Impact of microwave radiation on *in vitro* starch digestibility, structural and thermal properties of rice flour. From dry to wet treatments.

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Abstract

Microwave radiation (MW) is an environment-friendly technology used to physically modify flours. Rice flour was MW-treated at different moisture content (MC) (3%, 8%, 13%, 15%, 20% and 30%). *In vitro* starch digestibility was determined and related to the changes caused by MW treatment to flours' structure and thermal properties, which were influenced by MC. A reduction of 49% and 65% in the gelatinization enthalpy of samples treated at 20% and 30%MC denoted a partial gelatinization. A loss of granular crystallinity in treated samples was confirmed by XR-diffraction and FTIR, particularly at 15%, 20% and 30%MC. MW promoted the formation of random-coil, α-helix and β-turn protein structure, and the disappearance of LF-β-sheet. Morphological differences were found between samples treated at 8%MC (loss of polygonal structure, protein layer covering granules' surface and small holes) and 30%MC (rounded and aggregated granules, covered with exudate amylose). *In vitro* starch digestibility revealed that samples treated at 20% and 30%MC showed significantly higher rapidly digestible starch (40% and 47%), lower slowly digestible starch (48% and 70%) and lower resistant starch (90%) than the untreated flour. Flour MC in MW-treatment allowed the modulation of structural and thermal characteristics of rice flour and consequently its starch hydrolysis rate.

***Keywords**: Rice Flour Microwave Treatment; In vitro Starch Digestibility; Structural properties*

1. Introduction

Hydrothermal treatments are physical modification methods that change the physicochemical properties of starch. These treatments have been found to be responsible for amylose and amylopectin chains reorganization which causes changes of crystallinity, pasting, thermal, gelatinization and rheological properties, and final starch digestibility of starches and flours [1]. Heat-moisture treatment (HMT) refers to starch heating at temperatures generally from 90 to 140ºC for periods <24 h, at moisture content usually from 20% to 35% [2]. Conversely, in dry-heat treatment (DHT) the starch is heated in a dry state (<10% moisture content) at high temperature (>100 °C) for several hours [3,4]. To implement HMT or DHT on a large scale, alternative methods, such as microwave (MW), are used due to numerous advantages: the MW heating is fast and uniform as well as economical and environmentally friendly [5].

Rice (*Oryza sativa* L.) is one of the most widely consumed cereals in the world and represents an excellent source of energy. Although it is mostly consumed as cooked grains, rice flour production intended for baby formulas and gluten-free foods has recently increased [6]. However, rice and rice-based foods are often classified as having a high glycemic index, which may constitute a public health problem for populations who rely heavily on these foods [7]. Therefore, the *in vitro* starch digestibility is an important property to be established in a flour when a physical treatment has been applied to modify its techno-functional properties. Many factors, including surface organization (e.g. pores), granular architecture, starch composition, type of crystal polymorph, granular size, and the presence of compound granules, affect the rate and extent of digestion of starch granules. These factors are often interconnected and determine the fraction of dietary starch that is not digested in the small intestine of a healthy human, which is the resistant starch, RS [8]. Most of the available literature is focused on the effect of MW radiation on starch sources from different botanical origin at moisture contents ranging from 15% to 45% [9–15]. It was reported that MW treatment led to a reduction in the crystallinity of wheat and maize starches with 30% moisture content [13]. Villanueva et al. [14] reported that MW treatment changed the hydration properties and enhanced water absorption index and swelling power in potato starch samples, while a diminishment was determined in rice starch. They also found that MW promoted gel stability, led to lower viscometric profiles and resulted in enhanced viscoelastic moduli of potato starch. Guo et al. [15] informed that MW/HMT of *indica* rice starch led to a concave granule surface, loosened packing of chains in amorphous regions and somewhat disrupted crystallites, altering starch structure's susceptibility to hydrothermal effects, and modestly increasing the enzyme digestion rate of starch. Other authors evaluated the changes in total and damaged starch of rice starch following microwave treatment at 23% and 30% moisture content [16] and the digestibility and pasting properties of non-waxy and waxy rice starches heat-treated by microwave radiation at 20% moisture [17].

Although DHT has received a great deal of attention in recent years [3,4,18], there have been limited studies concerning the changes in the structure and properties of modified starches under DHT conditions by MW radiation.

Research has shown that the structure of starch granules is irrevocably altered by MW radiation, even at molecular level [19]. However, flour is a more complex matrix, and the changes that occur during MW treatments also depend on the reactions that take place between its components, mainly starch and protein [20], and the moisture content available in the system [21]. Generally, the literature reports the use of non-hermetic containers for the treatment of flour by MW, where it has been found that the moisture content (MC) of the flour is rapidly lost by evaporation during treatment [22]. Hence, the role of the MC in the achieved modifications has not been clearly determined, as in non-hermetic containers the MC it is not constant but progressively reduced leading to mixed HMT-DHT treatment conditions. Therefore, it is necessary to make a connection between the effect of MC and the final properties determined in the resulting modified flours. In the present study, a constant MC during treatment was ensured using a hermetic container. A previous study, also carried out at constant MC, concluded that rice flour treated by MW heating at 30%, 20% and 8% MC showed important changes in hydration, pasting and rheological properties [23]. However, a deeper study regarding the effect of controlled MC during MW treatment on the main biopolymers (starch and protein) in rice flour and its impact on starch digestibility has not been covered so far. Therefore, this study aims to determine the effect of MW radiation under HMT or DHT conditions on thermal, structural and digestibility properties of rice flour treated at constant moisture from 3% to 30%.

2. Materials and methods

2.1 Samples and chemicals

Indica rice flour (long grain) was kindly provided by Herba Ricemills S.L.U. (Valencia, Spain). The MC was 13%, ash 0.67 %, protein 7.6 % and lipid 0.82 %. The flour granulometry was as follows: 1% < 250 µm, 250 µm> 10-20% > 210 µm, 210 µm> 35-45% > 150 µm, 150 µm> 20-35% > 100 µm and 100 µm< 10-20% (data provided by manufacturer). This rice flour, without any treatment, was used as control flour. It was stored at 4ºC until utilization.

Pancreatin from porcine pancreas (8 × USP specifications; EEC No. 232-468-9), pepsin from porcine gastric mucosa (EEC No. 232-629-3), gum guar (EEC No. 232-536-8) and D-Glucose (EEC No. 200-075-1) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Amyloglucosidase (800 U/g; EEC No. 232-877-2) and invertase (200000 U/g; EEC No. 232-615-7) were gifted by Novozymes A/S (Bagsvaerd, Denmark). Glucose Oxidase/peroxidase was

provided by BioSystems S.A. (Barcelona, Spain). The remaining chemicals were all reagent grade and obtained from Panreac Quimica S.L.U. (Barcelona, Spain).

2.2 Microwave treatment of flour

Six batches at 3%, 8%, 13%, 15%, 20% and 30% ± 0.5% moisture content (MC) were prepared by lyophilization (3% MC sample), drying at 40ºC (8% MC sample) or spraying the calculated water to reach the levels of 15%, 20% and 30% MC from the original MC of the native rice flour (13%). Prepared samples were stored for 24 h at 4±2 ºC prior utilization for moisture equilibration. Water content was measured with the Official AACC Method 44-19.01 [24]. The procedure followed to perform the microwave treatment on the flour was described by Villanueva et al. [22] and Solaesa et al. [23]. Briefly, 50 g of flour, with the desired MC, was exposed to microwave radiation [900 W Sharp MW oven (R342INW)] for 480 s in cycles of 10 s radiation (on) and 60 s of rest (off) in a hermetic Teflon® container, which was continuously rotated by an external device at a speed of 60-70 rpm to ensure a uniform energy and temperature distribution during treatment. The temperatures reached by the flours during treatment varied with their MC as follows: 99 ± 1 ºC (3%), 173 ± 4 ºC (8 %), 138 ± 1 ºC (13 %), 141 ± 3 ºC (15 %), 136 ± 3 ºC (20 %), and 114 ± 3 ºC (30 %) [21]. Each treatment was carried out in triplicate and the flour obtained was sieved to <250 μm (60-mesh) for further analysis. Samples at 20% and 30% MC were firstly dried at 35ºC until getting back to their natural MC (~13%) to avoid spoilage.

