 14 h. For those comparative processes with lime introduced (excluding the trial with a reduced concentration, Trial 3), the same amount of lime was used as in the standard nixtamalization process. This allowed direct comparison of morphological changes and free-floating starch production among trials.

5.3. Sampling

For the nixtamalization and control trials (Trials 1–3), samples were taken at 0 (i.e., immediately following the pre-soak), 3, 5, and 7 h into the cooking time. Samples of maize, solution liquid, and the crust that formed on the surface of the solution were taken at each of these intervals. Soaking trials (Trials 4 and 5) were sampled at 24-h intervals, with the maize, liquid solution, and crust formation sampled at these intervals. For the boiling trials (Trials 6–8), maize samples were taken every 20 min, with samples of the solution taken at hourly intervals. Boiling samples were cooked for a total of 3 h.

Table 1
Summary of trial processes and sampling intervals.

Process	Trial Description(s) and Number	Sampling Interval
Nixtamalization	(1) Pre-soak, 100% lime concentration (conc.)	0, 3, 5, 7 h
Control	(2) Pre-soak, no lime	0, 3, 5, 7, hrs
Concentration	(3) Pre-soak, 50% lime conc.	0, 3, 5, 7, hrs
Soaking	(4) 100% lime conc. (5) no lime	Every 24 h, up to 144 h
Boiling	(6) Pre-soak, 100% lime conc. (7) Pre-soak, no lime (8) No pre-soak, no lime	Kernels every 20 min, solution and surface crust every hour, up to 3 h

5.4. Imaging: polarized light microscopy

The methodology for processing samples followed guidelines laid out by Pearsall (2015:368–70), briefly outlined here. Slides were prepared with a small amount of 1:1 glycerin-water solution; maize kernels were cut diagonally and starch from the kernel was scraped with a clean razor blade onto the prepared slide. For liquid samples, 30 μl of cooled solution was added to prepared slides. Coverslips were applied and their edges sealed with clear nail polish. Slides were analyzed with a Leica DM750P polarizing light microscope using 100x and 400x magnification. Photomicrographs were taken using Leica Acquire V4.9 software with a Leica DFC 290HD camera. Following Perry (2002), average measurements of starch particles were taken by measuring 10 granules per field of view, with five sets of coordinates per slide, yielding 50 length and width measurements per slide.

5.5. Imaging: scanning electron microscopy and SEM-EDS

Scanning electron microscopy was conducted at the Harvard University Center for Nanoscale Systems. Samples of starch solutions from the trials conducted were air dried in microcentrifuge tubes; the dry residue was scattered onto a standard stainless steel SEM stub covered with double-adhesive carbon tape. The stubs were coated in 80:20 platinum:palladium to a depth of approximately 10 nm using an EMS 150T S Sputter Coater. The coated stubs were imaged in a JEOL JSM-7900F Field Emission SEM, set to high vacuum mode, and imaging used a beam voltage of 5 kV and the lower electron detector. Elemental detection was achieved with an Oxford Instruments Ultim Max 100 sensor and the Aztec software package.

5.6. Staining tests

Following Long and Loy's (2005) protocol, a Congo Red 1mg/1 ml concentration solution was prepared in order to assess the degree of damage to experimentally treated starch granules. Congo Red does not stain unprocessed grains, unheated but physically damaged grains are stained a light red, and gelatinized grains are stained a bright red with an orange-red or green-gold glow. Here, starch solution was applied to microscope slides and allowed to dry, and coverslips affixed. Forty milliliters of the dye was placed on the edge of the coverslip and drawn across the surface of the slide through capillary action. After 15 min, 20 mL of 1M NaCl solution was applied to the edge of the coverslip and the saline solution drawn across the slide to clear the unbound dye.

Similar to Congo Red, Trypan Blue highlights damaged starch granules by staining the outer layers of processed starch blue. Undamaged starch granules will not accept the stain. Using a 0.4% solution, 20 mL of the stain can be applied to the edge of a coverslip and drawn across by capillary action, or applied directly to the slide and allowed to sit for 2 min before affixing a coverslip (Barton, 2007; Loy et al., 1992).

Although Lugol's is the most ubiquitous dye used to test for the presence of starch, standard protocols are difficult to find. Both peer-reviewed literature and public-facing articles are vague as to the concentration of solution used and do not include a suggested ratio of starch solution to Lugol's. In this study, 2 mL of starch solution was measured into a vial and 10 drops of Lugol's iodine were added. We used a 5% solution as suggested by Hostettler et al. (2016). The solution was then applied directly to a slide and sealed with a coverslip.

6. Results

6.1. Control sample morphologies

Control samples, those without the introduction of lime (Trials 2, 5, 7, and 8) exhibited morphologies consistent with those described in the literature for unprocessed/uncooked and cooked maize (Henry et al., 2009; Gomez et al., 1989). The starch displayed unaltered morphologies

and, when subjected to heating, those consistent with boiling and gelatinization. Morphologies associated with cooking damage include swelling and a loss of distinction of the outer borders of the starch granules as cooking times progress, as well as a loss of birefringence under cross-polarized light (Fig. 1). Starch from all control samples displayed the typical light blue color seen when viewed under cross-polarized light. In none of the control/non-alkaline samples were any nixtamalized morphologies (described below) observed.

