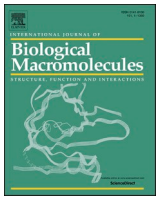
International Journal of Biological Macromolecules 316 (2025) 144395

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Molecular modifications in cereal and tuber starches induced by microwave treatment: A comprehensive analysis using asymmetric flow field-flow fractionation☆

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ARTICLE INFO

Keywords:
Asymmetric flow field-flow fractionation
Multi-angle light scattering detection
Physical modification
Molecular structure
Heat-moisture treatment
Modified starch

ABSTRACT

This study investigated the impact of microwave treatment (MWT) on the molecular structure of starches from cereal (normal and waxy rice, normal and waxy maize, and wheat) and tuber (potato and tapioca) sources using asymmetric flow field-flow fractionation (AF4) coupled with multi-angle light scattering and differential refractive index detectors. The starches were treated under the same conditions (7.5 W/g, 25 % moisture, 100 °C, 30 min). In addition to conventional parameters such as number- and weight-average molecular masses, radii, and dispersity, novel metrics, including mass-to-radius ratios, apparent density, and concentration profiles related to molecular mass, were employed to elucidate structural transformations. The results revealed that non-waxy cereal starches exhibited greater resistance to hydrolysis owing to higher structural compaction, whereas waxy cereals experienced homogenisation of molecular radii and apparent density. In contrast, tuber starches, particularly potato, underwent molecular aggregation, forming homogeneous structures with low dispersity and a high concentration of large particles. This study introduces a novel approach for evaluating and understanding the molecular structural modifications in starch induced by MWT and how the original matrix affects these modifications, providing valuable insights for future research and applications of this physical modification process.

1. Introduction

Starch is a key component in the food industry, serving as a primary source of energy and functioning as a thickening, stabilising, or gelling agent in food products [1]. Different types of starches have varying amounts and molecular structures of their main components, amylopectin and amylose, which determine their suitability for specific food applications [2]. For example, waxy starches, with low or negligible amylose content, are utilised in the preparation of sauces and fillings because their low retrogradation ensures texture maintenance during storage [3], whereas normal rice, normal maize, tapioca, and potato starches are widely used in gluten-free bakery products [4].

Although native starches are generally perceived as safe and natural by consumers, they have limitations under extreme processing conditions [5]. For instance, they present low stability at elevated temperatures and low pH levels and a tendency to retrograde at low temperatures, which can adversely affect the quality of the final product [5]. Physical modification of starch is one approach to overcome these problems, presenting the advantage of producing a safe and "clean label" ingredient with no legal restrictions on its use [4]. Microwave treatment (MWT) offers advantages over other physical processes, including reduced treatment time and improved yield and product quality [6]. Microwave-modified starches have been used to improve food characteristics, such as reduced bread hardening, decreased digestibility, increased thermal stability, reduced cooking times, and increased gluten-free product stability [7].

The characteristics of starch obtained after MWT differ depending on the treatment conditions and the intrinsic characteristics of the treated matrix, including its botanical origin, variety, crystalline structure, and amylose content [8,9]. Therefore, understanding the molecular and

☆ This article is part of a Special issue entitled: 'Starch and Starch Derivatives: S, P, & N.' published in International Journal of Biological Macromolecules.

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https://doi.org/10.1016/j.ijbiomac.2025.144395
Received 5 February 2025; Received in revised form 6 May 2025; Accepted 18 May 2025
Available online 19 May 2025

structural features of the original starches and the resulting alterations after MWT is crucial for their application in food processing. The fine molecular structure of starch can be correlated with its thermal, pasting, digestibility, and retrogradation properties, thus improving food product design [10,11]. In this sense, techniques such as size exclusion chromatography (SEC) allow for the accurate characterisation of parameters such as the average molar mass and size of debranched starches [12]. However, the importance of studying whole starch molecules should not be overlooked because of their impact on the techno-functional properties of starch. In this sense, SEC has limitations in measuring whole amylopectin molecules, as they can undergo shear degradation when passing through the stationary phase of the SEC column, resulting in structural alterations [13].

Asymmetric flow field-flow fractionation (AF4) emerges as a powerful alternative for the separation and characterisation of macromolecules. This technique enables the separation of analytes ranging from a few nanometres to micrometres without using a stationary phase [14]. By utilising two perpendicular flows, channel flow and crossflow, AF4 allows the separation of complex molecules, such as starch, while preserving their structural integrity [15]. AF4 has recently been employed to characterise starches from various sources [15–17]. However, to the best of our knowledge, this has not yet been used to determine the impact of the MWT. AF4 is particularly advantageous detecting molecular transformations, such as hydrolysis or aggregation, that may occur during MWT, as it substantially preserves the overall structure of macromolecules. In contrast to other separation methods, such as SEC, which can potentially alter treatment-induced molecular configurations, AF4 enables a more precise assessment of these events.

This study aimed to investigate the potential of MWT to modify the molecular structure of starches and how their original structure influences this impact, that is, which molecular characteristics of starches make them more vulnerable to MWT. Starches of different origins were included in the study: waxy rice, normal rice, waxy maize, normal maize, potato, tapioca, and wheat starch. Those starches were selected not only for their high industrial relevance as key sources of cereals and tubers, but also for their structural differences derived from botanical origin and amylose content, which influence their functionality. The AF4 technique, coupled with multi-angle light scattering (MALS) and differential refractive index (dRI) detectors, was applied in an innovative way for the molecular characterisation of starch. This approach allowed not only the quantification of molecular masses and radii but also the identification of starch fractions according to their location in the fractograms, as well as the calculation of concentration distributions, apparent density profiles, and mass-to-radius ratios. These parameters provided a novel assessment of the effects of MWT on starch molecular structure, enabled by new calculations applied to the AF4 results.

2. Materials and methods

2.1. Samples

The starches used in this study, namely normal maize (C*Gel 03401), waxy maize (C*Gel 04201), wheat (C*Gel 20,006), potato (C*Gel 30,002), and tapioca (C*CreamGel 70,001), were manufactured by Cargill Inc. (Minneapolis, MN, USA) and generously donated by Brenntag Química S. A. U. (Dos Hermanas, Seville, Spain). Normal rice starch (Remy DR) and waxy rice starch (Remyline XS) were procured from BENEO GmbH (Mannheim, Germany) and Ferrer Alimentación (Barcelona, Spain), respectively. The starches were $>$ 99 % pure on a dry basis. Moisture content (MC) was analysed using the AACC official method 44–19.01 for both untreated and treated starches [18].

