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Increase in the degree of substitution of cassava starches by dual modification processes

Incremento del grado de sustitución en acetatos de almidón de yuca implementando procesos de modificación dual

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Abstract
The dense packing of cassava starch determined by a semi-crystalline structure is responsible for obtaining low degrees of substitution in the acetylation processes. The present study evaluated the effect of dual modifications combining physical and chemical treatments on the degree of substitution (DS), structural, morphological, pasting and gelatinization properties of cassava starches. Four physical treatments such as annealing (ANN), heat-moisture (HMT), ultrasound (UTS) and homogenization (HMG) were used to subsequently perform the chemical modification of acetylation with acetic anhydride for each treatment. The modification was confirmed by infrared spectroscopy with the presence of the carboxyl group (C=O) characteristic of starch acetates. The modified starches did not change the type-A crystalline pattern, however, they presented decreases in the intensities of the peaks, relative crystallinity, as well as greater stability upon heating and less retrogradation. In conclusion, the dual physicochemical modification is an alternative to increase the efficiency of the chemical reaction, decreasing the use of chemical agents and increasing the GS achieved, which enables its application in the biofilm industry.
Keywords: Physical treatments, starch acetates, crystallinity, dual modification, morphology.

Resumen
El almidón de yuca presenta un denso empaquetamiento determinado por su estructura semicristalina, la cual es la responsable de la obtención de bajos grados de sustitución en los procesos de acetilación. El presente estudio evaluó el efecto de las modificaciones duales combinando tratamientos físicos y químicos sobre el grado de sustitución (GS), las propiedades estructurales, morfológicas, de empastamiento y gelatinización de almidones de yuca. Se utilizó cuatro tratamientos físicos como annealing (ANN), humedad-calor (HMT), ultrasonido (UTS) y homogenización (HMG) para posteriormente realizar la modificación química de acetilación con anhidrido acético para cada tratamiento. La modificación fue confirmada por espectroscopia de infrarrojo con la presencia del grupo carboxilo (C=O) característico de los acetatos de almidón. Los almidones modificados no cambiaron el patrón cristalino tipo-A, sin embargo, presentaron disminuciones en las intensidades de sus picos, en la cristalinidad relativa, así como una mayor estabilidad al calentamiento y menor retrogradación. En conclusión, la modificación dual fisicoquímica es una alternativa para aumentar la eficiencia de la reacción química, disminuyendo el uso de agentes químicos y aumentando el GS alcanzado, lo que posibilita su aplicación en la industria de biopelículas.
Palabras clave: Tratamientos físicos, acetatos de almidón, cristalinidad, modificación dual, morfología.

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1 Introduction

Cassava *(Manihot esculenta C)* is a transitory crop of great socioeconomic and agroindustrial importance in Colombia, grown for its edible tuberous roots and, minimally, for its leaves. Tuber is an important energy source, with starch as the main component, over 80% on a dry basis, making it a potential raw material for industrialization (Salcedo & Contreras, 2017). However, 95.4% of the production is destined for fresh consumption, and only 5.6% for processing into flours and starches (Ramos-Villacob *et al*., 2023). The cassava starch application in both food and non-food industries has been widely studied, due to its low cost, easy availability, non-toxic nature, renewability, biodegradable properties and available hydroxyl groups that can be modified (Ashogbon, 2017). Native cassava starch presents a smooth surface, free of pores with spherical or oval shaped granules of a regular size between 15-18 μm, low amylose content around 15 and 25 % w/w, and high amylopectin content between 80 and 83 % w/w, type-A polymorphism and a degree of crystallinity between 35 and 45, which determines its semi-crystalline nature and physicochemical properties (Colivet & Carvalho, 2017; Gutiérrez & Alvarez, 2018).

Native cassava starch presents limitations in film development due to its low mechanical strength, high hydrophilicity, and low thermal stability (Cui *et al*., 2021). To improve these drawbacks, physical, chemical, enzymatic or combined modifications are necessary to enhance its functionality and make it more suitable for its extensive use in these industries (Ochoa *et al*., 2017). Chemical modifications have been widely used for the development of starches with acceptable hydrophobic and thermal properties, among them, esterification by acetylation stands out, which is a process applied with relative ease and favorable results in its physicochemical and functional properties (Cuenca, *et al*., 2020; Trela *et al*., 2020). However, the dense packing, granular conformation and semi-crystalline nature of the starch molecule confers a high resistance to chemical modification, known as steric hindrance, which can be reflected by the low availability of free hydroxyl groups to react with acetyl groups, preventing the substitution of these groups, and therefore, the formation of starch acetates resulting in low degrees of substitution (Otache *et al*., 2021).

In addition, chemical modification processes require organic agents or solvents that increase the degree of substitution. However, these solvents can generate negative environmental impacts, therefore, it is necessary to propose industrially viable technological alternatives that lead to a reduction in the quantities of chemical agents. As a result, some studies have been oriented to implement physical treatments combined with chemical modification to improve GS, mainly because it is an environmentally friendly, low-cost technology, capable of altering the crystallinity of the granules, giving desirable characteristics to the starch and preparing it for chemical modification, and reducing the concentrations of chemical modifying agents (Schafranski *et al*., 2021). For this purpose, physical treatments such as heat-moisture and ultrasound promote starch chain interactions and alter properties such as gelatinization temperature, solubility, pasting, and morphological properties (Schafranski *et al*., 2021).

This research responds to scientific gaps in the scientific literature on dual modification processes using physical and emerging technologies such as annealing, heat-moisture, ultrasound, and homogenization; and chemical methods such as acetylation, with conditions below gelatinization. Therefore, the hypothesis presented is: The physical/chemical modification processes are feasible to increase the degrees of substitution of starch acetates for applications in biodegradable films, reducing the use of chemical agents applied. In this sense, the research aims to evaluate the effect of physical treatments on the degree of substitution of acetylated cassava starches.