2.3 Differential scanning calorimetry (DSC)

Starch gelatinization and retrogradation transitions were assessed by DSC (DSC3, Mettler Toledo, SAE, Barcelona, Spain). Rice flour samples, ~6 mg, were weighed into aluminum pans (40 μL) and distilled water was added to achieve a 30:70 (flour:water) ratio. The samples were scanned from 0 to 115 ºC at 5 ºC/min using an empty sealed pan as reference. The obtained thermograms were analyzed using STARe evaluation software (Mettler Toledo, Barcelona, Spain). Peak temperature (Tp, ºC) and enthalpy (ΔH, J/g flour dry basis, db) of endothermic transitions were reported. Two peaks were determined in the gelatinization transition (first scan), which were individually integrated (1^{st} and 2^{nd} peak). After the first run, the pan was stored at (4 ± 2) ºC for 7 days to allow starch retrogradation. Endothermic transition of retrograded starch was determined by a second run using the same procedure. Each sample was measured in duplicate.

2.4 X Ray Diffraction (XRD)

The diffraction assessment was performed using a Bruker-D8-Discover-A25 diffractometer (Bruker AXS, Rheinfelden, Germany) equipped with a copper tube operating at 40 kV and 40 mA, with CuKα radiation of 0.154-nm wavelength. To avoid the influence of humidity on the

values determined for relative crystallinity, the samples were equilibrated to 20 ± 0.5% MC using a saturated humidity chamber at 15 °C for 72 h. Equilibrated samples were scanned from 5º to 40º (2θ) at a rate of 1.2º/min, a step size of 0.02º, a divergence slit width variable of 5 mm and a scatter slit width of 2.92º and a nickel filter 0.02 to exclude the Kβ radiation. The relative crystallinity was determined from diffractograms based on the ratio between the reduced peaks area (assigned to the crystalline phase) and the global peaks area. The "search-match" software DifracEVA with PDF2-2004 and COD database was used for this purpose.

2.5 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of control and microwaved-samples [previously equilibrated at 15% MC in a constant climate chamber HPP260eco (Memmert GmbH, Schwabach, Germany)] were obtained using a FT-IR Nicolet iS50 spectrometer (Thermo Fisher Scientific, Massachusetts, USA) with an attenuated total reflectance (ATR) accessory. Spectra were obtained between 4000 and 400 cm^{-1} in absorbance mode. Each spectrum was collected with a resolution of 4 cm^{-1} by accumulation of 64 scans. Starch and protein secondary structure (amide I) analyses were conducted in the range of 1200 to 800 cm^{-1} and 1700 to 1600 cm^{-1}, respectively. The overlapped bands of Amide I were determined by Fourier-self deconvolution and second derivative techniques using OMNIC software (Thermo Fisher Scientific, Massachusetts, USA). The deconvoluted spectra were used for individual peak fitting following an iterative adjustment assuming Gaussian band shapes with Origin2019b software (OriginLab Corporation, USA). Peak assignment corresponds to high frequency (HF) β-sheet (1700-1690 cm^{-1}), β-turn (1690-1665 cm^{-1}), random structure and α-helix (1665-1640 cm^{-1}) and low frequency (LF) β-sheet (1640-1615 cm^{-1}) [25]. Samples were measured in triplicate.

2.6 Scanning electron microscopy (SEM)

A Scanning Electron Microscope model Quanta 200FEG (FEI, Oregon, USA) was used to study the microstructure of the control (native rice flour) and 8% and 30% MC treated samples. Each sample was mounted on an aluminum stub and sputter-coated with a 5 nm layer of gold using a sputter coater SCD-05 (Leica Microsystems, Wetzlar, Germany). The microstructure was visualized with an accelerating voltage of 5.5 keV in low vacuum mode using a secondary electron detector at magnifications of 500×, 1000× and 3000×.

2.7 Enzymatic hydrolysis of starch

In vitro starch digestibility was measured according to the Englyst method [26] with some modifications [27]. Briefly, 0.8 g of rice flour was mixed with 10 mL of pepsin-guar gum solution (0.5 g/100 mL of each in 0.05 M HCl) in a 50 mL centrifuge tube and transferred to a water bath

at 37ºC for 30 min. Then 10 mL of sodium acetate buffer solution (0.25 M pH 5.2) and 5 mL of enzyme mixture (2 g amyloglucosidase, 90 mg invertase and 13.5 g pancreatin per 100 mL) were added to each tube and agitated at 80 rpm in a shaking water bath at 37ºC. At time intervals of 0, 10, 20, 30, 40, 50, 60, 90, 120, 180, 240 and 360 min after enzyme addition, 0.2 mL sample solution was withdrawn and immediately mixed with 8 mL 66% ethanol to deactivate the digestive enzymes. Subsequently, the solution was centrifuged at 500 × g for 5 min. To measure the glucose concentration, 200 μL supernatant was mixed with 4 mL glucose oxidase/peroxidase reagent (GOPOD), followed by incubation at 37 °C for 20 min. The absorbance at 510 nm was measured against the blank reagent using a spectrophotometer (Lambda25 PerkinElmer, Madrid, Spain). The free glucose + glucose from sucrose content (FSG) and the total glucose content (TG) released from starch were determined following the optional rapid method described also by Englysh et al. [26]. The glucose concentration was then transformed to the starch digested ratio. From the glucose measured at 20 min (G20) and 120 min (G120) the rapidly digestible starch (RDS) = 100· 0.9· (G20 − FGS)/TS, slowly digestible starch (SDS) = 100· 0.9· (G120 − G20)/TS, resistant starch (RS) = 100· 0.9· (TG − G120)/TS, and total starch (TS) = 0.9· (TG − FGS) were calculated. The percentage of starch hydrolyzed in each flour was calculated based on the total starch content. A first order kinetic equation [$C = C\infty\ (1 - e^{-kt})$] was applied to describe the kinetics of starch hydrolysis, where C, C∞ and k were the hydrolysis degree at a time t, the maximum hydrolysis extent and the kinetic constant, respectively [28]. All measurements were made at least in quadruplicate.

2.8 Statistical analysis

Statistical analyses and the Pearson correlation study were conducted using the Statgraphics Centurion XVII-X64 software (Bitstream, Cambridge, MN, USA). The analysis of the variance (ANOVA) by Least Significant Difference (LSD) at p-value ≤ 0.05 was performed.

3. Results and discussion

3.1 Thermal properties

The thermograms obtained from the control and microwaved-flour samples are shown in **Figure 1**. The first (gelatinization) scan obtained from native flour (Fig. 1A) presented a main endothermic transition, between 60 – 87 ºC, corresponding to starch gelatinization, and a second smaller peak, at 95–100 °C, related to the dissociation of the amylose–lipid complex. The gelatinization transition presented a double peak (1^{st} and 2^{nd}) that could be due to a mixture of different *indica* rice varieties with different gelatinization temperature ranges conforming the studied rice flour. A similar thermogram, with a double gelatinization peak, was also found in rice flour by Cappa et al. [29]. Bao et al. [6] reported the existence of two types of rice starch

depending on the gelatinization temperature (GT), that in the case of rice accessions with low apparent amylose content (AAC) (< 20%) (such as the rice flour used in the present study, with an AAC of 16.4%), were classified into Low-GT (*Tp* from 65.8 to 71.0ºC) and High-GT (*Tp* from 77.6 to 79.8ºC). In the control rice flour, the first peak appeared at 67.6 ºC with a gelatinization enthalpy (*ΔH-gel-1st*) of 6.2 J/g db, while the second peak was determined at 75.7 ºC and presented a lower enthalpy (*ΔH-gel-2nd*), 3 J/g db (see **Table 1**). In general, gelatinization temperature increases with higher AAC, which might affect the water absorption and swelling behavior of starch [30]. There are, however, some exceptions [8,31,32]. Dhital et al. [31] studied eight rice varieties with AAC from 9 % to 19% and found that some of them, as *Basmati* and *Arborio*, despite having similar amylose content (~15%) presented a difference of 7°C between their gelatinization temperatures. That difference happens because starch gelatinization in flours does not depend exclusively on starch, but it is also influenced by other components (e.g., proteins and non-starch polysaccharides) as well as grinding process [31]. In the case of heat-moisture treated rice starch [33] and corn starch [34], it was suggested that the appearance of two peaks on gelatinization endotherm was due to the presence of two types of starch crystallites which caused a biphasic uptake of heat during gelatinization. In some cases, the second gelatinization endotherm is overlapped with the first one and it appears as a shoulder near the conclusion of the first peak. The appearance of this new endotherm was hardly appreciated in the treated samples. However, the thermal properties were strongly affected by the MC of the samples during the MW treatment (see **Table 1**), showing more marked differences to the native flour with higher MC (15%, 20% and 30% MC). The enthalpy of the first gelatinization endotherm (*ΔH-gel-1st*) decreased significantly in all microwaved samples except in that treated at 3% MC, while *ΔH-gel-2nd* only decreased in the samples treated at 20% and 30% MC. Since *ΔH-gel-1st* decreased in a higher degree than *ΔH-gel-2nd* in these two samples (20% and 30% MC), the ratio (*ΔH-gel-1st / ΔH-gel-2nd*) decreased from 2.1 in the control flour to 1.5 and 1.2 in 20% and 30% MC, respectively; so, the first peak was more sensitive to MW treatment at high MC than the second one, denoting a more thermolabile structure.