2.2. Sample treatment

Hydrated starch samples that underwent MWT were prepared using an MR-2 L Chopin mixer (Villeneuve-la-Garenne, France). The samples were supplemented with distilled water until the MC reached 25.0 $\pm$ 0.5 %, calculated based on the initial starch moisture. Subsequently, the samples were left for MC equilibration overnight at 4 °C, after which their MC was verified before being transferred to hermetically sealed bags. Finally, the bags were stored at −40 °C until further use. At the time of treatment, each sample was tempered at 25 °C, and 120 g was placed in a 250 mL hermetic and heat-resistant borosilicate glass container. The samples were subjected to heat treatment using a 900 W customised microwave oven (Sharp R-342(IN)W, Osaka, Japan) with computer-controlled temperature regulation. A 30-min program was used to achieve an even temperature increase from the initial value of 25 °C to the treatment temperature of 100 °C in the first 5 min. The temperature was maintained at 100 $\pm$ 3 °C until the end of the test. Temperature and treatment time were selected based on preliminary trials and prior studies on MWT [19], aiming to achieve structural modification while preventing visible damage or browning of the samples. The resulting starches were oven-dried at 35 °C to their original moisture content (~12 %). The samples were then sieved through an ASTM 250 μm mesh sieve. Finally, the samples were sealed in hermetic jars and stored at 4 °C until further testing.

2.3. Amylose content

The amylose concentration was determined by the percentage of glucose from amylose hydrolysis, obtained after selective precipitation of amylopectin with concanavalin A, in the total glucose concentration obtained from complete starch hydrolysis, according to the method described by Gibson et al. [20], using the Megazyme kit K-AMYL 06/18 (Megazyme, Wicklow, Ireland) under the conditions described by Mauro et al. [21]. Analyses were performed in duplicate.

2.4. Molecular mass and molecular radius measurement by AF4- MALS-dRI

The analytical instruments employed in this study included an Agilent 1260 Infinity II liquid chromatography system with an isocratic pump (Agilent Technologies, Waldbronn, Germany) and a vacuum degasser for delivering the carrier flow. The system was linked to an Eclipse WEC-04 (Wyatt Technology, Santa Barbara, CA, USA) which controlled the long-channel-type fractionation channel with the Dilution Control Module (DCM) port. The channel responsible for the molecular separation used a regenerated cellulose membrane with a nominal cut-off of 10 kDa (Merck Millipore), meaning that molecules with a mass below this value will be filtered out during separation and will not reach the detectors. A channel spacer with a nominal thickness of 400 μm (LC400WL, Wyatt) was used in this assay. Two consecutive detectors, MALS (Dawn WD3–04, Wyatt Technology) and dRI (Optilab WOP1–03, Wyatt Technology), were used, both operating at 658 nm. A 1 mg/mL solution of bovine serum albumin (BSA, 66.5 kDa, Sigma Aldrich, St. Louis, MO, USA) was used to verify the molecular mass calculations and adjust the alignment and band broadening of the detectors. A 10 mM sodium nitrate solution containing 200 ppm sodium azide was used as the mobile phase solvent [22]. The samples injected were prepared as follows: approximately 100 mg of each starch sample was dissolved in 3 mL of dimethyl sulfoxide (DMSO, HPLC grade, 99.9 %), heated with stirring at 100 °C for 1 h, and the volume was adjusted to 100 mL with mobile-phase solution. Each sample was analysed in duplicate, with two runs per replicate, and all materials in this study were processed under identical conditions. A blank solution with 3 mL DMSO to 100 mL of final volume was injected between samples (treated and untreated) and used to establish the baseline of the detector signal for the next sample. The method was optimized through preliminary trials to achieve accurate separation of starch macromolecules. Different injection volumes were evaluated, with 180 μL selected as providing optimal signal for medium and low molecular mass components without compromising

resolution of high molecular mass fractions (see supplementary Fig. S1). Several crossflow profiles were also tested, keeping the channel flow rate constant at 1 mL/min. Two profiles were selected for their good separation performance, one with an initial crossflow of 3.5 mL/min for 5 min followed by a linear decay to 0 mL/min over 25 min, and another with an initial crossflow of 4 mL/min for 5 min and an exponential decay over 25 min. The linear decay profile improved the detection of the amylose fraction, giving comparable molecular parameters of the amylopectin fraction to those of the exponential profile (see Supplementary Fig. S2 and Table S1). Therefore, the lineal profile was selected for data analysis. Data were processed using the ASTRA software (v. 8.1.2.1, Wyatt Technology, Santa Barbara, CA, USA). Signals from MALS and dRI were processed applying the first-order Berry formula to calculate the masses and radii [23]. The second virial coefficient (A2) was neglected, and a specific refractive index (dn/dc) of 0.151 mL/g was used [24]. All samples, both treated and untreated, were analysed under the same conditions, as no separation problems or detection errors were observed.

The obtained fractograms were used to calculate the number-average molecular mass (M_n), weight-average molecular mass (M_w), molecular dispersion or polydispersity ($Đ_M$), weight-average molecular radius (r_w), and number-average molecular radius (r_n). To confirm reproducibility, the coefficient of variation was calculated for each parameter and was considered valid if it was <10 %. M_n, M_w, and $Đ_M$ were calculated using the following equations [25,26]:

$$M_n = \frac{\sum N_i M_i}{\sum N_i} = \sum X_i M_i \quad (1)$$

$$M_w = \frac{\sum N_i {M_i}^2}{\sum N_i M_i} = \frac{\sum w_i M_i}{\sum w_i} = \sum W_i M_i \quad (2)$$

$$Đ_M = M_w / M_n \quad (3)$$

where N_i represents the number of molecules of specie i; M_i denotes the molecular weight of specie i, X_i corresponds to the mole fraction of specie i, w_i represents the weight of specie i, and W_i stands for the weight fraction of specie i. The values of r_w, r_n, and $Đ_r$ (radius dispersity, r_w/r_n) were calculated using the same equations, with M_i substituted with r_i.

The apparent density (ρ) for each molecular fraction (i) was calculated from the molecular mass (M_i) and radius of gyration (R_g), assuming a homogeneous distribution and spherical shape of the molecules. The apparent density of each molecular fraction ($\rho_{R_{g,i}}$) was calculated as [14]:

$$\rho_{R_{g,i}} = \frac{M_i}{N_A \times V(R_g)_i} \times q \quad (4)$$

where M_i expressed in kg/mol, $V(R_g)_i$ is the volume of the fraction i (in m^3), N_A is the Avogadro's constant, and q is a constant relating R_g to the hydrodynamic radius (R_h), resulting from the expression [14]:

$$q = \frac{V_{sphere}(R_g)}{V_{sphere}(R_h)} = \frac{{R_g}^3}{{R_h}^3} = \left(\frac{3}{5}\right)^{3/2} \quad (5)$$

2.5. Statistical analysis

Shapiro-Wilk normality tests and statistical analyses of the mean, standard deviation, and analysis of variance (ANOVA) with Tukey's comparison test were conducted using Statgraphics Centurion 19 - Version 19.4.01 (Statgraphics Technologies Inc., The Plains, VA, USA). The calculated average parameters (Amylose content, M_n, M_w, $Đ_M$, r_n, r_w, $Đ_r$, M_n/r_n and M_w/r_w) were statistically analysed by one-way ANOVA considering 14 samples (7 native or untreated and 7 treated). Differences between means were evaluated by Tukey's test at a significance level of $\alpha = 0.05$, with different letters indicating significantly different means. The results presented in the tables and graphs of comparison of means reflect this statistical treatment. The graphs and correlation study in this work were made using OriginPro, Version 2024b (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Amylose content