2 Materials and methods

2.1 Materials

Native cassava starch (*Manihot esculenta* cv M-TAI) was supplied by Almidones de Sucre S.A.S. Analytical grade reagents were used.

2.2 Simple modification process

2.2.1 Annealing treatment

The starch samples were treated following the methodology used by Yu et al., (2021) with slight modifications. Initially, a starch-water suspension was prepared under a 1:3 ratio, then the suspension was left under refrigeration for 24 h to reach equilibrium. The samples were then subjected to a hydrothermal process in a water bath at 60 °C with constant stirring at 250 rpm for 4 h.

2.2.2 Heat-Moisture treatment

Cassava starch samples were hydrothermally modified using the methodology described by Li *et al*., (2017) with some modifications. The moisture content of the samples was adjusted to 25 % w/w with distilled water

and mechanical mixing. Next, the samples were kept at room temperature for 24 h to reach equilibrium. After this time, the hydrothermal process was carried out in ovens with forced convection at 90 °C for 4 h.

2.2.3 Ultrasound treatment

Cassava starch samples were treated by ultrasound following the methodology used by (Zhang *et al*., 2019) with modifications. An ultrasonic bath (Elmasonic P 60 H, Germany) was used by adjusting the conditions to a frequency of 37 kHz and a power of 50 W. Suspensions of native cassava starch at 10 % w/v were prepared and subjected to the ultrasonic modification process at a temperature of 60 °C for 4 h.

2.2.4 Homogenization treatment

Samples of native cassava starch were suspended at 10% w/v in distilled water and treated using an ultra-turrax disperser (T25 Basic, IKA, Germany) with agitation at 3000 rpm for 4 h at 60 °C.

Native cassava starches (NCS) and modified by annealing (ANN), moisture-heat (HMT), ultrasound (UTS) and homogenization (HMG) were subsequently centrifuged at a rotational speed of 3402 RCF for 5 min. The precipitates were subjected to a drying process at 35 °C in a convective oven for 20 h until a moisture content of 10 % w/w was reached. Then, the samples were macerated, passed through a 200-mesh sieve, and finally stored in airtight bags for further characterization.

2.3 Dual modification process

2.3.1 Preparation of acetylated starches

Physically treated and native starch samples were acetylated following the methodology employed by Colussi *et al*., (2017) with some modifications. 10 g of starch were dispersed in 100 ml of distilled water with active stirring at 50 °C for 2 h. The suspension was cooled to 40 °C and the pH was adjusted to 8.0 with 1 M NaOH solution. Next, 5 ml of acetic anhydride was added dropwise to the stirred suspension and let to react for 2 h, the pH was maintained between 8 and 8.5 with 1 M NaOH. After the reaction time, the suspension was adjusted to pH 4.5 with 1 M HCl. The obtained starch was washed twice with distilled water and once more with 70 % ethanol and centrifuged at 3402 RCF for 5 min. Finally, the starch was dried in a convective oven at 35 ºC for 24 h and stored.

2.4 Degree of substitution of acetylated starches

The determination of acetyl groups (AC) was performed according to the methodology used by Salcedo *et al*., (2016). 1 g of modified starch on dry basis was transferred to a 250 ml Erlenmeyer, with 50 ml of distilled water and three drops of phenolphthalein. The sample was neutralized with 0.1 N NaOH until it remained a slightly pink color, and then 25 ml of 0.45 N NaOH was added, and the suspension was shaken vigorously for 30 min. After this time, the saponified samples were titrated with 0.8 N HCl. At the same time a blank was titrated using native starch. The percentage of substituted acetyl groups was determined based on the volume of acid spent (Eq. 1).

$$Acetyl(\%) = \frac{(\text{Blank ml -sample ml })*(\text{N HCl}*0.043\ *100)}{\text{sample g}} \quad (1)$$

Where, 0.043 are the milliequivalents of the acetyl group.

DS corresponds to the average number of acetyl groups introduced per unit of anhydroglucose, calculated according to Eq. (2):

$$DS = \frac{(162) * (\%\ acetyl)}{4300 - [42 * \%\ acetyl]} \quad (2)$$

2.5 Scanning electron microscopy and birefringence

The morphological characteristics of native and modified starch were observed by scanning electron microscopy (SEM) coated with a gold/platinum thin film under vacuum and placed on a double-sided Scotch tape (Figueroa-Flórez *et al*., 2019). The micrographs were obtained on a SEM microscope (JEOL, JSU-5600 LV, Japan) under operating conditions at 15 KV, 30 mA and an amplitude range of 1000 -11800X.

To observe birefringence, 7 mg of each starch sample were homogenized in 1 ml of deionized water. A 50 μL aliquot was poured onto a slide to examine morphology and birefringence of the granules using a trinocular polarized microscope (Optika, B-383POL, Italy). Microphotographs were acquired under bright and polarized light fields, at 40X magnification, using a high-resolution 10MP camera with USB 3.0 connection (Optika, CB10+, Italy) (Figueroa-Flórez, 2020).

2.6 Attenuated total reflectance Fourier transform infrared spectroscopy

Infrared spectra (FTIR-ATR) of starches were acquired on an infrared spectrometer (UATR, Perkin Elmer, USA), with a 1.5 mm diameter ZnSe/diamond crystal. The sample was placed on the crystal, until the entire diameter was covered, and each spectrum was recorded with a resolution of 8 cm^{-1} and 4 readout

scans in the range of 400 - 4000 cm^{-1}. The ratio of absorbances in bands 1047/1022 cm^{-1} and 925/1022 cm^{-1} was determined to assess the degree of molecular order (Yu *et al*., 2021).