6.2. Nixtamalized starch morphologies

6.2.1. Appearance under brightfield and polarized light microscopy

All samples of starch that had prolonged contact with an alkaline solution (Trials 1, 3, 4, and 6) contained structures that are far rounder, more symmetrical (spherical), and with smoother edges than typical polygonal maize starch granules, and when viewed under cross-polarized light have distinct Maltese extinction crosses, typified by arms that are narrow at the center and widen as they extend outward (Fig. 2B and C). With starch exposed to alkaline solution, the centers of the observed structures maintained a sharp Maltese cross throughout progressively longer cooking times, in direct contrast to the swelling and lack of contrast noted in samples that did not contain lime (Fig. 1B and D). The morphologies observed in starch exposed to lime were rounder than their native starch granules and starch cooked under nixtamalization conditions (i.e., at temperatures below boiling and in an alkaline solution, Trials 1 and 3) did not lose birefringence upon heating. This is in contrast with starch heated in the control trials with heat (Trials 2 and 8; Fig. 1B and D), where a loss of distinction in shape and morphology of both the extinction cross and the granule itself are characteristic as gelatinization occurs.

One of the most striking characteristics of the particles observed in nixtamalized starch samples (Trials 1, 3, and 6) is their rainbow morphology, where the particle displays bright multi-colored patterning. We observed this colorful birefringence under cross-polarized light without a tint plate. The most typical patterning observed is bright orange/yellow at the center of the starch granule, ringed by a bright blue or purple (Fig. 2A). Other variations in the rainbow morphology are observed, with some granules displaying bands of orange within the green-blue center, while others display centers of light blue and green with a thin ring of purple on the margin (Fig. 2B and C, respectively). This morphology was observed in all samples exposed to lime and was observed in combination with the additional nixtamalized morphologies described below.

The nixtamalized starch particles (Trials 1 and 3) also displayed features that are similar in appearance to fissures (Fig. 2G), often appearing at the boundaries of aggregated particles (Fig. 2J, K, and 2L). These demarcations can have sharp or uneven margins, sometimes appearing as an extra "arm" of the extinction cross (Fig. 2H, I, and 2J).

One notable difference between native maize starch granules and the structures produced under nixtamalization conditions is particle size. Maize starch used in these trials contained granules typically 15–20 μm in diameter, although some as small as 10 μm and as large as 22 μm were observed. In contrast, the structures produced by nixtamalization varied substantially in size, with some as small as 3.5 μm in diameter and others as large as 24 μm, although it is difficult to assess in some cases what should be counted as a single particle versus a connected chain of

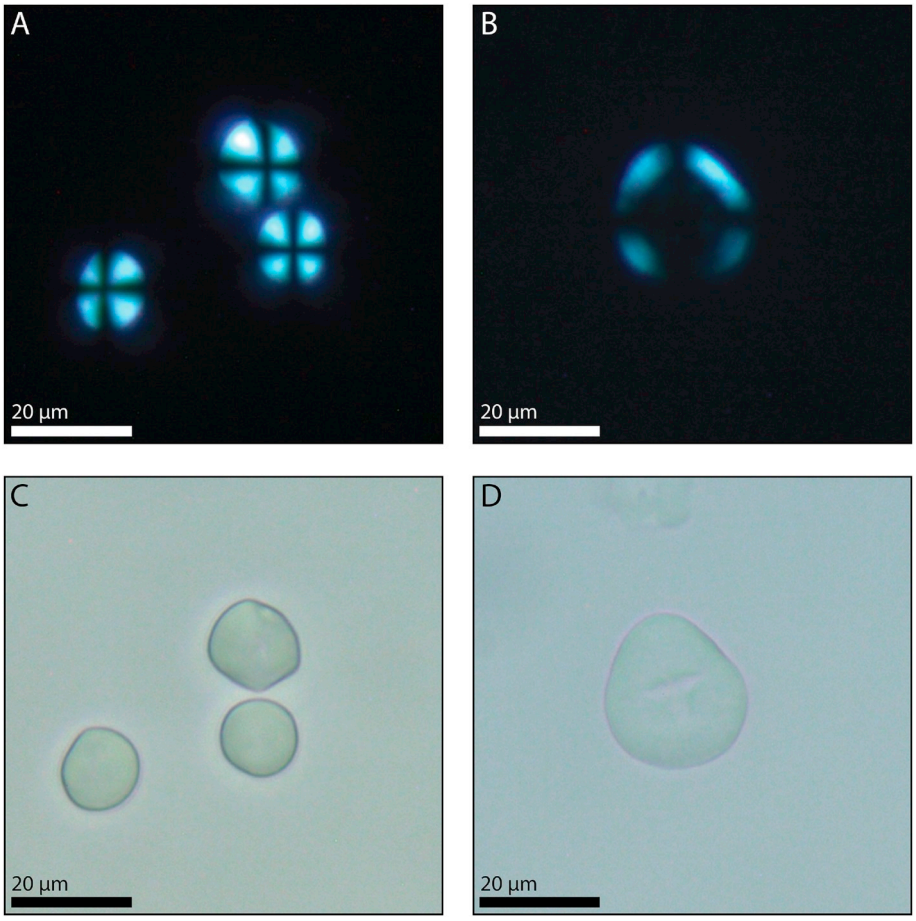

Fig. 1. Images A and C (Trial 2, 0 h) display unaltered maize starch morphologies in cross-polarized and brightfield light, respectively. Images B (cross-polarized) and D (brightfield) show a boiled starch granule displaying a typical "boiled" morphology including swelling, a loss of birefringence, and expansion of the hilum (Trial 8, 100 min).