The amylose content of the samples is presented in Table 1. The untreated samples exhibited amylose concentrations ranging from 2.8 (waxy maize) to 27.4 (potato), values consistent with those reported in previous literature for this method of measurement [1,20,27]. Normal maize, normal rice, tapioca, and wheat showed no significant variation in the amylose content following MWT, maintaining values between 19 and 24 %. For the waxy samples, a small, non-statistically significant variation was observed, with the amylose content ranging between 2 and 4 %. Other authors reported an increase in amylose content after MWT of tef flour [8] and taro starch [28]. These studies suggested that amylopectin chains were broken down during treatment, resulting in the formation of linear amylose chains. The lack of significant variation in amylose content observed after MWT in our study suggests that, in case of hydrolysis of the molecules due to MWT, this breakdown must have produced branched molecules from amylopectin, or a fractionation of amylose resulting in smaller linear dextrins without variation in the final weight of amylose content determined with Concanavalin A method. Potato starch was the only sample that demonstrated a statistically significant decrease in amylose concentration after MWT, dropping from 27.4 % in the untreated sample to 22.9 % in the treated one. Similar results were reported by Villanueva et al. [29] for potato starch subjected to MWT. These authors attributed the decrease in amylose content to the partial aggregation of amylose and amylopectin molecules after MWT, leading to their co-precipitation upon the addition of Concanavalin A, which was targeted to precipitate only amylopectin. This behaviour seems to be specific to potato starch and can be explained by two factors: (1) its less compact structure resulting from its B-type crystalline arrangement [30], and (2) the presence of phosphate groups [29,31]. The less compact crystalline structure of potato starch compared to other starches facilitates increased molecular interaction after treatment-induced restructuring, which favours aggregation (see Section 3.4) and consequently co-precipitation of amylose with amylopectin in the presence of concanavalin A. This causes an underestimation of linear dextrins and an apparent decrease in amylose content. In contrast, in native potato starch, this aggregation does not occur due to electrostatic repulsion between the phosphate groups, an effect that is significantly attenuated after treatment [29].

Table 1
Amylose content of untreated and microwave-treated starches.

Sample		Amylose content (%)
Normal Rice	Untreated	19.3 ± 0.9 C
	Treated	19.8 ± 0.1 CD
Waxy Rice	Untreated	2.9 ± 0.1 AB
	Treated	1.8 ± 0.0 A
Normal Maize	Untreated	23.7 ± 0.1 G
	Treated	23.0 ± 0.1 FG
Waxy Maize	Untreated	2.8 ± 0.0 AB
	Treated	4.3 ± 0.3 B
Wheat	Untreated	22.2 ± 0.9 EFG
	Treated	21.6 ± 0.6 DEF
Potato	Untreated	27.4 ± 0.3 H
	Treated	22.9 ± 0.3 FG
Tapioca	Untreated	21.6 ± 0.7 DEF
	Treated	20.8 ± 0.4 CDE

Data presented are means ± standard deviations. Different letters within each column indicate significant differences between means ($p < 0.05$, Tukey test).

3.2. Fractograms and molecular mass and radius profiles

3.2.1. Fractograms

Fig. 1 shows the fractograms of the untreated and treated samples obtained from AF4-MALS-dRI analysis. The fractograms were divided into three distinct zones for evaluation purposes, which can be distinguished in Fig. 1 by the different colours used in the molecular mass profiles. Zone 1 corresponds to the region between ~10 min and 20–30 min (depending on the starch) and is indicative of the amylose fraction. Zone 2 is an intermediate region containing unresolved fractions of both high-molecular-mass amylose and low-molecular-mass amylopectin, that begins at the end of zone 1 and goes up to zone 3.