2.7 *Apparent amylose content*

Apparent amylose content was determined by the standardized iodine spectrophotometric method (Li *et al*., 2017) with the following modification. 10 mg of the sample was dissolved/degreased in a 10 mL solution of 95 % DMSO and placed in a water bath for 15 min. The sample was reacted with I2/KI solution for 20 min in test tubes previously cover for light protection. Then, the absorbance value of this reaction was determined in a UV-VIS Pharo 300 spectrophotometer (Spectroquant®, Darmstadt, Germany) at 620 nm, according to a calibration curve of potato amylose (A0512) and corn amylopectin (10112) standard solutions from Sigma-Aldrich under concentrations of 0-100% w/w.

2.8 *X-ray diffraction*

X-ray diffraction (XRD) patterns of starch samples were obtained using a diffractometer (Panalytical, X'Pert MPD, Switherland) operated at 1.8 kW and 40 mA. Spectra were acquired in a range of 4-40°, at a scan rate of 2°/min and sampling interval of 0.02° (Figueroa-Florez, 2020). The degree of crystallinity (DC) was estimated as the ratio of the absorption peaks areas (crystalline zone) and the total diffractogram area, using numerical integration methods and MATLAB software (MathWorks, R2019a, USA).

2.9 *Pasting properties*

The pasting properties were determined using a rheometer (Anton Paar, MCR 302, Austria), according to the method used by Dar *et al*., (2018) with slight modifications. 8 % w/v starch suspensions were subjected to a temperature of 50 °C for 2 min, then increased to 95 °C, held at 95 °C for 5 min and cooled once again to 50 °C, and finally, held at 50 °C for 2 min. The ascent and descent rates were 7.5 °C/min for each stage. The different viscosity parameters were analyzed using RheoCompass software (v1.12, Anton-Paar, Austria).

2.10 *Gelatinization properties*

Gelatinization properties were determined using a rheometer (Anton par MCR 302, Austria), using the parallel plates geometry with a 25 mm diameter and a gap distance of 1.0 mm. After validation of the linear viscoelasticity region (LVR), 30% w/v starch suspensions were subjected to a temperature sweep between 30 and 90 °C with a constant frequency of 1.0 Hz and a 0.5 % deformation (Cham & Suwannaporn, 2010). The values of elastic modulus (G' [Pa]) and viscous modulus (G" [Pa]) were obtained. Results were processed using RheoCompass software (v1.12, Anton-Paar, Austria).

2.11 *Statistical analysis*

A unifactorial design was used with six (6) levels corresponding to the type of modification, as described in Table 1. Data were analyzed by analysis of variance (ANOVA) and Tukey's test for mean difference at a significance level of 5 % using Statgraphics statistical software (Centurion XVI.I, Statgraphics Inc., USA).

3 Results and discussion

3.1 *Degree of substitution of acetylated starches*

The percentage of acetyl groups (AC) and the degree of substitution (DS) of native and modified cassava starches are shown in Table 2. These parameters increased significantly ($p<0.05$) with dual modifications, being more evident in the HMTA and UTSA samples. According to Omodunbi Ashogbon (2021), the first modification could alter the granular surfaces and the bond strength between the chains, making the hydroxyl groups more available to be substituted by acetyl groups.

Table 1. Experimental design implemented in the modification of cassava starches.

Treatment	Nomenclature
Native cassava starch	NCS
Acetylated cassava starch	ACS
Starch modified by annealing and acetylation	ANNA
Starch modified by heat-moisture treatment and acetylation	HMTA
Starch modified by ultrasound and acetylation	UTSA
Starch modified by homogenization and acetylation	HMGA

Table 2. Percentage of acetyls and degree of substitution of starches with dual modifications.

Sample	AC (%)	DS
NCS	-	-
ACS	4.322±0.03^{a}	0.170±0.001^{a}
ANNA	5.618±0.19^{bc}	0.223±0.008^{bc}
HMTA	5.962±0.19^{c}	0.238±0.008^{c}
UTSA	7.109±0.19^{d}	0.287±0.008^{d}
HMGA	5.274±0.19^{b}	0.209±0.008^{b}

AC: Percentage of acetyls (%); GS: Degree of substitution; NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified by annealing and acetylation; HMTA: Starch modified by heat-moisture treatment and acetylation; UTSA: Starch modified by ultrasound and acetylation; HMGA: Starch modified by homogenization and acetylation. Same letters in the same column do not differ statistically ($p < 0.05$).

AC and DS of HMGA and ANNA treatments were lower compared to HMTA and UTSA treatments, possibly due to the higher steric hindrance and increase in the crystallinity cause by the homogenization and annealing processes, which reduces the availability of hydroxyl groups on the amylose and amylopectin chains for further acetylation. However, all these treatments were significantly higher than the single ACS modification. These results are above those reported by Zdybel *et al*. (2021), who obtained substitution degrees between 0.07 and 0.10 in dual-modified sweet potato starches by annealing and acetylation.

UTSA exhibited the most significant increase in AC and GS values, which could be attributed to the effect of sonification causing morphological changes in the granular surface as fractures, pores, and cracks, facilitating the penetration of the acetyl group (CH3CO-) during acetylation (Amini *et al*., 2015). Abedi *et al*. (2019) reported similar results, obtaining GS values between 0.2 and 0.5 in dual-modified wheat starches by ultrasound and acetylation. According to these authors, the variation of the ultrasonic frequency can alter the polymeric structure, reducing steric hindrance and increasing the interaction of the granule with the acetic anhydride. Likewise, HMT treatment could promote an increase in starch chain interactions, causing a separation of the double helix structure and a rearrangement of the crystal structure. Due to this separation, the granule becomes more susceptible and allows a better action of the modifying agents for the HMTA sample, achieving better efficiency in the reaction (Schafranski *et al*., 2021).