The total decrease in the gelatinization enthalpy of 20% and 30% MC samples was 49% and 65%, respectively, compared to the control flour, indicating a partial loss of crystallinity. In this case, this behavior seems to be due to the partial gelatinization of some less heat stable molecules [35]. The gelatinization enthalpy decrease due to HMT may be consequence of the disruption of double helices present in the crystalline and non-crystalline regions of the granule [36]. It might also be attributed to the transformation of the intercrystalline part into an amorphous phase, and thus the crystalline regions could melt more easily (requiring lower energy) [37]. Most published literature suggested a reduction of the gelatinization enthalpy as a result of HMT [22,35,37] and DHT [38], with increased reduction for higher treatment temperatures [3,4]. Chi et al. [38] reported that dry

heated-starch showed a slight decrease in *ΔH* but a remarkable reduction in average-Mw (g/mol), and proposed that dry heating mainly degraded α-1,6-glycosidic linkages.

The gelatinization temperature increased significantly in 15% (only the *Tp-gel-1st*), 20% and 30% MC samples, and decreased (1.2 ºC in *Tp-gel-1st*) in 3% MC sample (see **Table 1**). Lei et al. [3] observed that DHT applied by conventional heating on maize starch samples did not significantly affect *Tp-gel* until the temperature reached 180ºC, where it gradually decreased with increasing heating temperature. Comparing both peaks, the temperature increment was more pronounced in *Tp-gel-1st* than in *Tp-gel-2nd*, same trend as *ΔH-gel*, following the order: 30% > 20% > 15% MC samples, with a maximum increase of 6.1ºC in the sample treated at 30% MC. It seems that the increase in *Tp-gel* on HMT is due to improvement of amylose-amylose, amylose-amylopectin and amylose-lipid interactions. These interactions suppress the mobility of starch chains in the amorphous regions. Consequently, the amorphous regions would require a higher temperature to incur swelling that could contribute to the disruption of the crystalline regions. The extent of these interactions has been shown to be influenced by starch source, amylose chain length, and by the MC prevailing during heat-moisture treatment [36]. The results demonstrated that both treatments, HMT and DHT, caused different alterations to the internal granular structure.

Although gelatinization properties of treated samples were significantly different from those of the native flour, neither melting temperature nor enthalpy of the amylose-lipid complex dissociation was significantly affected by MW treatment (see **Table 1**), with the sole exception of the sample treated at 13% MC where the difference was also not quantitatively relevant. Therefore, the amylose-lipid complex structure quantified by DSC did not seem to be one of the most promoted interactions by this treatment.

After storage at 4 ºC for seven days, the second scan (**Figure 1B**) showed that the retrogradation endotherm of all treated samples appeared at lower temperatures (1ºC lower) than the control sample, with no clear differences between them. The melting enthalpy of retrograded amylopectin was similar in all samples, including the control flour, ~5.6 J/g db (**Table 1**). As Lim et al. [34] stated, this could be because starch crystals in both control and MW treated flours had fully melted before storage for recrystallization. Therefore, the differences in chain arrangements or matrix conformation induced by the hydrothermal treatments were mostly eliminated by the time recrystallization started. As it can be seen in **Table 1**, the enthalpy of the amylose-lipid complex dissociation in the second scan (*ΔH-am-lip* 7d) was similar for all the studied samples, and higher than the values determined in the first (gelatinization) scan. Eliasson [39] reported that the increased values of *ΔH-am-lip* usually found during a second scan are probably due to better conditions for complex formation after the first heating following the leaking of amylose from granules that can occur at temperatures above the gelatinization temperature range. The temperature of amylose-lipid complex dissociation increased significantly in all the treated

samples with respect to the control (up to ~3ºC), with more marked results in the samples treated at <15% MC.

3.2 X-ray diffraction

The most commonly used method to determine the type and degree of crystallinity in flours and starches is XRD. This method determines the starch long-range crystalline order, related to the packing of amylopectin double helices [32,40]. The XRD spectra of control and MW treated rice flours are shown in **Figure 2.** Due to the different moisture content of the samples, very low in some cases (3% and 8%), and in order to further analyze changes in starch crystallinity, moisture equilibration of the samples is required [41]. X-ray diffractograms were performed on the samples at equilibrated moisture content (20 ± 0.5%), where similar patterns were observed though differences in peak intensities were recorded (**Figure 2**). The main peaks of XRD spectra at 15º, 17º, 18º, 23º and 26º (*2θ*) indicated the typical A-type pattern [42] in agreement with the XRD pattern reported for most rice starches [31]. The patterns obtained for treated samples showed a similar arrangement to the native flour, which could suggest that the crystalline structure was not modified by MW treatments. The main difference was the diffraction intensity reduction observed in the samples modified by HMT (15, 20% and 30% MC), seen by the shape and size of the characteristic peaks, effect that seemed to be potentiated by increasing MC (**Figure 2**). The increase of temperature, combined with high MC, can disrupt the starch granules, promoting loss in native starch structure [43]. Previous studies have concluded that HMT of rice starch did not lead to changes of A-type polymorphism, but did cause a decrease of the determined relative crystallinity [1,22,44]. An additional peak at *2θ* = 20º, which is usually connected with V-crystallinity attributed to amylose–lipid complexes, was also observed in the control and microwaved samples [45]. The intensity of this peak was significantly increased in 20% and 30% MC samples, which could partially justify the non-decreased relative crystallinity values determined for these samples, in spite the evident intensity reduction of the other starch characteristic peaks **(Figure 2)**. Some authors observed that HMT at different moisture levels promoted the change of crystalline structure from A to A+V-type due to the amylose-lipid complexes formation [1]. Therefore, this peak could suggest an increase of the amylose-lipid complex in the samples with the highest water content, although this was not reflected in the DSC analysis (see section 3.1) as was also reported by Villanueva et al. [22]. This confirms the existence of two different amylose-lipid forms (polymorphs I and II) [46]. Form I, of low melting temperature, *Tm* = 95-100 °C, similar to that observed in the DSC assay, that is obtained under conditions favoring rapid nucleation and is described as an aggregated state where ordered (helical) chain segments have insufficient packing for crystalline order to be shown by X-ray diffraction (i.e. it gives an amorphous pattern). In contrast, form II (of high *Tm*, ≥120 ºC) is

described as a polycrystalline aggregate with well-developed long-range order, showing the typical reflections of the V-pattern by XRD [46]. Then, the peak usually measured by DSC as amylose-lipid complex at 90-100 °C refers to the amorphous form (form I) while that responsible for the V structure measured by XRD gives account of the polycrystalline aggregate (form II).

The diffraction intensities remained unchanged in 3%, 8%, and 13% MC samples and the crystallinity values were reduced by only 0.4-0.7% (**Figure 2**), which indicated that DHT at the studied treatment conditions hardly affected the packing of amylopectin double helices in the samples, and that the low MC during these treatments prevented any partial gelatinization. Qiu et al. [18] determined a crystallinity increase in glutinous rice flour heated for 4 h at 7% MC in an air oven, from 29 to 34 %, suggesting that DHT may contribute to the formation of new crystallites or recrystallization and perfection of the small crystalline regions of the starch granule. On the contrary, Lei et al. [3] reported a decrease in the crystallinity of DHT-maize starch from 28 to 22.4%. It is believed that in the present study said significant decrease was not observed because treatment time was much shorter (8 min) than that applied by Lei et al. [3] (2 h).