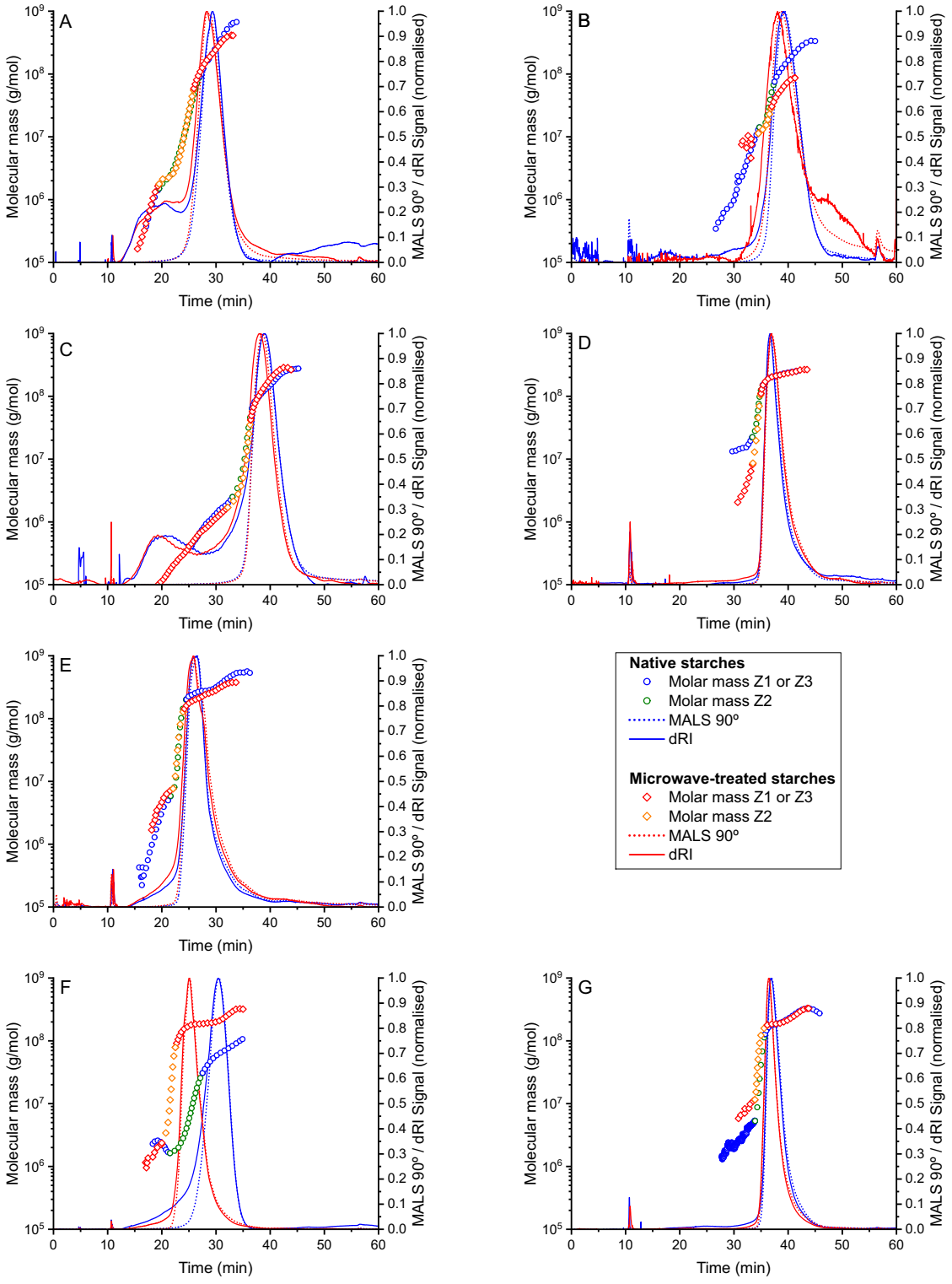

Fig. 1. Fractograms from AF4 showing molecular mass of zones 1, 2, and 3 (Z1, Z2, and Z3), as determined by MALS-dRI detection of untreated (blue/green circles) and modified (red/orange diamonds) starches in normal rice (A) waxy rice (B), normal maize (C), waxy maize (D), wheat (E), potato (F), and tapioca (G). The secondary axes display the normalised MALS (dotted line) and dRI (continuous line) signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Zone 3 corresponds to the high-molecular-mass molecules, which comprise the main amylopectin fractions of the starch sample. Zone 3 includes the main peaks of both detectors (MALS and dRI), as amylopectin was the molecule with the highest proportion and size in the samples measured. Recovery was always above 85 % for the samples tested. Untreated starches showed retention times like those of their corresponding treated counterparts, with slight variations in the signals of MALS and dRI detectors (see Fig. 1). The only exception was potato, which showed an earlier retention time for the main peak (MALS and dRI signals) of the treated starch.

Derived from the fractograms of zones 1 and 3, the M_n (number-average molecular mass), M_w (weight-average molecular mass), and $Đ_M$ (molecular mass dispersity or polydispersity) were obtained and shown in Fig. 2. Fig. 3 shows the parameters derived from the molecular radius including r_n (number-average molecular radius), r_w (weight-average molecular radius), and the $Đ_r$ (analogous to the molecular dispersity, $Đ_M$, but related to the average molecular radii instead of mass).

Despite the limitations of AF4 in characterising small amylose molecules, this technique offers a key advantage in detecting possible interactions between low molecular mass molecules after treatment. Although these interactions cannot be fully distinguished from amylopectin fragments of similar molecular mass, differences in apparent density and mass-to-radius ratio allow partial differentiation in this study, highlighting the potential of AF4 to provide relevant structural information.

3.2.2. Zone 3 molecular masses and radii

The results of the evaluation of the molecular mass of untreated starches in zone 3 (associated with amylopectin) are in agreement with previous studies reporting amylopectin molecular masses in the range of 10^7 to 10^9 g/mol determined by AF4 [14]. Although there is agreement in the order of magnitude, comparisons between studies must consider factors such as differences between varieties of the same species [16] and methodological aspects, including the technique used (SEC or AF4) [32], and sample pretreatment conditions [33]. In the comparison of amylopectin molecular masses obtained under similar analytical and sample preparation conditions, the values determined in our study are consistent with those previously reported for starches from maize (normal or waxy) [16,32], wheat [32], potato [1,32], and tapioca [1].

The MWT caused significant changes in the molecular masses and radii of the starches in zone 3, with different effects depending on the starch source. Except tapioca and waxy maize, that hardly varied with treatment, the rest of starches showed significant differences in the mass (Fig. 2D and E) and size (Fig. 3D and E) of amylopectin after the treatment.

The most widespread effect of MWT in zone 3 was a significant reduction in the molecular mass and/or radius, suggesting the amylopectin degradation during the treatment. Wheat, normal maize, waxy

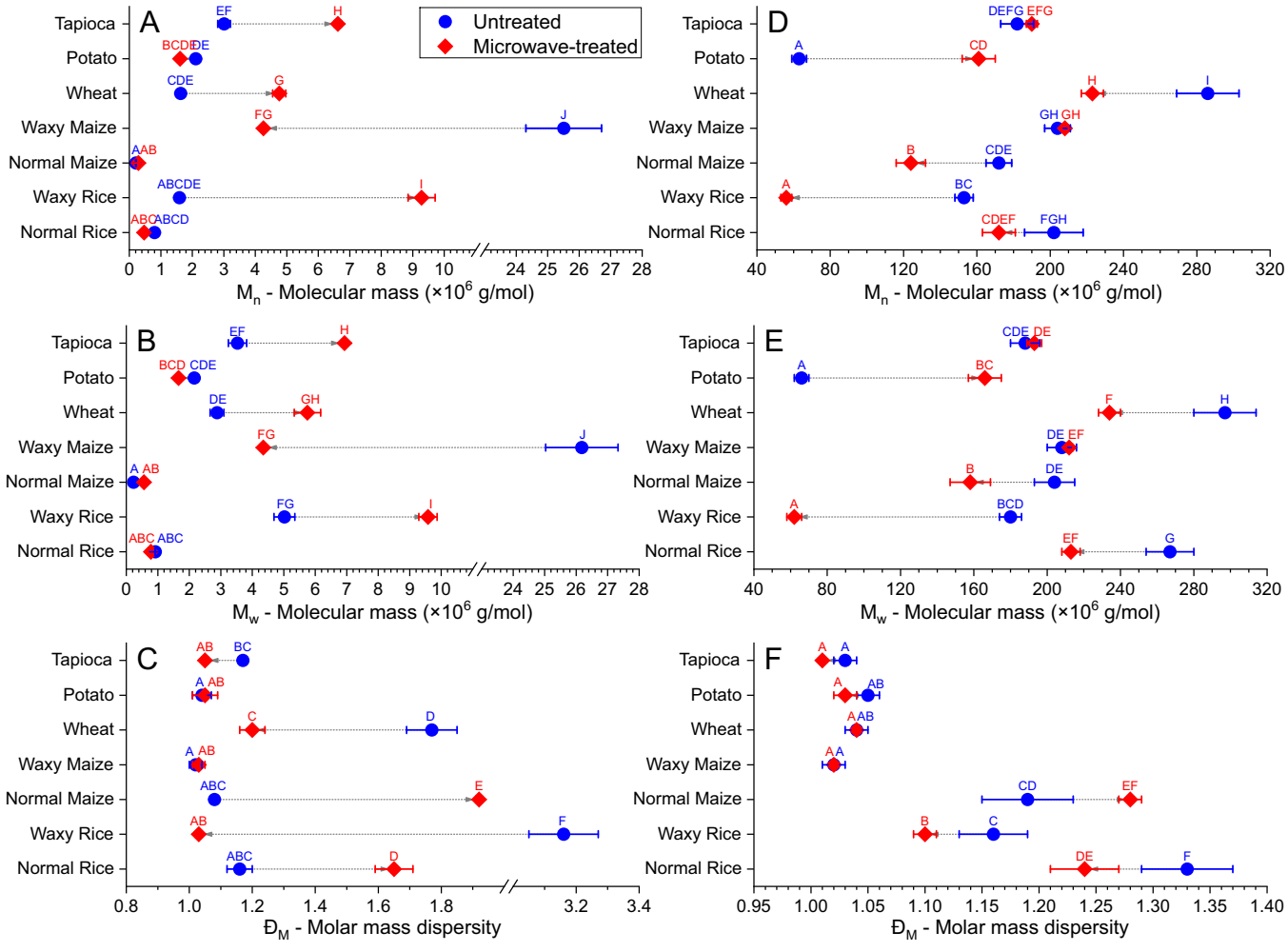

Fig. 2. Number and weight-average molecular masses (M_n and M_w, respectively) and molecular dispersity of untreated (blue circle) and microwave-treated (red diamond) starches in zone 1 (A, B and C) and zone 3 (D, E and F).The letters correspond to Tukey's test statistic, with different letters indicating statistically significant differences in the means at the $\alpha = 0.05$ level of significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