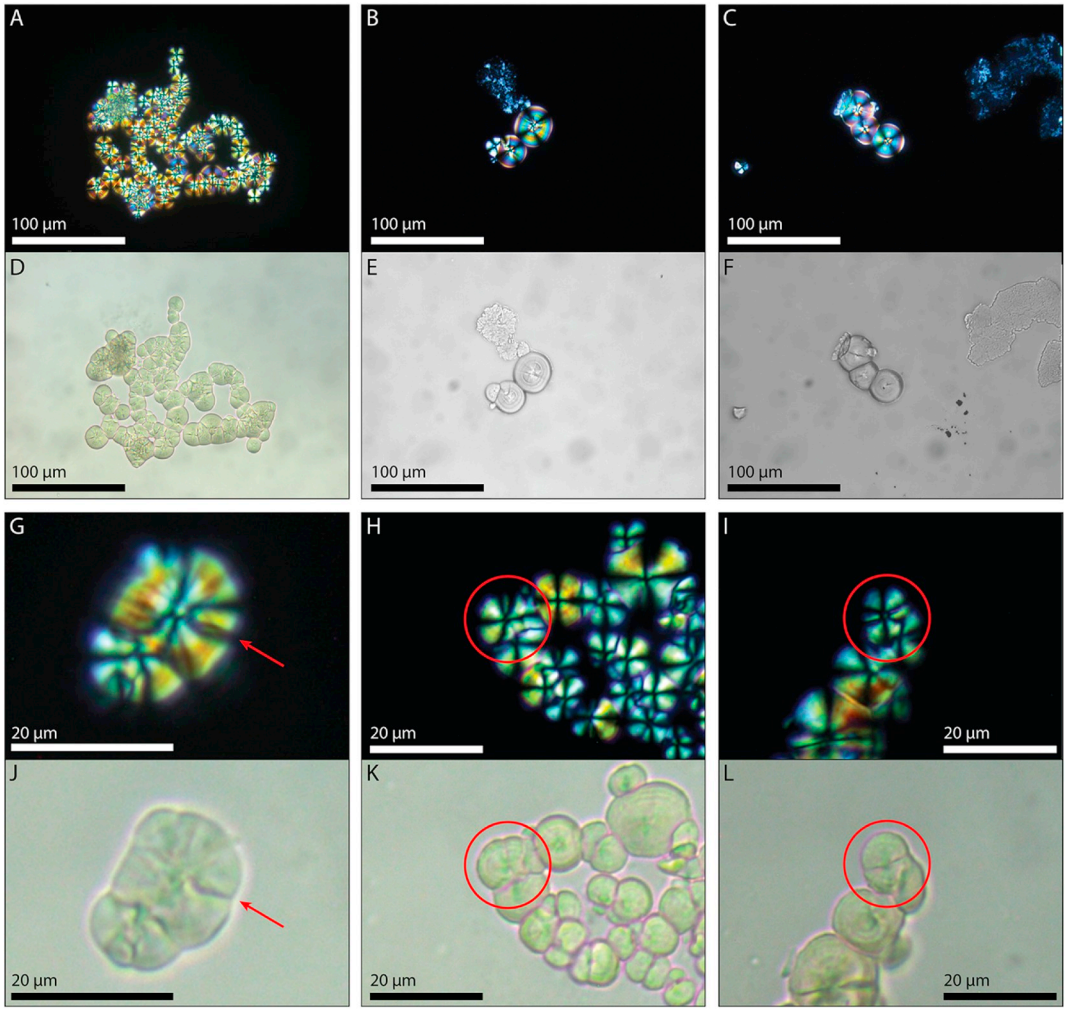

Fig. 2. Starch from nixtamalized samples displaying the rainbow nixtamalized morphology observed under cross-polarized light (A-C, G-I) and the same images in brightfield illumination (D-F, J-L). Images J and K show radial "fissures" as observed in brightfield illumination, which appear as additional "arms" of the extinction cross (though they are not a birefringence feature) in G and H. Image L shows a fissure that transects the entire particle, and image I the apparent refringence line corresponding to that feature. All images were sampled from Trial 1, surface lime/starch crust, 7 h into cooking.

particles. There does not appear to be a correlation between cooking time or lime exposure time and particle size, although we did not systematically quantify this parameter; however, particles of all sizes were observed in all samples, as demonstrated in Fig. 2.

In samples that were not exposed to heat but were soaked in an alkaline solution (Trial 4), free-floating starch exhibited nixtamalized morphologies identical to those noted in heated samples, including rainbow refringence (Fig. 3A). Here a time-dependent change in morphology was noted: the particles in the 120-h soak were rougher (Fig. 3D) and more likely to be fractured (Fig. 3B) than those removed at 48 h (Fig. 3A, C). Notably, however, some well-defined, round, smooth particles are also visible in the 120-h sample (Fig. 3D highlight). Despite the apparent degradation and severe fractioning of most observed starch structures, rainbow-type birefringence, including some Maltese extinction crosses, remained despite extreme distortion of the structures (Fig. 3B).

In both heated and non-heated experimental trials, however, maize kernels that were sampled directly (i.e., by sectioning and scraping with a razor blade) produced "normal" starch granules, indicating that the alkaline solution only modified the morphology of starch in direct contact with solution. The alkaline solution does appear, as discussed above, to have promoted the release of free-floating starch from soaking kernels, although we did not quantify this difference between experimental and control trials. Additionally, fully gelatinized starch was observed in all heating trials, both experimental and control, especially when the surface of heated maize kernels was sampled directly.

6.2.2. *Dye reactions*

The starch particles from all alkaline exposed samples did not accept common starch dyes, including Lugol's, Congo Red, and Trypan Blue. When Lugol's was added to vials of cooking solution from which starch particles were sampled, the solution turned the deep purple/black indicating the presence of starch; however, immediate sampling showed under brightfield light that the nixtamalized starch particles present all rejected the dye, and amorphous, gelatinized starch alone was stained (Fig. 4). Similar reactions were observed for Congo Red and Trypan Blue.