3.3 Fourier transform infrared (FT-IR) spectroscopy

Infrared spectroscopy was used to characterize the ordered structure of starch and changes to the protein secondary structure. Results of FTIR quantitative analysis are presented in **Table 2**. The FTIR spectra of starch typically shows bands at 3000–2900 cm^{-1} (C-H stretching), 1150–1100 cm^{-1} (C-O, C-C and C-O-H stretching) and 1100–900 cm^{-1} (C-O-H bending) [47]. The region between 1050 and 950 cm^{-1} has been shown to be sensitive to changes in starch structure on a molecular level (short-range order), particularly those bands at 995, 1022 and 1047 cm^{-1} [40]. While amorphous regions in starch are characterized by an absorbance band around 1022 cm^{-1}, the crystalline state is traced to the absorbance bands at 995 and 1047 cm^{-1}. This has led to the adoption of the band ratios at 1047:1022 and 1022:995 as measures of short-range ordered molecular structure [47].

Only few studies are based on powdered starch samples with controlled moisture contents [48]. Warren et al. [47] demonstrated the position shift of a hydration dependent peak in the FTIR spectra of starch associated with increased molecular order, but with complex and non-linear behavior. In the present study, samples' moisture content was previously equilibrated at 15% to avoid the influence of water in the rice flours' spectra. The determined 1047:1022 and 1022:995 ratios are presented in **Table 2**. A significant decrease of the 1047:1022 ratio was determined after MW treatment, indicative of disruption of short-range molecular order, showing lower values for higher MC. This trend could result from the dissociation and unraveling of double helices forming the crystalline array caused by the treatment [49], and the partial gelatinization

that occurred during treatment of the 20% and 30% MC samples (see Section 3.1 and 3.2), since the intensity of the band at 1022 cm^{-1} increases with gelatinization [40]. The 1022:995 ratio, on the other hand, increased in general with increasing MC during treatment, except for the sample at 3% MC. Higher values of this ratio represent a higher proportion of amorphous to ordered structure zones in the starch granules [50], suggesting an increased proportion of amylose chains after MW treatment. Both ratios were clearly influenced by MC during treatment. The band at 995 cm^{-1} is particularly sensitive to water content in starch (due to C-OH bending vibrations) [51], and could be related to the marked increase observed in the 1022:995 ratios of the 20% and 30% MC samples.

The Amide I (1700-1600 cm^{-1}) band has been extensively used to quantitatively evaluate changes in the protein secondary structure due to its intense protein signal and less influence of the side chains [25] (see Supplementary Figure 1). Individual peaks in the Amide I region were classified as high frequency (HF) β-sheet (1700-1690 cm^{-1}), β-turn (1690-1665 cm^{-1}), random structure and α-helix (1665-1640 cm^{-1}) and low frequency (LF) β-sheet (1640-1615 cm^{-1}), since it was found to be the best peak assignment from different scenarios available in the literature [25]. The percentage of each secondary structural feature was calculated by adding the area of the peaks found in the assigned band range and dividing it by the total area of all individual peaks. The obtained results are presented in **Table 2**. Results showed significant differences in all treated flours, without following a particular trend with MC. LF β-sheet structures were diminished after treatment (up to 24% with regard to the content in control sample), favoring the formation of random coil & α-helix (increased up to 20% after MW treatment) and β-turn (with an increase of 8%). Sun et al. [52] also reported an equivalent reduction of β-sheet with a consequent increase of random coil & α-helix after MW treatment of pigeon pea flour for 3 min. The decrease of the ordered structures (β-sheet) and increase of the disordered structure (random coil) after MW treatments indicated the formation of more unfolded structures and higher structural flexibility, which could contribute to increasing protein digestibility [52]. No significant differences were observed in the contents of LF β-sheet and β-turn among the treated samples, suggesting that the effect of MW treatment on these structures is independent of the moisture content of the sample. HF β-sheet were also increased after treatment, at a lower degree, showing higher values for the higher studied MC (15% – 30%). As can be seen below (Section 3.5) no correlation could be inferred between the observed changes in protein secondary structure as a result of the MW treatment, which were independent of flour MC, and the quantified changes in starch digestibility.

3.4 Microstructure

Scanning electron micrographs (SEM) of selected samples (control and MW treated rice flours at 8% and 30%) are displayed in **Figure 3.** These MW treated samples were selected for illustrative

purpose because they presented the more marked morphological changes for treatments performed under dry (DHT at 8% MC) and moistened (HTM at 30% MC) conditions. Rice starch granules have an angular and polygonal shape with smooth surface as it can be seen in the control rice flour (**Figure 3A**). The native granules are packed very tightly in the rice grain cells being entwined with globular protein bodies and lipids [22]. In the case of 8% MC sample (**Figure 3B**), the surface was not smooth, and it appeared cracked and cut, probably caused by dehydration. Micrographs showed no discernible granules and no polygonal structure probably due to proteins' unfolding, which spread over the starch granule surface during treatment. This suggests that starch reacted with other components present in the flours during DHT process, consistent with previous reports [53]. Numerous small holes can also be observed, which may also be caused by dehydration, making the surface to become porous. The 30% MC sample, however, presented a much more degraded microstructure (**Figure 3C**). The granules were more aggregated, and their integrity was lost, presenting a much rounder and smoother surface than the native sample, and their polygonal shape disappeared. The starch granules surface degradation caused by HMT might be attributed to partial gelatinization of the outer layer of the granules due to high moisture in the surface exposed directly to high temperatures [37,44,55]. Starch granules seemed to be bound together as a cluster, with no grooves between them. Amylose exudation during the thermal treatment could be responsible for this external bounding [22]. Furthermore, some cavities could be clearly observed in the particles of the treated flour. This could be explained as a molecular structure rearrangement of the granular weaker tissues resulting from the pressure and heating suffered by the sample during MW treatment [1,54,55].

3.5 *In vitro* starch digestibility

In vitro starch digestibility of control and MW treated rice flours at different moisture contents was measured. **Table 3** shows the starch fractions calculated based on the rate of hydrolysis by the digestive enzymes. Digestibility of rice starch is influenced by several factors, including the content of amylose and amylopectin, the crystalline structure and the size of the particles [44]. In the case of rice flour, the degree of crystallinity and digestibility are not always well correlated, and its digestibility depends, among others, on the content and structure of non-starch components present in the flour, such as proteins, lipids and fiber [1,8].

As it can be seen in **Table 3**, the control flour presented lower values of FSG and RDS, and higher values of SDS and RS than the MW treated rice flours. The content of TS was about 85.5% in all samples. Compared with the control flour, the 3% and 8% MC samples did not present significant differences in FSG and SDS, as well as the 13% and 15% MC samples in RDS and SDS. In contrast, the changes determined for the samples treated at the highest MC were very pronounced. The RDS content in 20% and 30% MC samples increased by 40% and 47%, respectively, while

the SDS decreased by 48% and 70%. The degradation of the starch granules during HMT treatment, evident in the micrographs (Figure 3C), would be related to these results. RS decreased greatly and in a similar extent in both samples (around 90%). RS was also decreased in MW treated samples at 8%, 13% and 15% MC, but to a lesser extent, between 70% and 50%. It is believed that starch containing a higher amount of free water was more easily changed by the treatment and became more accessible to digestive enzymes. According to Gunaratne & Hoover [56], the HMT could promote the disruption of double helices present in crystalline and non-crystalline regions of the granule under the conditions prevailing during the treatment as evidenced by decreased gelatinization enthalpy (**Table 1**) and short-range order crystallinity (**Table 2**). The crystalline regions are more resistant to enzymes action, in other words, the amorphous areas of starch granules are more easily degraded by bacterial and pancreatic α-amylase than the crystalline areas [44]. Disruption of crystallites by HMT may have exposed α-1,4 and/or α-1,6-glycosidic linkages that were hidden within the starch crystallites in its native arrangement, and thus made them readily accessible to enzyme attack [49]. Chung et al. [57] also explained that the increase in RDS and decrease in SDS after HMT may be due to disruption of double helices forming the starch crystallites at the granule surface and/or to crystallite reorientation, in agreement with the decreased gelatinization enthalpy values obtained in the present study (**Table 1**) and decreased amount of ordered crystalline domains favoring the amorphous domains (1047:1022 and 1022:995 ratios in **Table 2**). The gelatinization enthalpy ($\Delta H\text{-}gel\text{-}1^{st}$ + $\Delta H\text{-}gel\text{-}2^{nd}$) was negatively correlated with RDS ($r=-0.998$, $p<0.001$) and positively correlated with SDS ($r=+0.996$, $p<0.001$) and RS ($r=+0.814$, $p<0.05$). At the same time the 1022:995 ratio was positively correlated to RDS ($r=+0.962$, $p<0.01$) and negatively correlated to SDS ($r=-0.963$, $p<0.01$) while the 1047:1022 ratio was not significantly correlated to the starch fractions contents. The results are consistent with those obtained by Kweon et al. [58] and Zavareze et al. [44] who observed a trend toward increasing starch digestibility with an increase in moisture content of the HMT. However, it is difficult to reach a consensus on the effect of hydrothermal treatments from previous research on *in vitro* starch digestibility due to differences in enzyme source and concentration, time of hydrolysis, conditions of DHT or HMT, starch source and cultivar [57,59]. In fact, works can be found where RS and SDS increased while RDS decreased in HMT starches [1]. Concerning DHT, Chi et al. [38] showed that treatment of maize starch (130 ºC for 2 h) led to unchanged RDS but increased SDS and reduced RS content. They also reported that dry heating exerted a serious action on amylopectin amorphous lamellae and mainly degraded α-1,6-glycosidic linkages [60]. In addition, Lei et al. [3] showed that the proportion of short-amylose chains increased while the medium- and long-amylose chains were degraded with the increase of heating temperature in DHT. This might explain unchanged or slightly changed RDS and SDS contents after DHT samples, while RS was significantly