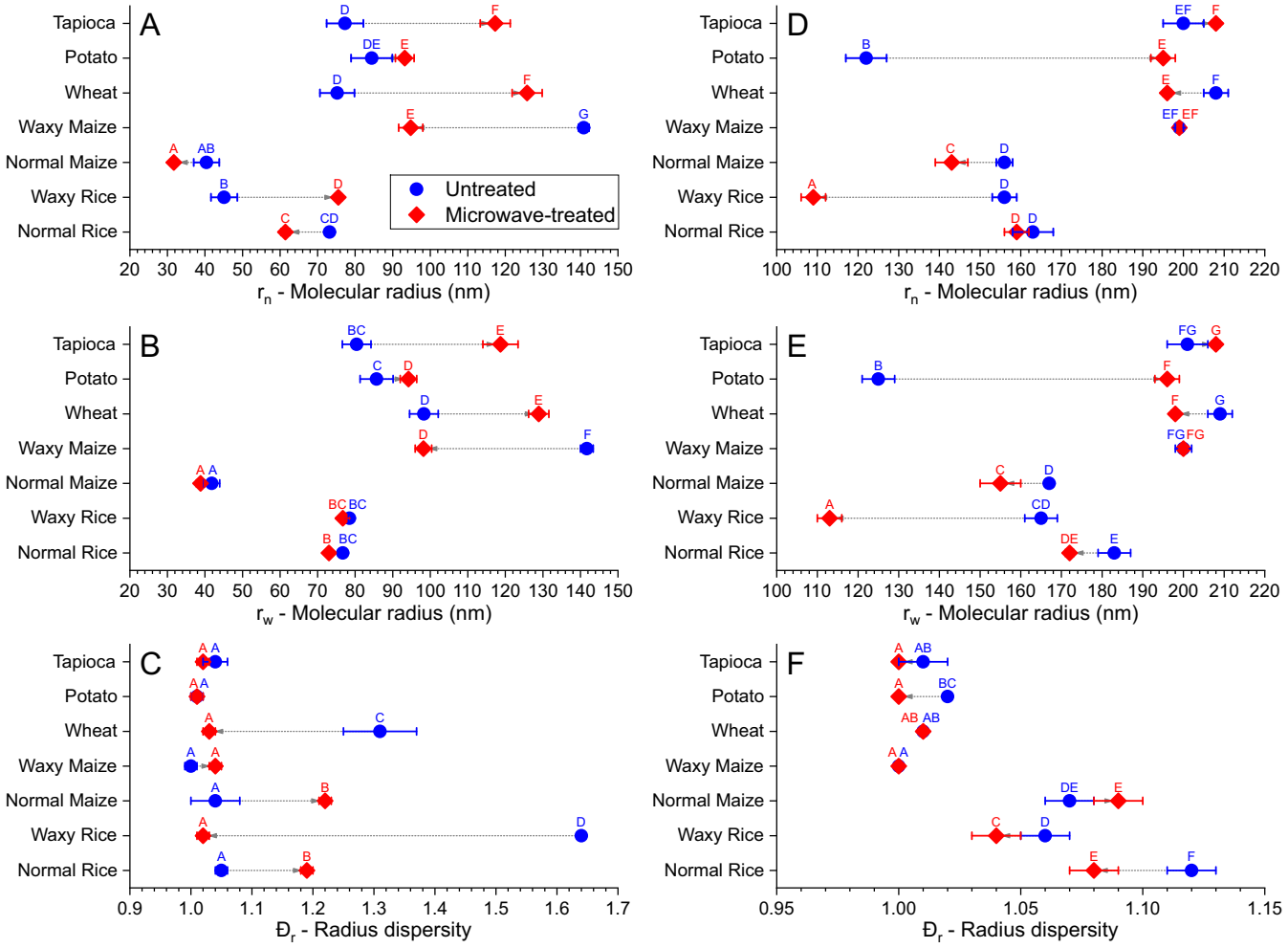

Fig. 3. Number- and weight-average molecular radius (r_n and r_w, respectively) and r_w/r_n ratio of untreated (blue circle) and treated (red diamond) starches in zone 1 (A, B and C) and zone 3 (D, E and F). The letters correspond to Tukey's test statistic, with different letters indicating statistically significant differences in the means at the $\alpha = 0.05$ level of significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rice, and normal rice showed reduced zone 3 molecular masses (Fig. 2D and E) and radii (Fig. 3D and E) after MWT. In the case of normal rice, this reduction was only significant in terms of molecular mass. Waxy rice experienced the greatest decrease in amylopectin average molecular mass that varied from 153×10^6 to 56×10^6 g/mol for M_n, and from 128×10^6 to 46×10^6 g/mol for M_w (Fig. 2D, E and F), as well as the largest decrease in average radii, highlighting the greater susceptibility of this sample to be modified by the MWT.

The effects of MWT on starches have been reported to vary depending on factors related to the sample to be treated, such as botanical origin, amylose content, proportion and length of amylose molecules, and amylopectin structure, as well as MWT conditions like hydration, time, and temperature. Despite variations in results due to these factors, in general, a consistent reduction in molecular mass of amylopectin has been reported [6,34,35], which aligns with our findings. Previous studies have described that amylopectin hydrolysis occurs first on the internal chains, then within the amorphous region, and subsequently on the external chains, in the crystalline region. Furthermore, a preferential cleavage of α-(1,6)-glycosidic bonds over α-(1,4)-glycosidic bonds has been observed, probably due to the lower steric strength of the former [6]. Additionally, the capacity of the resulting fragments to reorganise into new structures played a significant role in the obtained modifications [35]. Our study shows that the susceptibility of amylopectin to hydrolysis and its tendency to aggregate after MWT are influenced by the resistance of the sample to modification. These susceptibilities are further analysed through factors like molecular compactness and apparent density (see Section 3.4) and initial characteristics (see Section 3.5).

The waxy and normal samples of rice and maize were used to evaluate the role of amylose in MWT-induced structural modifications of amylopectin. Waxy samples showed greater modification than their normal counterparts, suggesting that the presence of amylose reduces susceptibility to MWT-induced hydrolysis. This could be attributed to the reduction in granule hydration capacity due to the presence of linear amylose molecules [21,36]. However, differences were noted between the modifications in zone 3 of the waxy rice and waxy maize samples. Waxy rice underwent greater modification, while waxy maize only showed a reduction in the concentration of high molecular mass molecules (see Section 3.3), with minimal changes in average molecular mass. These differences could be related to the granular characteristics and hydration properties of each starch. Previous studies have shown that waxy maize has the lowest water absorption capacity and swelling power, while waxy rice has the highest of the starches studied [21]. This observed higher susceptibility to MWT-induced transformation could be linked to the internal structure of the amylopectin molecules in each starch and correlated with its water absorption capacity. Maize starch, with higher proportion of long amylopectin chains (>36 glucose units), presents a more rigid structure, higher relative crystallinity, and more stable double helices, resulting in granules that are more resistant to swelling, than rice starch, which has a higher proportion of short

amylopectin chains (6–24 glucose units) [36].