3.2 Scanning electron microscopy and birefringence

The morphological properties of native and modified starches are presented in Fig.1(A, B). NCS samples exhibited starch granules of spherical or oval shape, smooth and relatively uniform surfaces, as reported for native cassava starches by Figueroa-Flórez *et al*. (2019) . Regarding the particle size, the average diameter of granules is between 15-18 μm. However, some fragmented granules with truncated ends were observed, possibly due to the extraction process of the starch. Similar results were reported for native cassava starches by other authors (Javadian *et al*., 2021).

ACS treatment did not exert significant changes in the morphology and size of starch granules compared to their native counterparts, possibly due to the low concentrations of the esterifying reagent. However, small agglomerations were observed among the granules, which could be related to the presence of hydrophilic groups in the starch molecule. Sodhi & Singh, (2005) and Singh *et al*., (2004) reported similar results for acetylated rice, corn, and potato starches, respectively.

Starch showed changes in the morphological surfaces after dual modification. HMTA sample presented roughness, fissures, cavities, and holes. These variations could be caused mainly by the combination of the physical and chemical modification methods, altering the internal molecular structure of the starch and evidencing these changes in morphology (Subroto *et al*., 2022). Majzoobi *et al*. (2016) found agglomerations and morphological irregularities in hydrothermally treated rice starch granules, which were more significant when the time of HMT treatment was increased.

In UTSA samples were visualized notable changes in the surface microstructure and size of the starch granules, such as erosion and fragmentation, as well as granules with partial loss of their native morphology. This phenomenon could be attributed to the high shear generated by the rupture of the cavitation bubbles generated by the ultrasound treatment that eroded the starches surfaces, altering both the size and the surface of the starch granules (Yang *et al*., 2019). Khurshida *et al*. (2021) found small lacerations in samples of cassava starches dual modified by ultrasound and acetylation, which intensified with increasing ultrasonic treatment, suggesting the effect exerted by ultrasonic pretreatment.

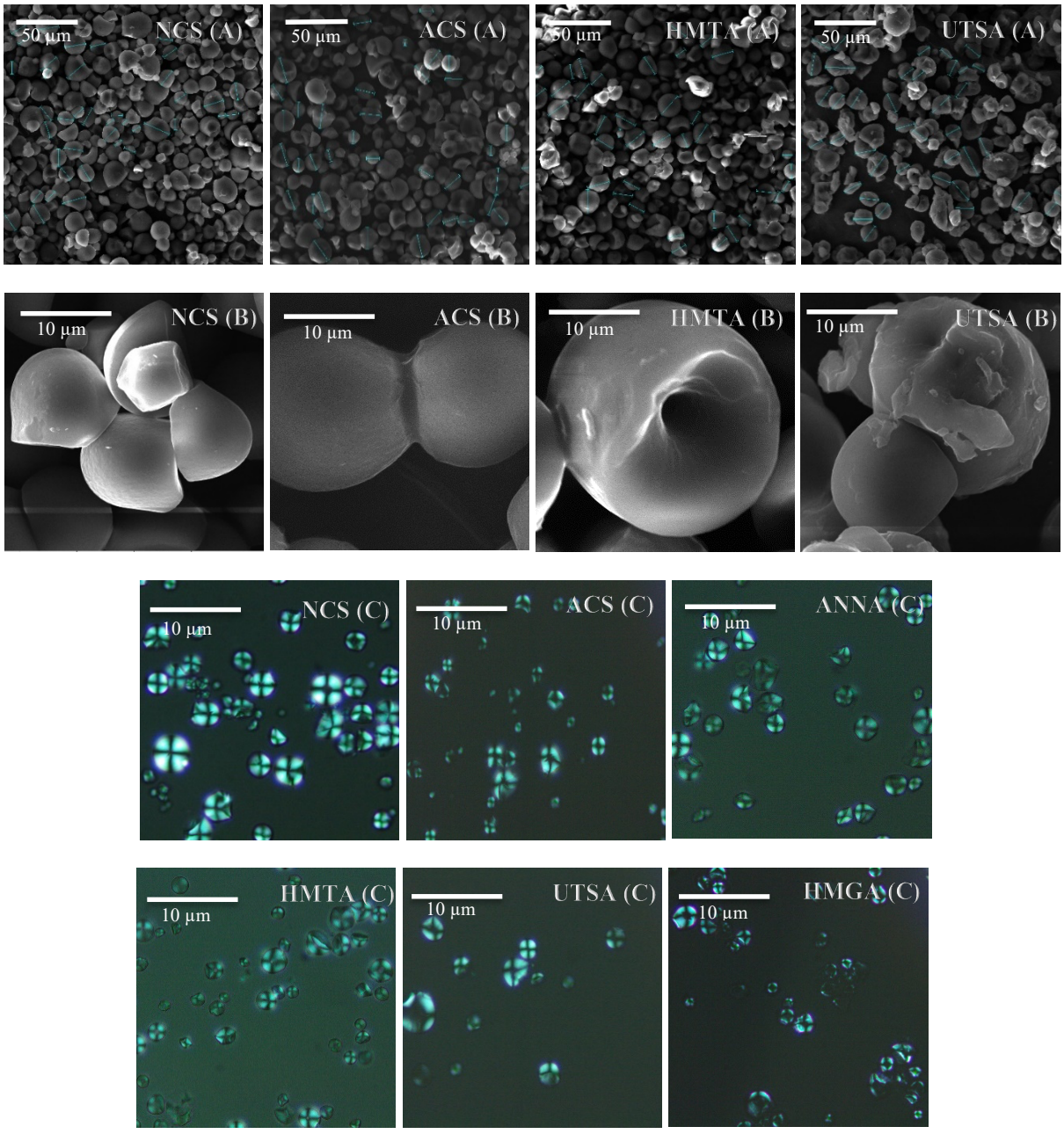

Fig. 1. Scanning electron microscopy images (A, B) and polarized field (C) of native and modified cassava starch. (A) Micrographs at 1000X magnification; (B) Micrographs at 11800X magnification. NCS: Native cassava starch; ACS Acetylated cassava starch; HMTA: Starch modified with heat-moisture treatment and acetylation; UTSA: Starch modified with ultrasound treatment and acetylation.