6.2.3. *SEM imaging*

SEM images of the experimentally produced nixtamalized starch particles give insight into the composition and formation of the objects, while also allowing us to assess the hypothesis that these structures may be composed of calcium carbonate, as are dung spherulites (Canti, 1997).

Upon examination at low magnifications under SEM, the nixtamalized starch structures appear much as they do under brightfield illumination (Fig. 5A). The structures are clustered in aggregate, often grouped in sets of multiples touching each other, with individual

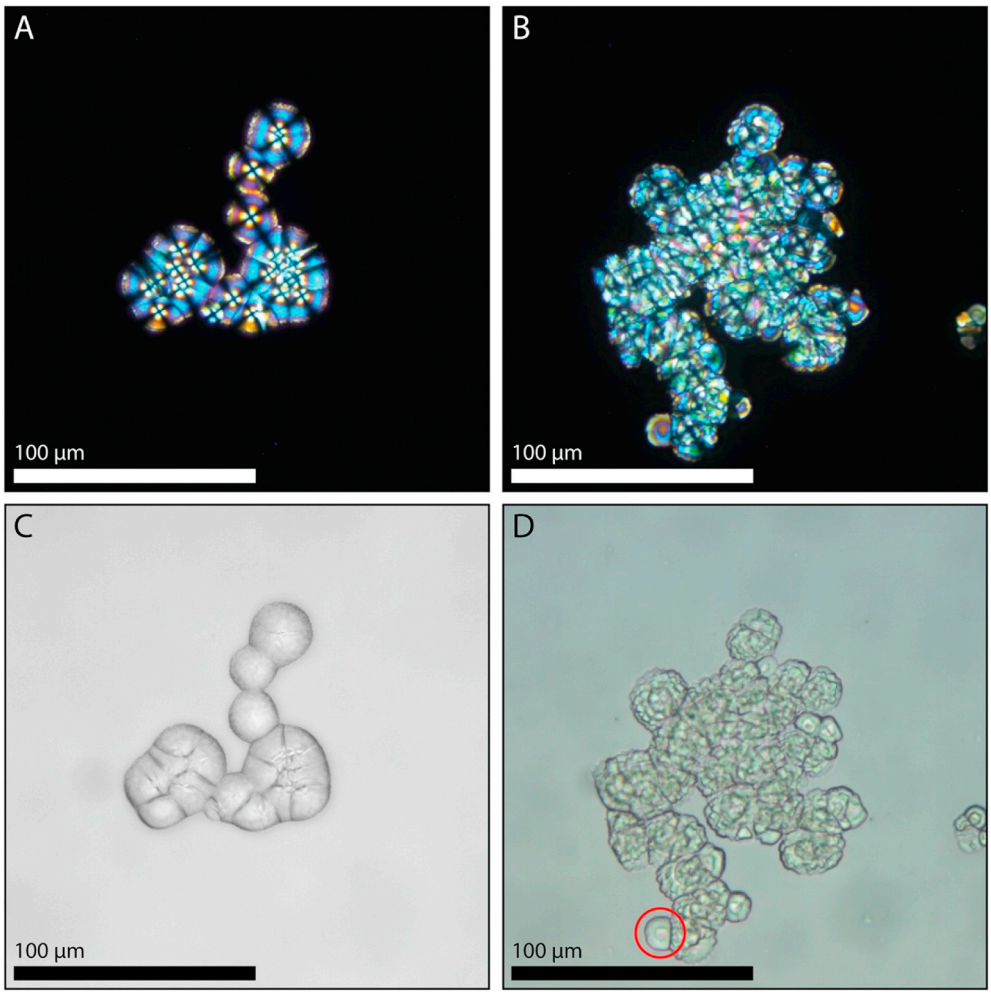

Fig. 3. Maize starch structures skimmed from the surface of an alkaline solution after 48 h (A, C) and 120 h (B, D) of soaking with no heat (Trial 4). Highlighted particle is smoother and appears "newer" than other particles visible in D.

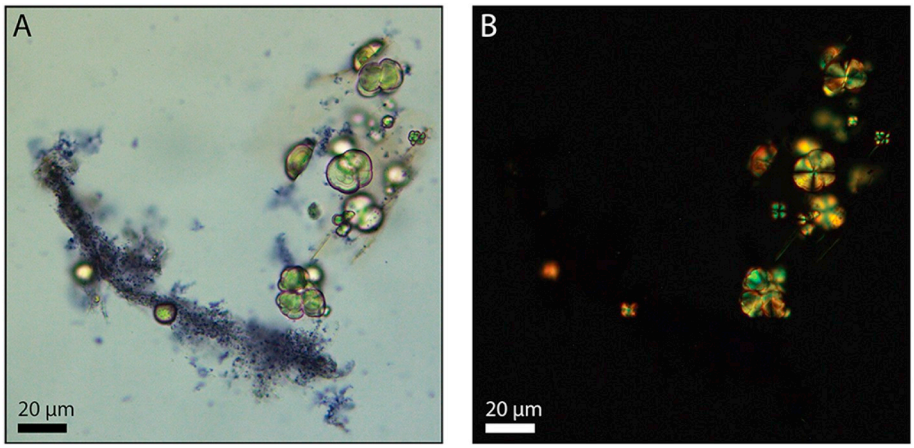

Fig. 4. A nixtamalized starch sample (Trial 3, 7 h) exposed to Lugol's solution. Under brightfield illumination, gelatinized starch stains blue-purple, while all spherical structures repel the stain (A). In cross-polarized light, these structures retain their birefringence properties, though the color is shifted due to the presence of the yellow iodine solution surrounding them (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

boundaries more difficult to distinguish in polarized light (e.g., Fig. 2D, K) than in SEM images (e.g., Fig. 5A). The starch particles, when viewed under SEM, appear to grow directly off of one another, with a range of sizes perhaps indicating different stages in their formation and growth.