decreased, except in the sample treated at 3% MC, where the temperature reached during treatment was only 99 ºC [23].

The hydrolysis curves of selected samples are presented in **Figure 4**. Total hydrolysis of all samples increased with prolonged digestion time. High MC samples (20% and 30%) presented a higher degree of hydrolysis than the control and those modified at lower MC, especially during the first 60 min, showing increasing values with increasing moisture content. Zavareze et al. [44] reported that heat treated starches at 25% MC had a higher enzymatic susceptibility in the first two hours of digestion as compared to native starches, and explained that the rearrangement caused by the hydrothermal treatment facilitated the accessibility of enzymes to the amorphous areas. Figure 5 summarizes the effect of MW treatment on rice starch granular structure depending on flour MC and its impact on the *in vitro* starch digestibility.

Starch hydrolysis, which followed first order kinetics ($0.94 < R^2 < 0.99$), proceeded at different rate for each MW-treated rice flour depending on its MC during treatment (**Table 3**). The kinetic constant (k, min^{-1}) of starch hydrolysis increased in microwaved-samples, although no statistical difference was found between the 3%, 8%, 13% and 15% MC samples and the control flour (0.028 min^{-1}). However, in samples treated at 20% and 30% MC, k increased by 65% and 77% respectively. This evidenced that the hydrolysis kinetics of starch depend strongly on the MC of the flour during the thermal treatment [28]. The starch hydrolysis kinetic constant k was negatively correlated to the gelatinization enthalpy ($\Delta H\text{-}gel\text{-}1^{st}$ + $\Delta H\text{-}gel\text{-}2^{nd}$) (r=-0.981, p<0.001) and positively correlated with the 1022:995 ratio (r=0.926, p<0.01). The FSG content was also positively correlated to the kinetic constant k (r=+0.926, p<0.01) denoting that FSG, which also increased with MC in the treated samples, had a positive impact on the hydrolysis rate. C∞, that corresponds to the maximum hydrolysis extent of starch, varied between 84% and 85% in all the studied samples (**Table 3**). Thus, all MW-treated samples showed a similar maximum starch hydrolysis value, statistically equal to that of the untreated/control sample. Similar results were obtained by Chung et al. [57] in their starch samples treated at 30% moisture in a forced air oven at 100 and 120 ºC for 2 h. During the early stages of hydrolysis (0–40 min), HMT rice flours (20% and 30% MC samples) were hydrolyzed to a greater extent than the rest of samples (**Figure 4**). At 40 min, 70% and 80% of the starch had already been hydrolyzed in 20% and 30% MC samples, respectively. At 120 min, these samples approached the equilibrium percentage of hydrolyzed starch, $C\infty$. At 180 min all samples had hydrolyzed all the digestible starch.

4. Conclusions

The effect of MW on *in vitro* starch hydrolysis depended strongly on the moisture content (MC) of the flour during the thermal treatment, performed always under restricted water conditions. *In*

vitro starch digestibility assays revealed that samples treated at low MC (< 15%) were similar to the control sample while those treated at high MC (20% and 30%) showed higher rapidly digestible starch (RDS), and lower values of slowly digestible starch (SDS) and resistant starch (RS) than the untreated rice flour, with a starch hydrolysis rate four times higher than the value determined for the native flour. This behavior could be explained by a partial starch gelatinization in the samples treated by 900 W microwave radiation for 8 min at 20% and 30% MC as demonstrated the lower gelatinization enthalpy values obtained by DSC tests and the lower short-range crystallinity confirmed from FTIR spectra. In light of the obtained results, it can be confirmed that the rate of starch hydrolysis in the flour can be controlled by the moisture applied during HMT treatment. If it is desired to increase the starch digestibility of the flour, MW-HMT treatments (20%-30% MC) should be preferred. However, if it is desired to significantly modify the functional properties of the flour without significantly change the hydrolysis kinetics, MW-DHT treatments are recommended.

Therefore, flour MC in MW-assisted treatment allows the modulation of structural and thermal characteristics of rice flour and consequently of its starch hydrolysis rate. The combination of HMT and DHT treatments as those performed by MW in non-hermetic containers, where moisture can decrease in a controlled way and pass from HMT to DHT conditions, can represent an easy alternative combining advantages of both treatments to apply at industry level.

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Author contributions

Angela García Solaesa and Felicidad Ronda conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; wrote the paper. Angela García Solaesa, Marina Villanueva and Antonio Vela performed the experiments; analyzed and interpreted the data. Felicidad Ronda: Funding acquisition, conceptualization, methodology, resources, investigation, visualization, supervision, writing - review & editing, project administration.

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Table 1. Thermal properties of aqueous dispersions (30% solids) of control and microwaved-rice flours treated at different moisture contents (MC)

	Control	**MC 3%**	**MC 8%**	**MC 13%**	**MC 15%**	**MC 20%**	**MC 30%**
First run							
ΔH-gel-1st (J/g db)	6.2 ± 0.0 d	6.1 ± 0.1 d	5.5 ± 0.0 c	5.6 ± 0.1 c	5.6 ± 0.1 c	2.8 ± 0.1 b	1.7 ± 0.2 a
Tp-gel-1st (ºC)	67.6 ± 0.6 b	66.4 ± 0.0 a	67.8 ± 0.0 bc	68.1 ± 0.1 bc	68.3 ± 0.0 c	70.3 ± 0.3 d	73.7 ± 0.1 e
ΔH-gel-2nd (J/g db)	3.0 ± 0.1 c	2.7 ± 0.2 c	2.8 ± 0.1 c	2.8 ± 0.2 c	2.8 ± 0.0 c	1.9 ± 0.3 b	1.5 ± 0.0 a
Tp-gel-2nd (ºC)	75.7 ± 0.0 ab	75.0 ± 0.4 a	75.8 ± 0.5 b	75.8 ± 0.0 b	75.9 ± 0.3 b	77.1 ± 0.0 c	79.6 ± 0.3 d
ΔH-gel-1st / ΔH-gel-2nd	2.1 ± 0.1 cd	2.3 ± 0.1 d	2.0 ± 0.1 cd	2.0 ± 0.1 c	2.0 ± 0.1 c	1.5 ± 0.2 b	1.2 ± 0.2 a
ΔH-am-lip (J/g db)	0.9 ± 0.0 a	0.9 ± 0.0 a	0.8 ± 0.1 a	1.0 ± 0.0 b	0.9 ± 0.1 a	0.9 ± 0.1 a	0.9 ± 0.1 ab
Tp-am-lip (ºC)	97.8 ± 0.5 a	97.9 ± 0.2 a	98.7 ± 0.6 ab	98.9 ± 0.3 b	98.3 ± 0.1 ab	98.3 ± 0.7 ab	97.9 ± 0.2 a
Second run (after 7 days at 4 ºC)							
ΔH-retro-7d (J/g db)	5.8 ± 0.3 bc	5.3 ± 0.1 ab	6.1 ± 0.0 c	5.7 ± 0.2 abc	5.3 ± 0.4 a	5.7 ± 0.1 abc	5.3 ± 0.0 ab
Tp-retro-7d (ºC)	49.8 ± 0.5 d	48.6 ± 0.1 ab	48.4 ± 0.4 a	48.5 ± 0.3 ab	49.2 ± 0.1 bcd	48.9 ± 0.3 abc	49.4 ± 0.1 cd
ΔH-am-lip 7d (J/g db)	1.3 ± 0.0 a	1.3 ± 0.3 a	1.5 ± 0.3 a	1.4 ± 0.1 a	1.5 ± 0.2 a	1.4 ± 0.0 a	1.2 ± 0.1 a
Tp-am-lip 7d (ºC)	97.4 ± 0.5 a	99.8 0.1 cd	100.1 ± 0.5 d	100.2 ± 0.6 d	99.1 ± 0.0 bc	98.9 ± 0.4 bc	98.6 ± 0.2 b