On the one hand, birefringence is the ability of starch granules to refract polarized light in two directions. The birefringence patterns of native and dual-modified samples of cassava starches are exposed in Fig.1C. The native forms of NCS starches exhibited a birefringence pattern with a centralized "malt cross" typical of native cassava starches crystal structure (Arroyo-Dagobeth *et al*., 2023). In contrast, the modified forms of the starches presented variations in cross-polarization. All modifications significantly decreased the birefringence patterns, causing a more opaque appearance of the starch hilum structure (Alcázar-Alay & Meireles, 2015). In this context, weak patterns indicate disorganization of the crystalline regions, and loss of birefringence is associated with deformation of the granule due to the various modifications performed.

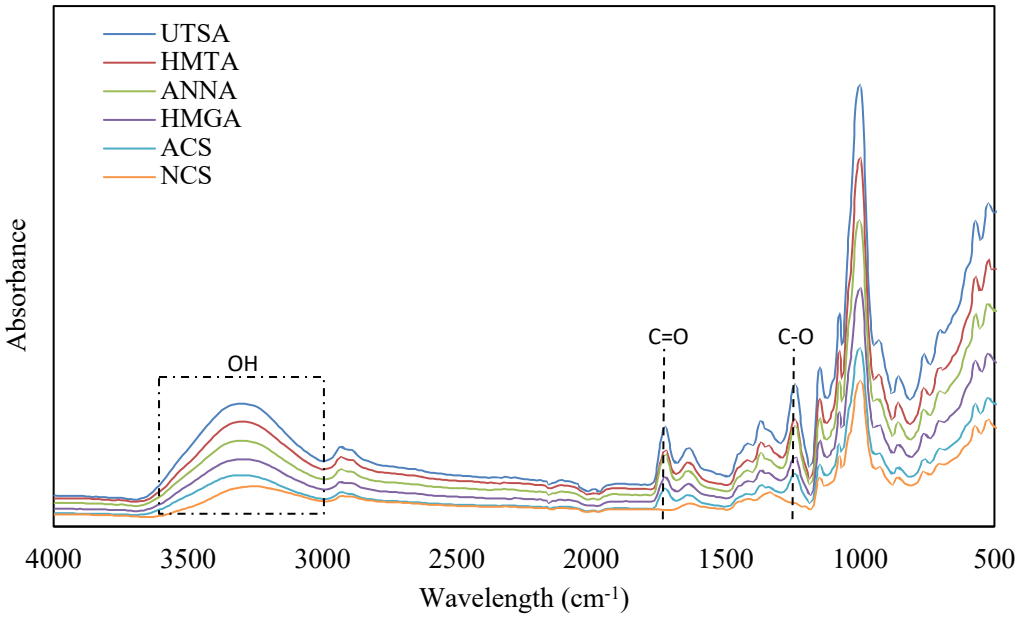

Fig. 2. FTIR-ATR spectra of native and modified cassava starches. NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified with annealing treatment and acetylation; HMTA: Starch modified with heat-moisture treatment and acetylation; UTSA: Starch modified with ultrasound treatment and acetylation; HMGA: Starch modified with homogenization treatment and acetylation.

3.3 Attenuated total reflectance Fourier transform infrared spectroscopy

FTIR-ATR spectroscopic analysis provides information on short-range conformational variations in amylaceous materials, indicating changes in chain conformation, semicrystalline order and molecular ordering (Hoover, 2009). The FTIR-ATR spectra of native and modified starches are shown in Fig. 2. These spectra show significant changes in the intensities of the absorption bands of all starch samples, both in the diagnostic zone (4000 - 1500 cm^{-1}) and in the fingerprint zone (1500 - 500 cm^{-1}). The absorption bands in the range 3000-3600 cm^{-1} correspond to the vibration of the O-H bond associated with the hydroxyl groups. A variation in the spectra of the modified starches compared to the native control was observed due to the substitution of O-H groups by acetyl groups in the polymeric chains, causing a decrease in these bands (Salgado-Delgado *et al*., 2022;Cuenca, Ferrero, *et al*., 2020; Figueroa-Flórez *et al*., 2016). Additionally, in the 2940 cm^{-1} region, related to the C-H bonds of the glucose units, a variation was observed in the vibrations of the absorbance peaks of modified starches compared to their native control, possibly originating from changes in the ratios of their amylose/amylopectin contents (Kizil *et al*., 2002).

In the ACS treatment was observed a new band at 1740 cm^{-1} related to the C=O group, intensified in dual-modified starches such as ANNA, HMTA, UTSA, and HMGA, and was completely absent in the native counterpart (NCS). Olagunju *et al*. (2020) found similar results observing changes in these same bands for pigeon pea acetylated starches with different DS. The absorption band near 1650 cm^{-1} is correlated to the strongly bound water present in the starch structure (Trela *et al*., 2020). A decrease in absorbance of these bands in modified samples compared to NCS. When a water molecule strongly interacts through hydrogen bonds, its bending vibration requires higher energy to undergo vibrational excitation, resulting in reduced absorption of the band vibration, considering a strong water-starch interaction for modified starches (Kumar *et al*., 2018).