At greater magnification, it can be seen that these structures are microcrystalline spheres made up of crystalline structures radiating from a central point (Fig. 5D); this radial structure is especially visible when particles are broken in cross section (Fig. 5B). Images focusing on the surface of the structures highlight their irregular surface; this is due to the ends of the crystalline needles that make up the particle (Fig. 5C).

6.2.4. *SEM-EDS: elemental composition*

The elemental composition of these nixtamalized starch structures was evaluated using SEM-EDS; the spectrum of a transect across the

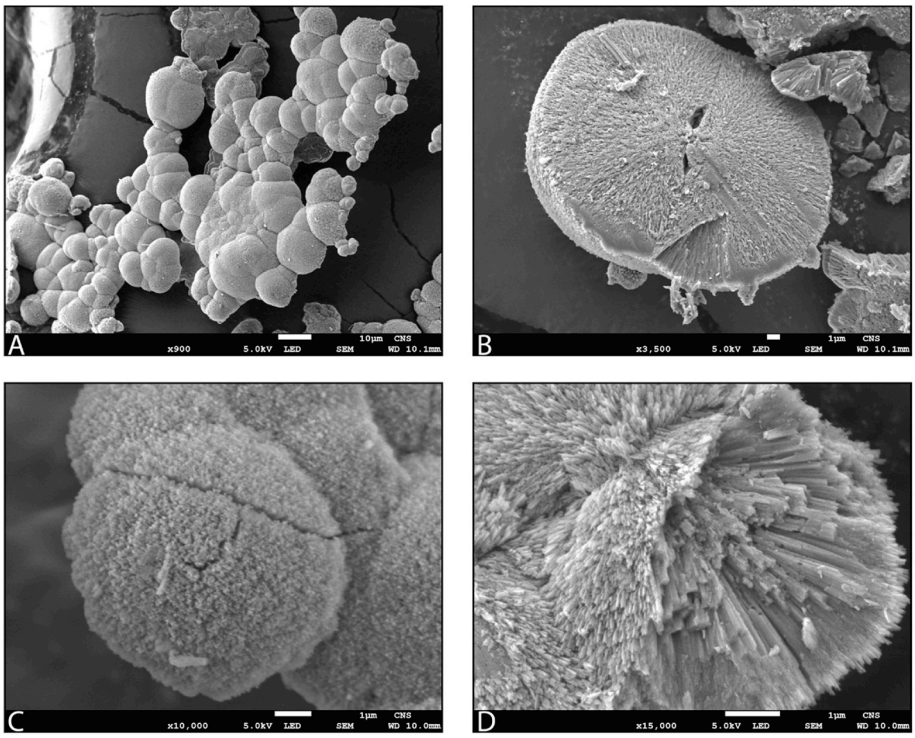

Fig. 5. Nixtamalized starch particles observed under scanning electron microscopy. Entire structures (A), and their surface texture (C), are revealed as radial bundles of linear crystalline structures at higher magnifications (D) and when particles are broken open (B).

interior surface of the particle shown in Fig. 5B is shown (Fig. 6). Elements detected include platinum and palladium (from coating), and carbon, oxygen, and calcium, which appear to be intrinsic to the particle. Notably, hydrogen, which we hypothesize to be present in a starch-based particle, cannot be detected by this EDS instrument. In the spectrum presented in Fig. 6B, the left-to-right decline in oxygen (and, to a lesser extent, carbon) and the notable "dip" in O and C at 25.8 μm are due to angular effects of the X-ray beam on the slanted and imperfectly smooth sample. These results indicate that the sample is homogenous in elemental composition across the diameter of the structure.

Elemental weight ratios were calculated using single-point semi-quantitative EDS across a grid of eight points spanning the sectioned particle pictured in Fig. 6A. The averaged relative weights of carbon (19.3%), oxygen (49.1%), and calcium (31.6%) yield an approximate molar ratio of 1Ca:2C:4O. The formula for starches (amylose and amylopectin) is $[C_6H_{10}O_5]_n$, so if this particle were entirely starch, we would not expect Ca to be present and we would expect a 6:5 M ratio of C:O. The formula for lime is $Ca(OH)_2$, which would produce a Ca:O molar ratio of 1:2; carbon would not be present. Finally, a third option is that this particle could be a "dung" spherulite of calcium carbonate ($CaCO_3$). In that case the molar ratio of Ca:C:O would be 1:1:3. The observed molar ratio of 1:2:4 Ca:C:O indicates that this particle is neither pure starch nor pure calcium carbonate, but contains a hybrid chemical structure. As it has long been demonstrated that calcium is absorbed by maize starch under nixtamalization conditions (Trejo--González et al., 1982), this is not a surprising finding.

6.3. Starch spherulite production

Maize kernels introduced to both heat and lime produced far more free-floating starch than kernels under other conditions. The presence of lime in the solution caused a lime "skin" to form on the surface that appears to trap free-floating starch. The development of this skin and its collection of free-floating starch caused a thick residue to form on the side of the cooking vessel (Fig. 7). Sampling of this crust in nixtamalization trials (1 and 3) produced abundant starch spherulites with the characteristic morphologies noted above. Pflugfelder et al. (1988) note that the solution resulting from nixtamal production—nejayote (occasionally "nexayote")—contains abundant suspended solids, 20% of which is composed of starch.