Data are the mean ± standard deviation (n = 2). Values with a letter in common in the same line are not significantly different ($p<0.05$). db: dry basis

Table 2. Starch band analysis and secondary structural content of proteins in Amide I region of control and microwaved-rice flours treated at different moisture contents (MC).

		Control	MC 3%	MC 8%	MC 13%	MC 15%	MC 20%	MC 30%
Starch bands	**IR 1047:1022**	0.717 ± 0.001 f	0.705 ± 0.001 e	0.695 ± 0.001 d	0.686 ± 0.002 c	0.679 ± 0.008 b	0.667 ± 0.003 a	0.672 ± 0.001 a
	IR 1022:995	0.865 ± 0.001 a	0.871 ± 0.003 c	0.868 ± 0.001 b	0.868 ± 0.001 b	0.877 ± 0.001 d	0.905 ± 0.002 e	0.905 ± 0.001 e
Protein secondary structure (%)	**LF-β-sheet**	40.8 ± 0.3 a	31.9 ± 0.9 b	31.4 ± 0.9 b	31.7 ± 0.4 b	31.8 ±0.7 b	31.9 ± 0.2 b	31.1 ± 0.8 b
	Random coil and α-helix	36.9 ± 0.6 a	42.8 ± 0.9 bc	44.1 ± 0.9 c	42.1 ± 0.9 b	42.1 ± 0.4 b	42.7 ± 0.1 bc	42.4 ± 0.9 b
	β-turns	20.9 ± 0.5 a	22.6 ± 0.5 b	22.1 ± 0.3 b	22.5 ± 0.9 b	22.5 ± 0.5 b	22.1 ± 0.2 b	22.5 ± 0.3 b
	HF-β-sheet	1.5 ± 0.2 a	2.7 ± 0.9 bc	2.4 ± 0.3 b	3.6 ± 0.9 cd	3.6 ± 0.5 cd	3.2 ± 0.1 bcd	3.9 ± 0.1 d

IR 1047:1022 and IR 1022:995: Intensity rate of 1047 cm^{-1} and 1022 cm^{-1} and 1022 cm^{-1} and 995 cm^{-1} bands, respectively. LF: Low Frequency; HF: High Frequency. Data are the mean ± standard deviation (n = 3). Values with a letter in common in the same line are not significantly different ($p<0.05$).

Table 3. Free sugar glucose (FSG), rapidly digestible starch (RDS), slowly digestible starch (SDS), resistant starch (RS) and total starch (TS) contents and kinetic parameters obtained by applying a first order kinetic equation [$C = C\infty\ (1 - e^{-kt})$] to describe the kinetics of starch hydrolysis of control and microwaved-rice flours treated at different moisture content (MC).

MC	FSG	RDS	SDS	RS	TS	$C\infty$ (%)	k (min^{-1})
Control	0.09 ± 0.01 a	38.1 ± 1.7 a	42.9 ± 2.8 c	5.7 ± 1.2 c	86.3 ± 1.3 a	84 ± 1 a	0.028 ± 0.001 a
MC 3%	0.10 ± 0.03 a	39.4 ± 3.0 a	41.5 ± 4.0 c	4.5 ± 2.5 c	85.7 ± 2.6 a	85 ± 1 a	0.030 ± 0.003 a
MC 8%	0.11 ± 0.01 a	43.1 ± 2.5 b	40.3 ± 3.3 c	1.7 ± 0.1 ab	85.1 ± 2.3 a	85 ± 2 a	0.033 ± 0.002 a
MC 13%	0.21 ± 0.03 b	40.3 ± 2.0 ab	42.2 ± 3.2 c	2.8 ± 0.6 b	85.0 ± 0.5 a	84 ± 2 a	0.031 ± 0.002 a
MC 15%	0.21 ± 0.04 b	42.5 ± 2.0 b	39.5 ± 4.2 c	3.0 ± 0.9 b	85.3 ± 1.5 a	85 ± 2 a	0.038 ± 0.007 a
MC 20%	0.23 ± 0.01 b	63.6 ± 2.6 c	22.0 ± 1.9 b	0.5 ± 0.1 a	86.1 ± 0.6 a	84 ± 2 a	0.079 ± 0.008 b
MC 30%	0.47 ± 0.04 c	72.5 ± 2.8 d	12.8 ± 3.4 a	0.8 ± 0.2 a	85.6 ± 2.4 a	84± 2 a	0.121 ± 0.014 c

Mean values ± standard deviation. Within columns, values (mean of three replicates) with the same following letter do not differ significantly from each other ($p > 0.05$). $C\infty$ is equilibrium concentration of starch and k is the starch hydrolysis kinetic constant.

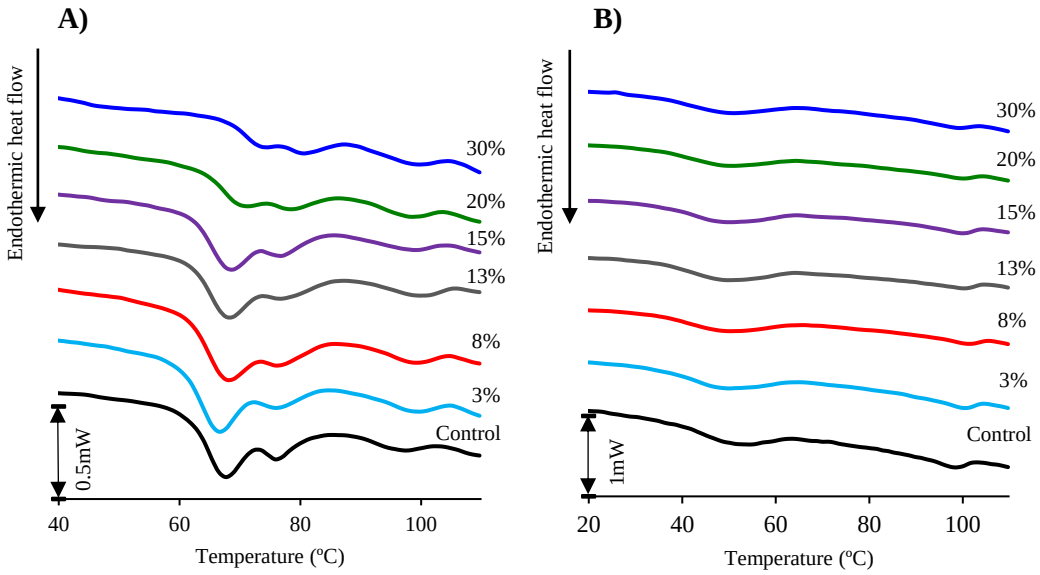


Figure 1. Thermograms of control (black) and microwaved-rice flours treated at 3% moisture content (MC) (light blue), 8% MC (red), 13% MC (grey), 15% MC (purple), 20% MC (green) and 30% MC (dark blue). A) First run (gelatinization) and B) Second run after 7 days at 4ºC (retrogradation).