A stretch in the 1245 cm^{-1} bands, corresponding to the C-O group, was observed in the fingerprint region between 1500 - 500 cm^{-1} of modified starches whereas absent in native starch. The presence of this peak could be related to the acetyl group and the effects of hydrothermal processes (Salcedo-Mendoza *et al*., 2016). In this same region, it has been shown that the absorption bands at the wavelength of 1047 and 1022 cm^{-1} are associated with ordered and amorphous starch structures, respectively (J. Figueroa-Flórez, 2020). Thus, the relationship between the heights of the bands at 1047 and 1022 cm^{-1} has been used to quantify the degree of molecular order (MO) in starchy materials. As listed in Table 3, the MO_1 values presented a significant decrease for the UTSA, HMGA, and ANNA treatments compared to the NCS sample, this could be caused by the dissociation and unwrapping of the double helices that form the crystalline matrix to a greater extent by the

Table 3. Degree of molecular order, apparent amylose content and degree of crystallinity of native and modified starch samples.

Sample	MO_1	MO_1	CA (%)	DC (%)
NCS	0.721±1.06E-03^{ab}	1.231±3.97E-03^{a}	21.785±0.216^{a}	48.613±0.313^{a}
ACS	0.723±3.53E-04^{bc}	1.151±1.24E-02^{b}	12.639±0.213^{b}	39.456±0.578^{b}
ANNA	0.718±2.41E-05^{d}	1.137±2.35E-03^{bc}	16.854±0.330^{c}	37.498±0.432^{c}
HMTA	0.727±3.52E-03^{c}	1.169±2.35E-03^{d}	14.407±0.643^{d}	35.540±0.298^{d}
UTSA	0.708±1.82E-03^{e}	1.124±3.75E-03^{c}	11.634±0.351^{be}	27.580±0.160^{e}
HMGA	0.712±3.91E-04^{e}	1.152±3.15E-05^{b}	10.939±0.928^{e}	31.560±0.229^{f}

MO_1: Molecular order based on the 1047/1022 cm^{-1} ratio related to the "semi-crystalline order", MO_2: molecular order based on the ratio 995/1022 cm^{-1} related to "swelling capacity", CA: Percentage of amylose (%), DC: degree of crystallinity (%), NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified by annealing and acetylation; HMTA: Starch modified by heat-moisture treatment and acetylation; UTSA: Starch modified by ultrasound and acetylation; HMGA: Starch modified by homogenization and acetylation. Same letters in the same column do not differ statistically ($p < 0.05$).

physical treatments, which could affect the molecular structure and increase the introduction of acetyl groups, correlating with the increase of DS (Hoover, 2009). In turn, HMTA presented a significant increase in these values, suggesting a molecular rearrangement in the starch structure and changes in crystallinity (Yu *et al*., 2021). Likewise, MO_2 values decreased significantly for all modified treatments compared to NCS, results that could be attributed to conformational changes of the starch molecule after the modifications, possibly due to the substitution of O-H groups.

3.4 Apparent amylose content

The apparent amylose content (AC) of native and modified cassava starch samples is listed in Table 3. The NCS treatment shows an amylose content between 20 and 22 %, similar to results found by Hoover (2001) and Toae *et al*. (2019) in native cassava starches of variety M-TAI. CA decreased significantly after modifications ($p<0.05$). Amilose decreased in ACS treatment (41.98 %) compared to NCS counterpart. Such behavior could be attributed to the interference of acetyl groups affecting iodine uptake during amylose determination (Betancur *et al*., 1997; Kumar *et al*., 2019). Lawal *et al*. (2015) reported a decrease in the amylose content of acetylated starches from cassava, maize, and sweet potato compared to native controls, suggesting it could be attributed to the leaching of amylose during modification processes.

The UTSA and HMGA treatments showed a reduction of 46.59 and 49.78 %, respectively, in the percentage of amylose content compared to the native sample. Chen *et al*., (2004) and Wang *et al*., (2022) explained that a higher content of amylose is in the amorphous zone of the starch granule, indicating that the dual modifications occurred to a greater extent in this region, causing a decrease in the CA. These results could induce variations in the crystallinity patterns determined by XRD.

The amylose content decreased between 33.86 and 31.81 % in HMTA and ANNA treatments. In previous studies, hydrothermal treatments reduced the apparent amylose content, possibly due to conformational changes in the polymeric chains of amylose-amylose and amylose-amylopectin interactions. This phenomenon results in a decrease in the number of helical turns, decreasing the intensity of the characteristic color of the amylose-iodine complex (Hoover, 2009; Jin *et al*., 2023; Nakazawa & Wang, 2003; Paraginski *et al*., 2016). Consequently, the decrease of CA in dual-modified starches could be explained by a combined effect of physical and chemical modifications that would cause leaching of amylose and/or depolymerization of the chains, decreasing their ability to form a complex with iodine (Hoover, 2009).

3.5 X-ray diffraction

The crystalline patterns of native and modified starch granules are shown in Fig. 3. Native cassava starch exhibited an A-type monocyclic polymorphism with strong reflection at Bragg 2θ angles around 15.04°, 17.03°, 18.00°, 23.035° and a small peak around 20° (Figueroa-Flórez et al., 2023). These peaks represent the ratio of the amylopectin side chains and amylose chains arranged in a helix shape (crystalline zones) to the unstructured components (amorphous zone) (Martens *et al*., 2018). Although tuber starches usually show B-type diffraction patterns, previous studies also reported a characteristic A-type pattern in cassava starches (Tupa *et al*., 2013; Van Soest & Vliegenthart, 1997).