7. Discussion

The results presented here illustrate that cooking maize in an alkaline solution produces particles that are morphologically distinct from those produced without an alkaline solution, that these particles are formed of a starch-calcium complex, and that they share morphological and optical characteristics with starch spherulites produced experimentally and described in the food science literature. These starch spherulite structures are not present in starch sampled from the center of nixtamalized maize kernels, where the lime solution had not fully saturated the seed; the distinct spherulite structures were only seen in solution, in the crust formed on the surface of the cooking liquid, or when starch was sampled from the outermost, saturated portion of the maize kernel. However, actions such as stirring and ladling, which were not performed in these experimental analyses, are likely to have been performed in the actual cooking of nixtamal. These actions would have agitated the maize, breaking open kernels and exposing a greater number of starch granules to the alkaline solution. Additionally, processing the cooked maize via grinding would ensure that starch granules that were not modified by the alkaline solution would be incorporated into residues with abundant starch spherulites. Therefore, our results indicate that starch spherulite residues are the product of several steps in nixtamalization, including extended soaking of maize kernels in alkaline solution, heating of the same, and processing of nixtamalized kernels for masa. Starch spherulites are thus expected to be found in cooking vessels, where they may form thick concretions, on grinding stones used to produce masa, and even in select soil deposits where nejayote, the cooking solution that is a caustic byproduct of nixtamalization, was discarded.

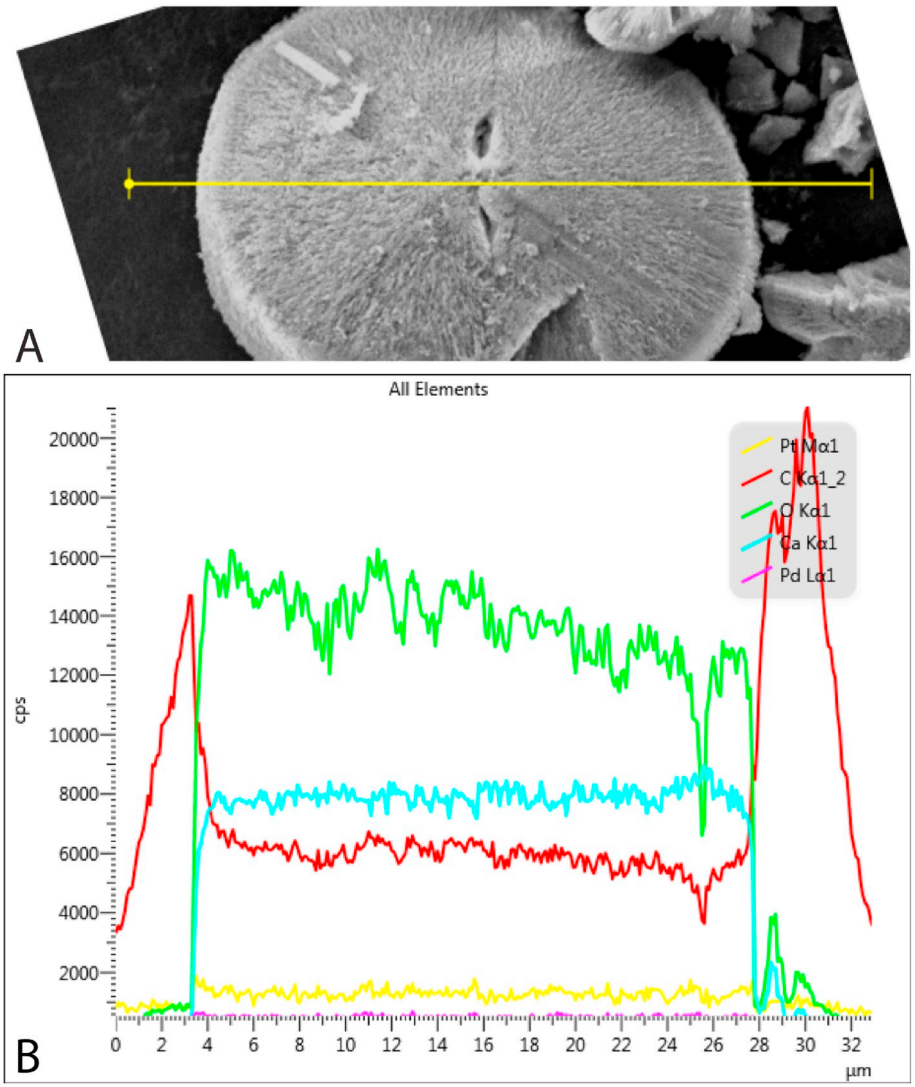

Fig. 6. SEM-EDS image (A) and spectrum (B) of nixtamalized starch particle shown in Fig. 5B. Pt and Pd are present due to sample coating; carbon readings below 4 µm and beyond 28 µm are measurements of the carbon tape on the stub. Hydrogen cannot be detected by this instrument.

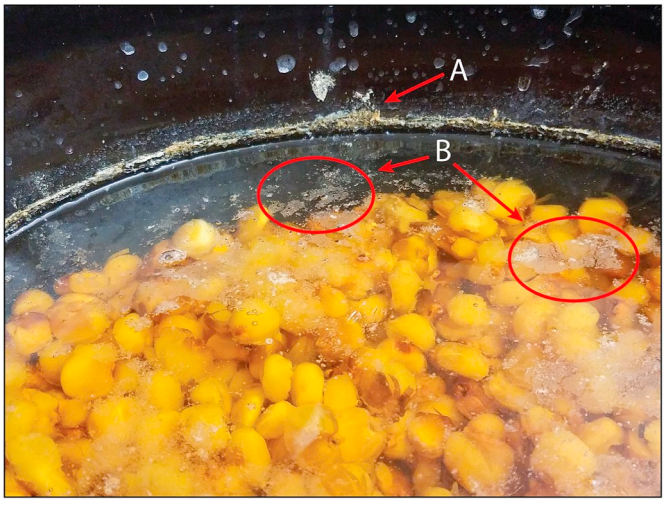

Fig. 7. Image of cooked sample of nixtamalized maize. Note the starch-crust formation on the side of the vessel (A) and the lime and starch skin on the surface of the solution (B). This lime-starch skin was sampled in all trials, including controls.