Potato starch showed the most remarkable modification, but with the opposite effect compared to the rest of the samples, showing higher molecular masses (Fig. 2D and E) and radii (Fig. 3D and E) in zone 3 after MWT. For instance, the r_n and r_w increased with MWT from 122 nm to 195 nm. It is worth noting that potato presented the lowest molar mass (Fig. 2D and E) and molar radius (Fig. 3D and E) in zone 3 among the untreated starches, despite having a large starch granule size [1]. The increase in mass and size could be attributed to treatment-induced aggregation of amylopectin molecules, which would be consistent with reduced hydrolysis [35,37]. The low compaction of the B-type structure present in potato starch would facilitate the interaction between the molecules and their aggregation (see Section 3.4). In addition, potato starch has one of the lowest swelling power values among the samples [21], which could contribute to the low impact of irradiation and the limited extent of hydrolysis.

The impact of MWT on $Đ_M$ (Figs. 2F) and the $Đ_r$ (Figs. 3F) for zone 3 was also observed to be different depending on the starch studied. $Đ_M$ was unaffected by MWT for samples with an initial value close to 1 (tapioca, potato, wheat, and waxy maize). However, for samples with higher initial molecular dispersity (1.15–1.35), $Đ_M$ varied significantly after MWT. Normal and waxy rice starches showed a reduction in molecular dispersity, whereas normal maize starch showed an increase. A decrease in molecular dispersity because of MWT indicates that molecules at the boundaries of the mass distribution, that is, larger and smaller molecules, are more susceptible to modification by MWT. Conversely, the increase in molecular dispersity as happened with normal maize, indicates a more random rupture of the molecules. The $Đ_r$ in zone 3 was closer to one (1.00–1.12) for all untreated starches, denoting that their molecular sizes were more uniform than their masses. Only slight reductions in the $Đ_r$ were recorded for potato, waxy rice, and normal rice after MWT, which is in line with their molecular dispersity results.

3.2.3. Zone 1 molecular masses and radii

The molecular masses found in zone 1 (amylose-related) were like those previously reported for amylose molecules of starches, between 10^5 and 10^6 Da [14,16,38]. The normal maize, normal rice and, to a lesser extent, wheat starch, exhibited a clear amylose peak formed in the zone 1, as evidenced by the dRI signal (continuous lines in Fig. 1A, C, and E). The untreated normal maize and normal rice samples showed the lowest M_n (0.21×10^6 and 0.80×10^6 Da respectively) and M_w (0.23×10^6 and 0.92×10^6 Da) values in zone 1 among the samples analysed (see Fig. 2A and B). On the opposite, the small amount of zone 1-molecules in the untreated waxy maize sample presented molecular sizes between 24 and 26 $\times 10^6$ Da (Fig. 2A and B), significantly higher than expected for amylose molecules. Consistent with this observation, the r_n and r_w values of waxy maize, 141 and 142 nm, respectively (Fig. 3A and B), were also much higher than those obtained for the rest of starches studied in zone 1. These molecules can likely be attributed to very small amylopectin populations with structural differences sufficient to distinguish them from those in zone 3. Additionally, these results may also reflect the presence of amylose molecular aggregates or amylose-amylopectin interactions. $Đ_M$ and $Đ_r$ in zone 1 were close to 1 in all untreated samples except in wheat and waxy rice, which values were 1.3 and 3.15 (for $Đ_M$) and 1.31 and 1.64 (for $Đ_r$), respectively (Figs. 2C and 3C).

MWT caused diverse effects in zone 1 molecular mass, radius and dispersity depending on the starch source. Potato, normal maize and normal rice did not show significant changes in zone 1 as result of treatment (Figs. 2A, B, 3A and B). This would be the expectable result because most of the MWT-mediated hydrolysis occurs at branching points and not in linear segments [39]. However, tapioca, wheat and waxy rice starches showed an increase in their masses and sizes as result of MWT. This could be related to the appearance of fragments of larger molecules formed during MWT, or to the possible aggregation between amylose molecules induced by the treatment (Fig. 2A and B). The latter is plausible for linear molecules, which can form double helices more easily than branched molecules [40]. MWT affected the size of molecules of zone 1 for tapioca, wheat and waxy rice in a similar way to how it affected to their masses, with the sole exception of waxy rice, which maintained its r_w value (Fig. 3A and B). These starches also showed a decrease in zone 1 of the molecular dispersity, both in terms of mass and radius ($Đ_M$ and $Đ_r$) (Figs. 2C and 3C) denoting higher uniformity in molecular masses and radii after the treatment.

After MWT, waxy maize showed a significant decrease ($p < 0.05$) in average masses and radii in zone 1 (Figs. 2A, B, 3A and B). Since there was no significant change ($p > 0.05$) in the molecules of zone 3 (Figs. 2D, E, 3D and E), the changes were likely due to hydrolysis or disaggregation of large molecules in zone 1. This is particularly evident in the fractograms of this starch (Fig. 1D), where zone 1 suffered the greatest reduction in mass after treatment. This phenomenon may be explained by the unique characteristics of the molecules in this fraction, which may be associated with particularly small amylopectin molecules or amylose aggregates. Hydrolysis may have been facilitated by the lower density of these large structures, together with their possible greater exposure to hydration and the hydrolytic effect of microwave radiation.

Normal maize and normal rice samples showed significant ($p < 0.05$) increase in $Đ_M$ and $Đ_r$ after MWT, despite showing no significant effect on molecular mass and radius ($p > 0.05$). The molecular masses and radii in zone 1 for these samples were among the lowest of the studied samples; so small and non-significant variations in M_w, M_n, r_w, or r_n may have led to a significant change in their relationships (M_w/M_n and r_w/r_n).

It has been suggested that the α(1,6) linkages in starch molecules, predominantly present in amylopectin, are the most susceptible to hydrolysis [39]. In contrast, the α(1,4) linkages have a lower cleavage rate, probably due to their involvement in the formation of single or double helices, giving them greater resistance to degradation and a greater tendency to form aggregates after treatment [35]. The limited hydrolysis of amylose, the presence of amylopectin fragments with a molecular mass similar to amylose, the potential aggregation detected, and the limitations of AF4 in measuring very low molecular mass molecules, limits direct comparations of MWT effects in this study with those reported in the literature. This factors also require caution in interpreting the results. Despite these limitations, AF4 proves valuable in detecting treatment-induced aggregates that other techniques might miss.