Modifications did not change the crystalline pattern of the starch granules but induced significant decreases in the intensities of the peaks, resulting in variations in the degree of relative crystallinity. UTSA sample presented the maximum decrease in the characteristic peaks with respect to NCS, showing almost the loss of the small peak around

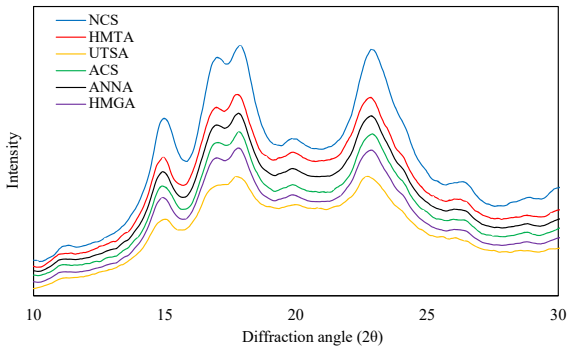

Fig. 3. X-ray diffraction patterns of native and acetylated cassava starches. NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified by annealing and acetylation; HMTA: Starch modified by heat-moisture treatment and acetylation; UTSA: Starch modified by ultrasound and acetylation; HMGA: Starch modified by homogenization and acetylation.

20°. According to Bartz *et al*. (2015), during the acetylation, the unsubstituted free hydroxyl groups manage to rearrange themselves, forming new hydrogen bonds, thus maintaining the characteristic crystalline peaks of starch but with reduced intensity.

The reduction of crystalline peaks caused by molecular rearrangement in modified samples resulted in significant decreases in the degree of crystallinity (DC) compared to the native sample (Table 3). DC decreased from 48.613 % for NCS to 39.456 % for ACS. Similar findings were reported by Palavecino *et al*., (2019) for native and acetylated starches from cassava and sorghum. This effect was more significant for dual-modified samples. UTSA presented a higher reduction in DC (27.58 %) among all treatments. Zhang *et al*., (2012) argue that intramolecular and intermolecular hydrogen bonds when partially replaced by acetyl groups, lose their ability to form hydrogen bridges. Although they are rearranged and retain their crystalline pattern, lowering the intensity of the peaks results in the loss of crystallinity. Furthermore, the action of physical treatments prior acetylation increased changes in the conformation of the polymeric chain, semicrystalline order, and molecular ordering of modified starches, according with results obtained through FTIR-ATR infrared spectroscopy.

3.6 Pasting properties

Figure 4 shows the behavior of the pasting properties during the heating and cooling of suspensions of native and modified cassava starches. All modified starches presented a significant reduction of pasting temperature (PT) compared to NCS, being the lowest for ACS, ANNA, and HMTA. This decrease could be explained by a possible insertion of acetyl groups in the amorphous region of the starch molecules,

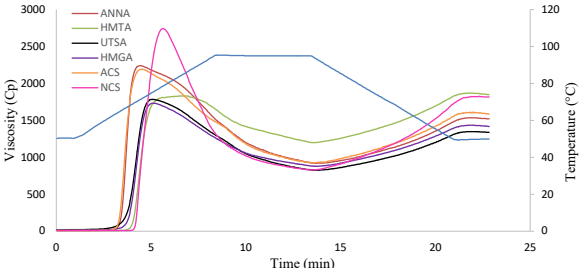

Fig. 4. Pasting properties of native and modified cassava starches. NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified with annealing treatment and acetylation; HMTA: Starch modified with heat-moisture treatment and acetylation; UTSA: Starch modified with ultrasound treatment and acetylation; HMGA: Starch modified with homogenization treatment and acetylation.

weakening the integrity of the granule and facilitating water absorption during the heating process, thus decreasing the pasting temperature of the modified samples (Saartrat *et al*., 2005). Khurshida *et al*., (2021) observed similar behaviors in dual heterogeneous ultrasound and acetylation modifications of cassava starches associated with changes in morphological properties and swelling power. These results could be verified previously in the results of apparent amylose content, finding a significant decrease in dual modifications.

During the heating phase, the starch granule swells and increases volume until the structure collapses, leading leach of amylose and an increase in viscosity until the peak viscosity (PV) is reached (Hoover, 2010). ACS treatment presented a significant PV decrease compared to NCS, suggesting fewer unbroken swollen granules, possibly indicating substitutions of O-H groups by other functional groups (Khurshida et al., 2021). Such effect was more significant in dual-treated samples, showing lower values in the PV peak, suggesting a crystallinity decrease, lower amylose leaching and higher substitutions of the O-H groups, according to the DS, FTIR, and CA results. Bello-Pérez *et al*. (2010) reported decreases in PV for acetylated barley starches with different GS. This behavior implies a tendency to decrease viscosity peak by all modifications, correlating with those results obtained for amylose content, and may as well interfere with other physicochemical properties such as swelling power (SP) and solubility in cold water (SCW).

The breakdown viscosity (BV) is related to the stability of starch granules against shear force with temperature action and depends on the difference between the peak viscosity (PV) and the minimum viscosity (MV) during heating at constant temperature (Aaliya *et al*., 2021). The modification of starch significantly reduced the values of BV, improving the stability of the pastes of starches. The most stable were

HMTA, UTSA, and HMGA, with lower values of BV. This behavior could be related to depolymerization of starch chains during dual modifications and partial alteration of the granular structure due to the introduction of new functional groups, which contributed to the increase in hydrophobicity and decrease in apparent amylose content, as well as changes in crystallinity values (Khurshida *et al*., 2021; Saartrat et al., 2005). Similarly, Hong *et al*., (2016) observed similar behavior in heterogeneous modifications of potato starches with acetylation assisted by pulsed electric fields.