7.1. The argument for identification of nixtamalized starch particles as starch spherulites

The morphological changes we observed in starch granules that have been nixtamalized suggest that the resulting starch is not simply damaged, but that it has undergone a transformation into a starch spherulite. The size, shape, and smooth exterior of the particles produced in this study closely mirror those of starch spherulites produced from maize starch in other experiments (e.g., Davies et al., 1980; Fanta et al., 2002:163; Nordmark and Ziegler, 2002a:441). The variety of sizes among observed particles in a given sample may be a facet of their duration of growth, with larger spherulites having begun growth earlier than their smaller neighbors. This hypothesis may be supported by the soaking experiment (Trial 4) and the rougher, more fragmented state of spherulites in samples left for longer periods (Fig. 3D). The "fissures" seen in particles produced in this study represent growth boundaries of spherulites, both edges of adjacent spherulite structures growing against one another (Fig. 2K highlight) and internal "seams" that form equatorially from the poles of primary growth among spherulites (Fig. 2L highlight; Canti and Nicosia, 2018:33).

The multi-color birefringence observed here is similar to that seen with other spherulites under polarized light (e.g., Yun et al., 2006a; 2006b). Yun et al. (2006b) note that the formation of crystalline lamellar

bundles within the spherulite is correlated with the formation of banded spherulites (e.g., Fig. 2B and C), and that the interaction of polarized light with these structures is responsible for the spherulite's colorful birefringence. Such a phenomenon is an expected consequence of a reorganization of the crystalline structure, for example from the A-type to the V-type helical conformation (Fanta et al., 2002; Gomez et al., 1992; Mondragon et al., 2004). Gránásy et al. (2005:2) note that this type of formation can leave the spherulite with two "eyes" on either side of the central growth point; elsewhere, this formation is described as an infilling dumbbell shape or a "wheatsheaf" (Canti and Nicosia, 2018:33). Published illustrations indicate a structure identical to that observed in the cross-sectioned spherulite imaged using SEM, in which two holes bracketing the center remain (Fig. 5B). Finally, the refusal of all starch dyes by these particles is consistent with the behavior of a crystalline spherulite, rather than (damaged) native starch.

Even under conditions of extreme temperature, pressure, and gelatinization, starch granules retain smooth margins at high magnification and lack the crystalline growth patterns typical of all spherulites, including the particles produced in this study (Kaur et al., 2004; Zhang et al., 2007). The experimental starch spherulites are also distinct from dung or fecal spherulites viewed under SEM. Dung spherulites, while overall spherical in shape and with similar optical properties to starch spherulites, have rough and uneven surfaces under SEM (Canti, 1997; Canti and Nicosia, 2018), in contrast to the even surface texture and shape seen in Fig. 5A and C. Elemental analysis through EDS also confirms that the starch spherulites produced here are comprised solely of neither starch nor calcium carbonate, but suggest instead a biopolymer that incorporates Ca into its structure. With optical microscopy only (without SEM and EDS), we do not believe it is possible to distinguish definitively between dung and starch spherulites: they share a similar range of sizes (~5–25 μm; Canti, 1997:222; Dunseth and Shahack-Gross, 2018:118) and morphology at low magnifications. Fortunately, the societies known to have practiced nixtamalization in North America and Mesoamerica lacked ruminant domesticates prior to the Spanish introduction of bovines, reducing the possibility of confusing the two in pre-Columbian contexts. SEM would be necessary to distinguish the two in post-contact contexts.

7.2. A proposed mechanism for the formation of starch spherulites

It is known that an extremely basic (~10.5 pH) lime solution will break down starch granules into their composite polymers, amylose and amylopectin, gelatinizing the starch (Israkarn et al., 2014; Israkarn and Hongsprabhas, 2017). These gelatinized starches then have the potential to restructure themselves as semicrystalline starch spherulite structures under the right conditions (Davies et al., 1980:149–150). Hongsprabhas et al. (2014) note that for mungbean (*Vigna radiata*) and cassava (*Manihot esculenta*), an alkaline solution promoted the leaching of amylose and amylopectin into solution upon disintegration of the starch granule and facilitated formation of starch spherulites at temperatures of 75 °C, much lower than those required at neutral pH (Davies et al., 1980:157; Nordmark and Ziegler, 2002a:440) and consistent with those reached in our study and in traditional nixtamalization cooking practices. It is clear that with whole maize kernels heat alone is not enough to cause the restructuring of the starch granule into a spherulite, as no evidence of starch spherulites was observed in control trials (Trials 2, 5, 7, 8); similarly, the production of starch spherulites has not been described in the food science literature without temperatures unachievable using traditional cooking techniques.

Heat is, however, not required to form the spherulites—the prolonged presence of the lime solution alone caused the dissociation of starch granules and the formation of spherulites (Trial 4). Therefore, it appears that prolonged contact with the alkaline solution (>24 h) is sufficient to gelatinize starch granules enough to allow amylose to recrystallize into a new spherulitic form in the presence of a divalent cation, such as calcium. The literature indicates that divalent cations (Ca, although Mg also works; Trejo-González et al., 1982:254–256) are a necessary component that allows cross-links among starch molecules to form, enabling crystalline restructuring (into V-type conformations; Mariscal-Moreno et al. 2017; Mondragon et al., 2004) and spherulite formation (Bryant and Hamaker, 1997; Hongsprabhas et al., 2014).