3.3. Molecular concentration profiles

Fig. 4 shows the concentration of the starch molecules, expressed in g/100 g, as a function of molecular mass for each sample. Previous studies have performed this type of representation with SEC chromatograms, generally using a calibration curve with pullulan standards, and estimating the concentrations of the fractions using the Mark-Houwink equation. To the best of our knowledge, this representation has not been performed in AF4, as the concentrations have usually been calculated with respect to the retention time, rather than the molecular mass [16].

The untreated starch samples showed diverse concentration profiles. Waxy rice and maize starches exhibited a high concentration of high molecular mass fractions (>10^8 Da) and very low concentrations of molecular masses below 10^8 Da, as expected for samples with low amylose content (~3 %) (Fig. 4B and D). Normal cereals (rice, maize, and wheat) and potato starches showed significant concentrations in the lower molecular mass fractions (10^6–10^8 Da), as expected for their amylose content (19–27 %) (Fig. 4A, C, E, and F). However, tapioca starch showed a low concentration of the lower molecular mass fractions (Fig. 4G), despite its high amylose content (22 %). The absence of an appreciable concentration of low molecular mass molecules may be indicative of a certain degree of aggregation between molecules, leading to higher concentrations of higher molecular masses. Some amylose

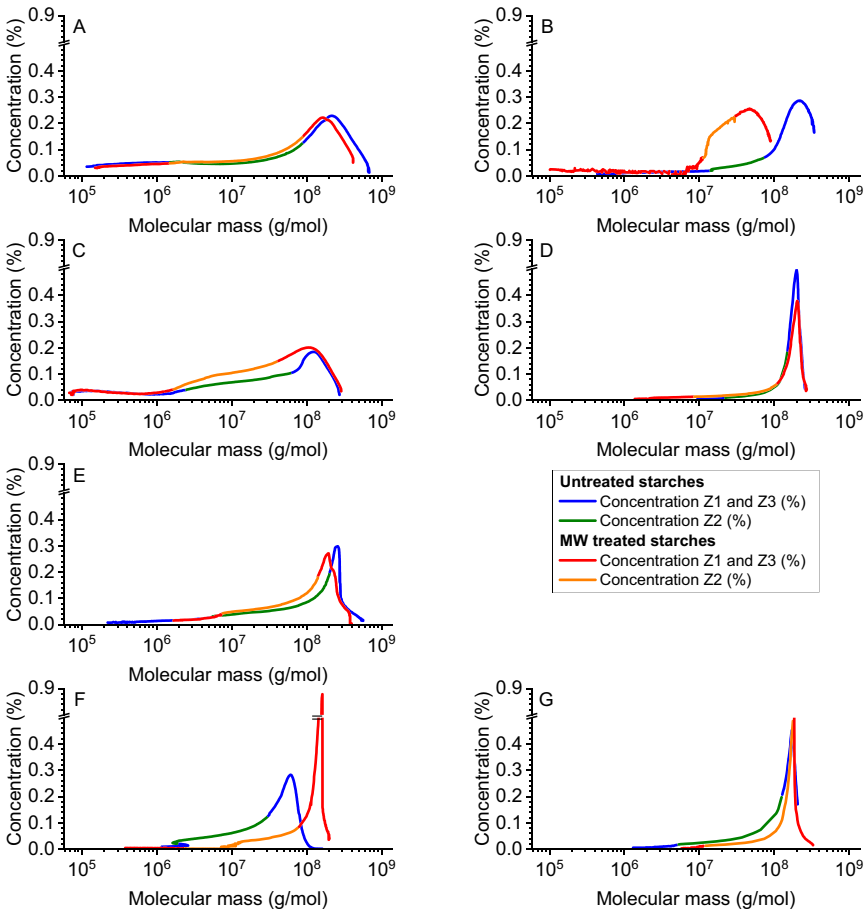

Fig. 4. Concentration of molecules as a function of molecular mass for untreated and microwave-treated starches of normal rice (A), waxy rice (B), normal maize (C), waxy maize (D), wheat (E), potato (F), and tapioca (G).

molecules aggregate more easily because the linear structures have less steric hindrance to assembly [41].

The effect of MWT on starch concentration profiles varied significantly depending on the starch source. Cereals exhibited in general (except normal maize) a decrease in the concentration of the molecules of higher molecular mass (~10^8 Da) (Fig. 4A, B, D and E) and at the same time (except waxy maize) a shift of the peak towards the left, denoting a reduction of the molecular mass of the larger molecules. This shift was more marked for waxy rice starch, to the extent that the initial higher-molecular-mass fractions disappeared in the treated samples (Fig. 4B). This agrees with the observations made in the Section 3.2, where a strong reduction of >60 % was observed in the average molecular mass of zone 3 in waxy rice. Waxy maize (Fig. 4D) showed the most marked reduction in maximum peak height, but without varying its position. However, normal maize showed a particular behaviour among cereal starches, with a relative increase in concentration in the region between 10^6 and 10^8 Da, because of the relative reduction in the molecules of the highest and lowest molecular masses (Fig. 4C). This could be related to the combination of hydrolysis and aggregation of the molecules in agreement with the increase in the molecular mass dispersity of zone 3, as observed in Fig. 2F and discussed in Section 3.2.

In contrast to the cereal samples, potato and tapioca showed increased concentrations in the highest molecular mass fraction (Fig. 4F and G) after MWT. At the same time, potato starch showed a shift of the peak towards the right, denoting the appearance of higher molecular mass molecules at a relatively high concentration (Fig. 4F). This observation, along with the increased average molecular masses in zone 3 (Fig. 2D and E), corroborates that MWT caused aggregation of the molecules in the case of potato starch. In tapioca, the increase in the concentration of the main peak occurred at the expense of a decrease in the concentration of the lower molecular mass fractions, analogous to what was observed in potato starch. This may be explained by a greater tendency for aggregation in these samples, facilitated by MWT. In tapioca, the maintenance of the maximum concentration peak at approximately the same molecular mass fraction as in the untreated sample is consistent with the conservation of the average molecular masses of zone 3 (Fig. 2D and E).

3.4. Apparent density and molecular mass-to-size ratio profiles

The apparent densities as a function of molecular mass of untreated and treated samples are plotted in Fig. 5. The study of apparent density is a useful parameter for assessing the structure-function relationship of the analysed material [15]. This provides information on the compactness of the molecule, and it has been suggested that a higher apparent density may correlate with increased antidiabetic activity, as the more compact molecules are less accessible to the enzymes that break down starch and release glucose into the bloodstream [42]. The relationships between the average molecular masses and the average molecular radii (M_n/r_n and M_w/r_w) of zones 1 and 3 were also calculated and are shown in Table 2. These ratios were used as additional and simpler parameters to analyse the degree of molecular compaction and as valid indicators of molecular density. The M_n/r_n and M_w/r_w ratios were higher in zone 3 (mainly referred to highly branched amylopectin molecules) compared to zone 1 (mainly linear amylose molecules) for all starches. This agrees with previous measurements of molecular density that demonstrated highly branched structures have a higher degree of compaction than lower branched ones [43].