Setback viscosity (SV) is defined as the difference between the final viscosity (FV) and the minimum viscosity of pastes in the cooling phase. The magnitude of SV is the tendency of the pastes to retrograde (Wang et al., 2015). The dual modifications significantly reduced the SV, mainly in UTSA, HMGA and ANNA samples. This phenomenon could be explained by a lower leaching and the decreases in amylose caused by the dual modification, producing a lower regrouping reflected in lower SV (Aaliya *et al*., 2021). In contrast, Sindhu *et al*., (2021), report a non-significant increase in SV values of acetylated starches compared to native samples, as a result of leached amylose, granular size, and the presence of rigid and non-fragmented swollen granules. Final viscosity (FV) also decreased significantly in the modified samples compared to native control, exception for HMTA which showed a significant increase, possibly due to depolymerization of leached amylose and amylopectin.

3.7 Gelatinization properties

Temperature sweep tests have been used to study the behavior of native and modified cassava starches during the gelatinization process. The results show that the elastic moduli (G') of the starch hydrogels predominated over the viscous moduli (G"), suggesting an elastic structure for the hydrogels of starch (Serna-Fadul, 2022). The onset temperatures (To), peak temperatures (Tp), termination temperatures (Tc) and temperature range (Tc-To) of gelatinization of cassava starches in their native and modified forms were estimated through dynamic-oscillatory tests as a function of temperature and are presented in Figure 5. Initially, the ACS treatment showed a significant decrease for To, Tp and Tc compared to NCS. This behavior could be explained by a weakening of the internal hydrogen bonds of the granule, due to the reduction of hydroxyl groups partially substituted by hydrophobic acetyl groups (Hong *et al*., 2016). Results are similar to previous reports estimated by differential scanning calorimetry (DSC) on cassava starches acetylated with acetic anhydride (Osundahunsi & Mueller, 2011).

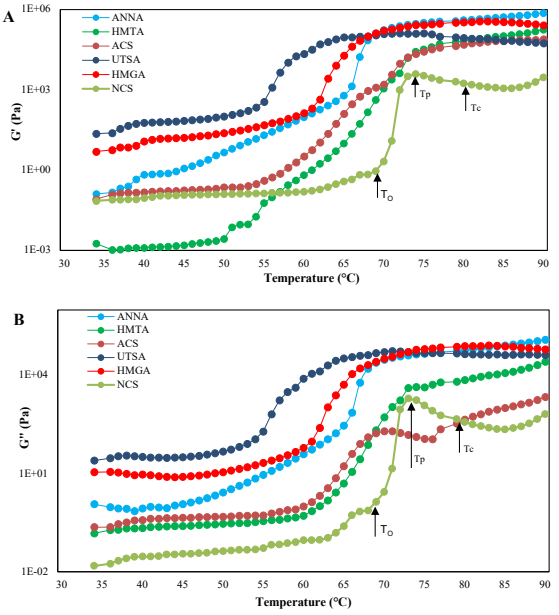

Fig. 5. Gelatinization properties estimated by oscillatory temperature sweeps on native and modified cassava starches. A) Elastic modulus behavior (G'); B) Viscous modulus behavior (G''). NCS: Native cassava starch; ACS Acetylated cassava starch; ANNA: Starch modified with annealing treatment and acetylation; HMTA: Starch modified with heat-moisture treatment and acetylation; UTSA: Starch modified with ultrasound treatment and acetylation; HMGA: Starch modified with homogenization treatment and acetylation.

On the other hand, it has been found that hydrothermal modifications can change the gelatinization temperatures of starches, depending on the moisture level of the sample, the starch source, and the amylose content. This has been attributed to short-range changes at structural level within the starch granules involving amylose-amylose and amylose-lipid interactions (Adebowale *et al*., 2009). Similarly, it has been reported that acetyl groups added to the polymer chains cause a decrease in the energy required to hydrate the granule, leading to a decrease in transition temperatures and an increase in swelling by the reduction of the crystalline structures (Osundahunsi & Mueller, 2011). Which in turn, decreased To, Tp and Tc of dual treated samples with hydrothermal pretreatment ANNA and HMTA, behavior reported for potato starches treated with annealing and acetylation (Zdybel *et al*., 2021).

Significant decreases in starch gelatinization temperature parameters were also observed for the UTSA and HMGA treatments, compared to the control sample. This could be explained by a loss in the crystallinity after the dual modification because water molecules require less time to penetrate the crystalline area of the polymeric chains. As a consequence, a lower temperature would be required

to produce the swelling of the granule (Moorthy-Subramony, 2002; Odeku & Picker-Freyer, 2007). These effects were observed previously in cassava starches doubly modified with acetylation and electric fields (Gagneten *et al*., 2023).

Conclusion

Physical and chemical treatment combinations allowed the obtention of dual-modified cassava starches with short- and long-range conformational changes. Physical treatments prepared the granular surface of the starch for its subsequent acetylation, achieving better penetration of the esterifying reagent and increasing the degrees of substitution. The results reveal that the HMTA and UTSA treatments were the most suitable to obtain a higher efficiency in the acetylation reaction, leading to reduced steric hindrance and increasing the GS. Moreover, dual modification reduces up to 49% of the apparent amylose, to 56% of the relative crystallinity, and changes in granular morphology, pasting, and gelatinization properties of cassava starch.

These results suggest that dual-modified cassava starches could be suitable for biomaterial applications. The significant decrease in apparent amylose content and relative crystallinity indicates that these starches may have improved properties in terms of flexibility and degradability, which are desirable characteristics in bioplastics. In addition, changes in granular morphology and gelatinization properties may favor the formation of more uniform and stable films, thus increasing their applicability in the biodegradable packaging industry. Furthermore, these findings suggest potential applications in the textile industry, improving the quality and strength of natural fibers. In the field of adhesives, the modified properties of these starches could result in products with enhanced adhesion and water resistance capabilities.

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