7.3. Archaeological implications of starch spherulites as a product of nixtamalization

By conducting experimental cooking trials to mimic the ancient process of nixtamalization, we approximate the morphologies that would have been deposited in the archaeological record by residue buildup on cooking vessels, on processing implements such as grinding stones and griddles, and through discard of cooking liquid (nejayote). Given the durability of dung spherulites in archaeological sediments (Shahack-Gross, 2011) and the presence of starch granules in such contexts (Henry, 2014; Zarrillo et al., 2008), we expect that starch spherulites should be preserved in sediments/residues and can be recovered using standard microbotanical recovery techniques (e.g., Pearsall, 2015; Smith et al., 2019). Thus, one location to sample for evidence of nixtamalization would be grinding stones thought to be used for the production of masa from nixtamal; starch spherulites ought to accompany maize kernels through this process and be recovered from grinding stones. A second context to sample would be areas where nejayote might have been routinely discarded.

The cooking process causes a thick crust of free-floating maize starch to form on top of the solution, producing an accumulation of starch and lime on the inner circumference of the cooking vessel. Should porous ceramic vessels be used repeatedly for alkaline soaking or cooking, these accumulations would build up further, and likely persist despite potential cleaning. This observation suggests that residue analyses of cooking vessels focused on starch recovery have the potential to reveal evidence of maize treated with an alkaline solution. The depictions of specific cooking vessels, such as the *olla* in the Codex Mendoza, used to soak and cook nixtamalized maize may provide a guide for the specific types of vessels most likely to have been used for this process. Starch spherulites adsorbed onto the inner surfaces of semi-porous ceramic cooking vessels should further increase the chance that these particles would be preserved, as is the case for starch granules (Henry, 2014; Zarrillo et al., 2008). Thus, a third location to sample for evidence of nixtamalization is the upper portion of vessels thought to be used in the nixtamalization process, especially those with evidence of any visible lime accretion.

In the Americas, nixtamalization is a common cooking process that extends across time and space. The current lack of comparative studies of nixtamalized starch granules represents a significant gap in paleoethnobotanical investigations of ancient maize processing across this vast region. The specific nixtamalization process implemented in this study is one of several methods of producing nixtamal (Beck, 2001; Briggs, 2015), and some experimental evidence indicates that Mesoamerican practices, based on $Ca(OH)_2$, would be more likely to produce spherulites than traditional Eastern Woodlands practices in which wood ash leachate (lye: NaOH or KOH) was used as the alkalizing agent, due to the presence of a divalent cation (Trejo-González et al., 1982). However, further experimental work with lye solutions is necessary to confirm this theory. It is not well understood if other grains in Mesoamerica were also cooked in alkaline solutions. Gomez et al. (1989) indicate that sorghum is used in some Central American countries in modernity as an alternative to maize, but sorghum was not introduced into the Americas until well after Spanish contact. There is no evidence that other sources of starch, such as beans, were cooked under nixtamalizing conditions in the past. In other areas of the world, plant sources of ash have been added to rice, beans, and sorghum to produce effects similar to nixtamalization and to enhance flavor (e.g., Che-lin et al., 1942). The lack of paleoethnobotanical analysis focused on these foodways highlights the need for detailed analyses of how these cooking methods might be

represented in the archaeological record.

8. Conclusions

By utilizing experimental cooking techniques, this study addresses the ways in which the culturally significant process of nixtamalization affects the morphology and transformation of native starch granules into starch spherulites. Control samples in which maize was cooked without lime failed to produce spherulite morphologies, while all samples of maize treated with lime (even in the absence of heat) produced starch spherulites. Nixtamalized maize starch did not exhibit the morphology of gelatinized starch typical of prolonged exposure to heat and moisture, but instead produced structures that were spherical, with a prominent Maltese cross and multicolored birefringence under polarized light. While under cross-polarized light starch spherulites can resemble fecal spherulites, SEM imaging and elemental composition analyses reveal distinct differences. Thus, starch spherulites resulting from nixtamalization have a unique set of characteristics that distinguish them from other common microbotanical objects. Finally, nixtamalized maize samples also produced free-floating starch and starch spherulites at a significantly higher proportion than cooking in water alone: this is promising for the archaeological recovery of starch spherulites from archaeological sediments and porous pottery.

Nixtamalization has played a long and significant role in the history and foodways of native peoples in the Americas. To date, however, there has not been a method developed to identify nixtamalization directly in the archaeological record. Although starch grains are preserved in a variety of sites and contexts in the Americas, and work has been done previously in archaeology to understand how specific cooking practices affect the appearance of starch granules, no research published to date identifies specific effects of nixtamalization on starch granules. Although the analyses performed in this study illustrate that nixtamalized starch spherulites should be identifiable in the archaeological record, further experimental work needs to be conducted in order to understand how the diagnostic morphologies resulting from nixtamalization may change due to taphonomic processes, differences in maize preparation, and the differing levels of amylose and amylopectin present in different varieties of maize such as flint, flour, and popcorn. The morphologies analyzed here were still present on prepared slides more than one year after initial cooking, but it is less clear how these structures will appear after prolonged exposure to soil conditions, including acidity and microbial activity. This study lays the groundwork for the identification of nixtamalization directly from the archaeological record, offering invaluable insight into the inception and expansion of nixtamalization throughout Mesoamerica.

Author contributions

E.S.J. conceived the study and designed the experimental protocol, with advice from J.M.M. E.S.J. conducted the experiments and light microscopy analysis. J.M.M. conducted dyeing, SEM, and EDS analyses. Both authors contributed to the literature review and writing of the manuscript.

Declaration of competing interest

The authors have no competing interests.

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