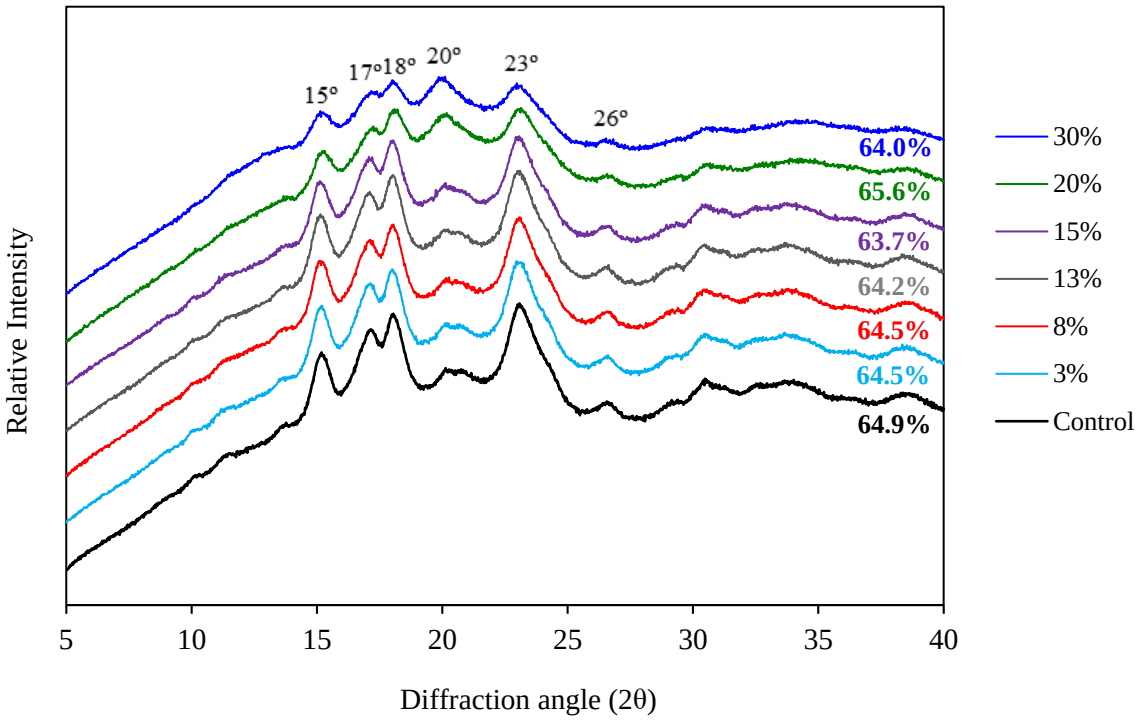


Figure 2. X-ray diffraction patterns of control (black) and microwaved-rice flours treated at 3% moisture content (MC) (light blue), 8% MC (red), 13% MC (grey), 15% MC (purple), 20% MC (green) and 30% MC (dark blue) (all samples were equilibrated in saturated humidity chamber until 20 ± 0.5% MC). Crystalline peaks and percentage relative crystallinity of individual samples are shown. Data have been offset for clarity.

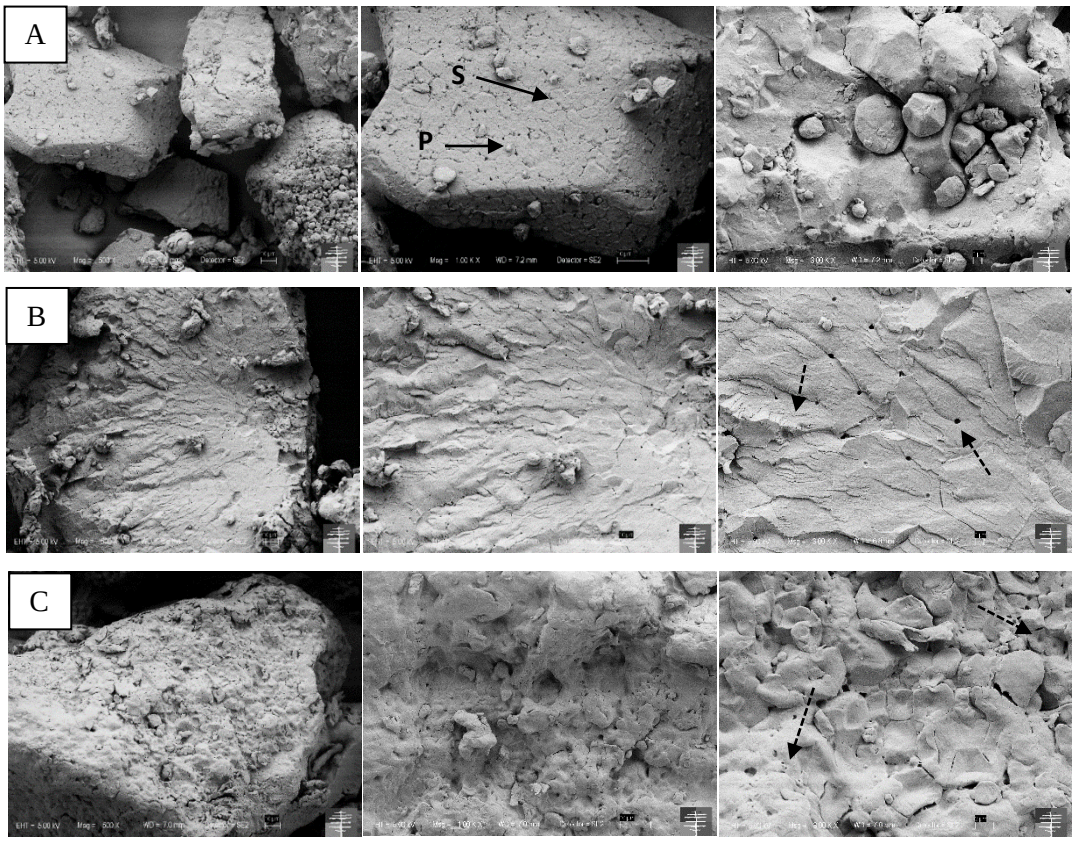


Figure 3. SEM images of control flour (A), microwaved-rice flour treated at 8% moisture content (MC) (B), microwaved-rice flour treated at 30% MC (C). Magnification 500× (left), 1000× (center) and 3000× (right). "P" indicates protein and "S" refers to starch granule. Dashed arrows show the most marked morphological changes.

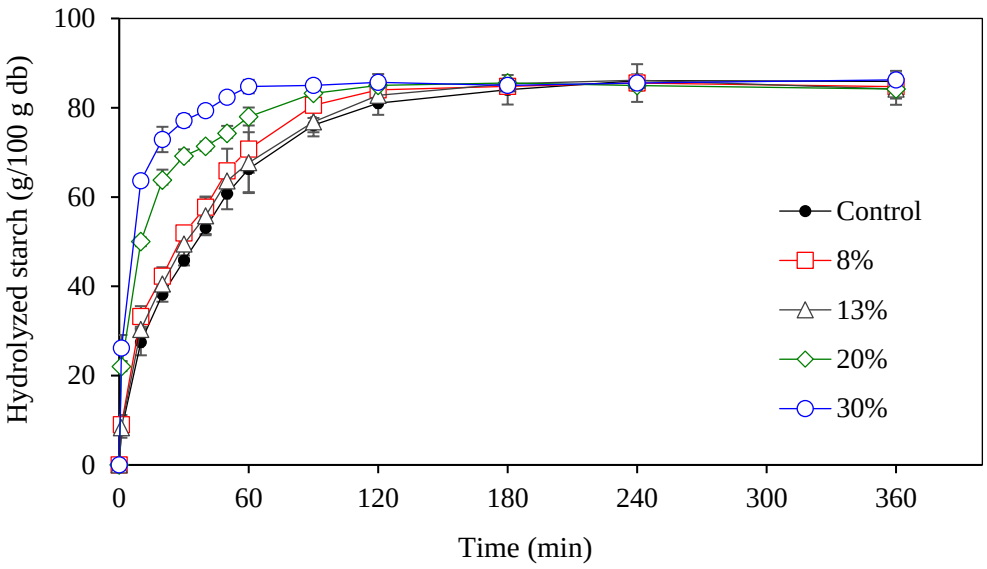


Figure 4. Hydrolyzed starch of control (solid circles) and microwaved-rice flours treated at 8% moisture content (MC) (squares), 13% MC (triangles), 20% MC (rhombus) and 30% MC (circles).

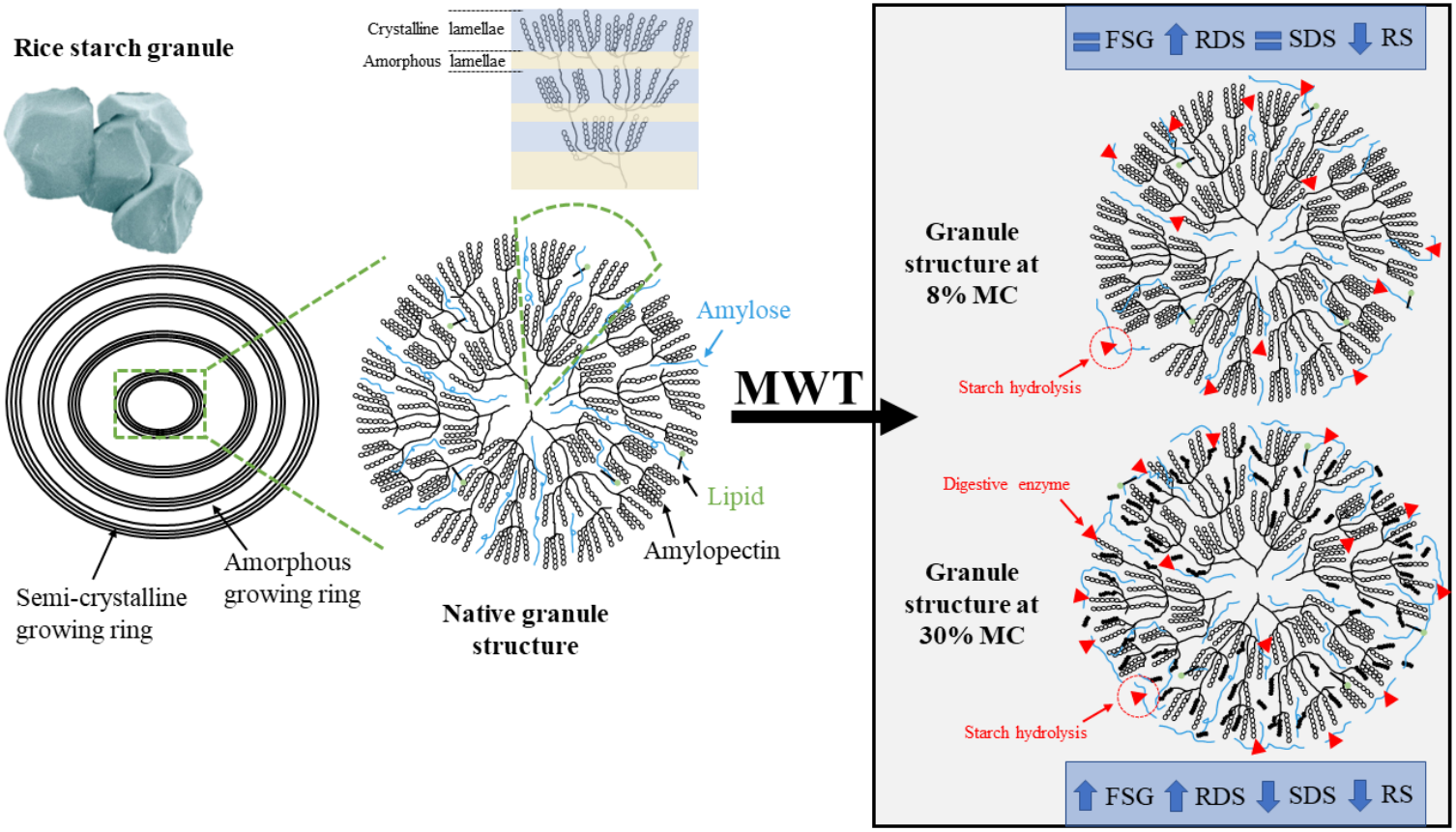
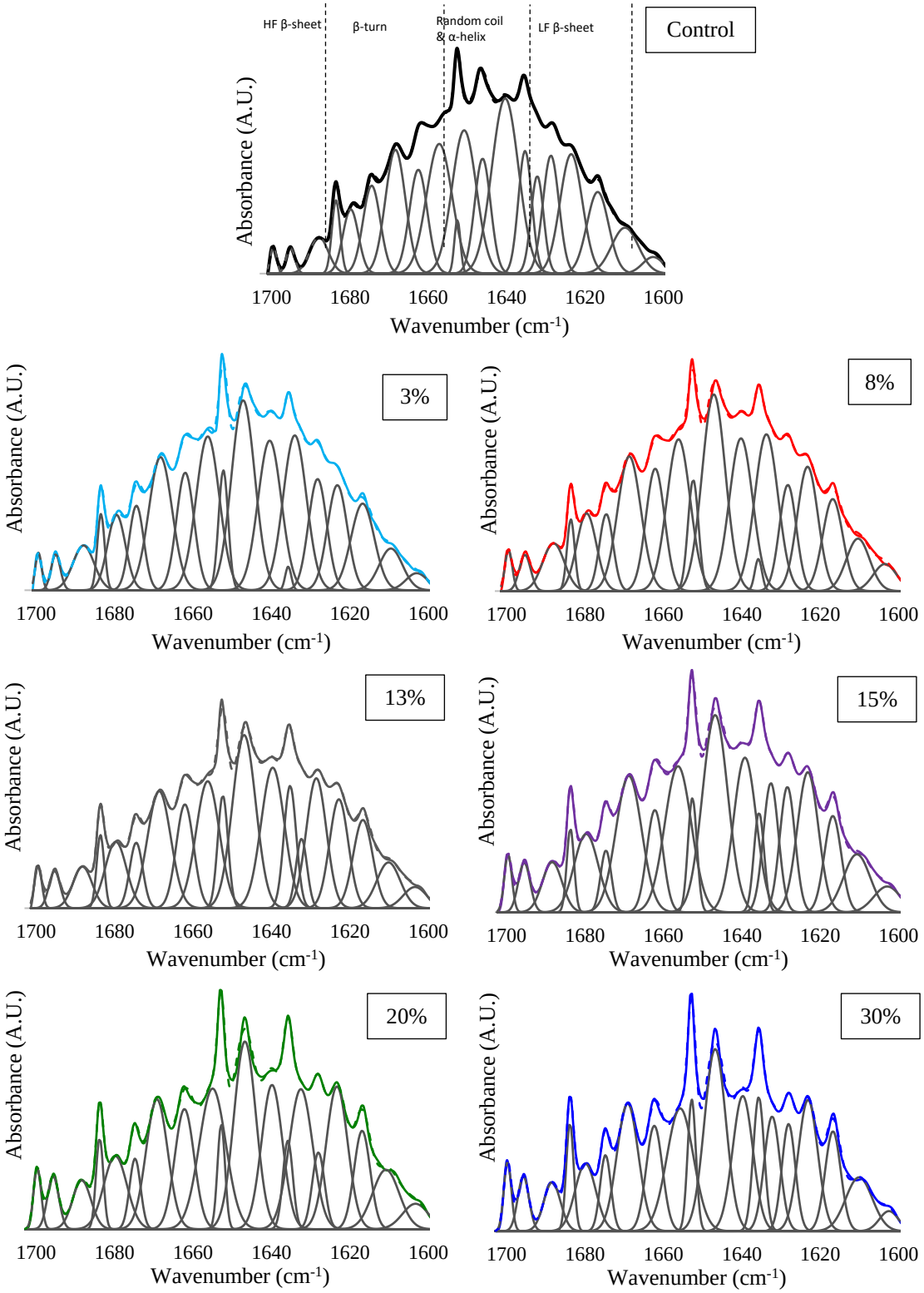


Figure 5. Schematic representation of rice starch internal granule structures. A. Granule structure in native rice flour, with aligned double helices within crystalline lamellae and amylopectin branch points within amorphous lamellae and amylose molecules distribution. B. Granule structure of rice flour microwaved at 8% MC, with increased proportion of amylose chains. C. Granule structure of rice flour microwaved at 30% MC, with increased proportion of amylose chains, increased amount of amylose-amylose, amylose-amylopectin and amylose-lipid interactions, and disruption of crystallites and partial gelatinization of some less heat stable molecules.



Supplementary Figure 1. Deconvoluted Amide I bands of the studied flours. Band assignment correspond to: β-sheet (high frequency) (1700 – 1690 cm^{-1}), β-turns (1690 – 1665 cm^{-1}), random coil & α-helix (1665 – 1640 cm^{-1}) and β-sheet (low frequency (1640 – 1615 cm^{-1}). The continuous line corresponds to the deconvoluted spectra and the discontinuous line to the fitted curve.