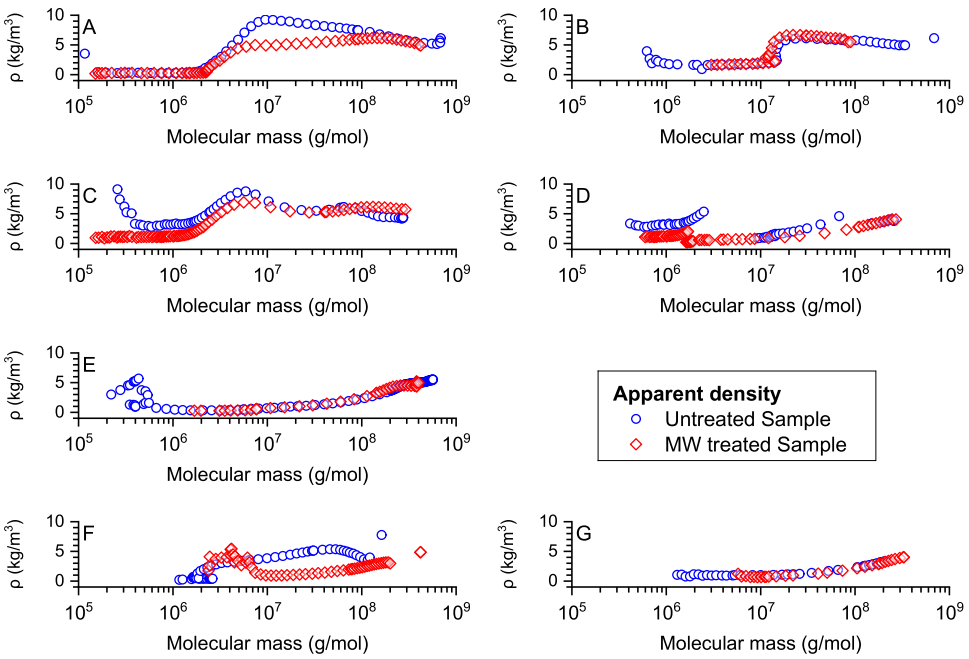

Fig. 5. Distribution of apparent densities (ρ) as a function of molecular mass for untreated and treated starches of normal rice (A), waxy rice (B), normal maize (C), waxy maize (D), wheat (E), potato (F), and tapioca (G).

Table 2
Relationship between molecular masses and sizes of amylose and amylopectin.

Sample		M_n/r_n (Z1)	M_w/r_w (Z1)	M_n/r_n (Z3)	M_w/r_w (Z3)
Normal Rice	Untreated	10.9 ± 0.1 AB	12.0 ± 0.1 AB	1233 ± 55 G	1459 ± 39 G
	Treated	7.7 ± 0.1 AB	10.7 ± 0.1 AB	1081 ± 34 DEF	1236 ± 19 F
Waxy Rice	Untreated	35.3 ± 1.7 D	67.1 ± 0.0 E	979 ± 10 CD	1084 ± 8 DE
	Treated	123.0 ± 4.2 F	124.9 ± 3.5 F	516 ± 17 A	547 ± 21 A
Normal Maize	Untreated	5.3 ± 0.3 A	5.6 ± 0.2 A	1102 ± 50 EF	1223 ± 69 F
	Treated	9.1 ± 0.3 AB	14.3 ± 0.3 AB	866 ± 30 BC	1020 ± 41 CD
Waxy Maize	Untreated	180.9 ± 6.7 G	184.8 ± 5.9 G	1025 ± 25 DE	1040 ± 30 D
	Treated	44.9 ± 1.4 D	44.4 ± 0.2 D	1047 ± 7 DEF	1063 ± 9 D
Wheat	Untreated	21.7 ± 0.5 C	29.9 ± 1.7 C	1377 ± 58 H	1417 ± 58 G
	Treated	37.8 ± 1.1 D	44.6 ± 3.6 D	1142 ± 25 FG	1185 ± 25 EF
Potato	Untreated	23.8 ± 0.6 C	24.4 ± 0.1 BC	513 ± 18 A	528 ± 16 A
	Treated	17.0 ± 0.1 BC	17.5 ± 0.4 B	822 ± 34 B	845 ± 34 B
Tapioca	Untreated	40.4 ± 1.7 D	45.1 ± 3.0 D	908 ± 25 BC	934 ± 19 BC
	Treated	56.1 ± 0.8 E	58.3 ± 2.1 E	916 ± 12 BC	926 ± 14 BC

M_n: number-average molecular mass, r_n: number-average molecular radius, M_w: weight-average molecular mass, r_w: weight-average molecular radius. Z1: zone 1 region, Z3: zone 3 region. The values in the table are expressed in kDa/nm. Data presented are means ± standard deviations. Different letters within each column indicate significant differences between means ($p < 0.05$, Tukey test).

In zone 1, the lowest mass-to-radius ratio in untreated starches were observed in non-waxy cereals, in the order: normal maize < normal rice < wheat (Table 2). Potato showed values similar to wheat, while tapioca and waxy cereals had the highest ratios. Waxy maize values were significantly higher in zone 1, nearly 450 % for M_n/r_n and over 300 % for M_w/r_w, compared to the second highest, tapioca. This very high value of the mass-to-radius ratio in the case of waxy maize reinforce the hypothesis that the molecules found in zone 1 contains populations of smaller amylopectin molecules, fragments of amylopectin or small aggregates.

In zone 3, the untreated non-waxy cereal samples exhibited the highest amylopectin-related M_n/r_n or M_w/r_w values, in the order: wheat > normal rice > normal maize. This is consistent with their A-type crystal structure with a compact orthorhombic organisation [44]. After them, the order was waxy maize > waxy rice > tapioca > potato. The lowest Mw/r_w and M_n/r_n ratios in the amylopectin zone for potato starch can be related to the presence of B-type crystallinity with a less compact hexagonal organisation. In potato starch, the presence of high amounts of short and long amylopectin chains, together with low amounts of intermediate chains, may explain its less compact packing, as the short chains cannot form double helices and the long chains enter the crystalline zone, causing a change in structure [30].

MWT led to different changes in mass/size ratios depending on starch source and starch fraction, confirming the different susceptibilities of the starch molecules to modifications due to MWT. Changes in mass-to-radius ratios or apparent densities can occur for several reasons: (1) hydrolysis of large molecules, such as amylopectin, may result in a loss of mass but with preservation of the size; (2) some molecules that have resisted hydrolysis may have lost their crystalline structure and become deformed and enlarged, i.e. same mass in increased volume; (3) non-hydrolysed molecules may have rearranged their structure and become more compact.

Following MWT, an increase in the mass-to-radius ratio in zone 1 is expected due to the potential generation of branched fragments from zone 3 and/or the aggregation of small linear molecules. Conversely, in zone 3, this ratio may undergo a decrease because of fragmentation at

branching regions α(1,6) glycosidic bonds. However, in the absence of fragmentation, structural reorganisation with molecular aggregation and compaction could occur.

The mass-to-size ratio in zone 3 significantly decreased after MWT for normal rice, waxy rice, normal maize, and wheat, while it increased for potato (Table 2, $p < 0.05$). In contrast, waxy maize and tapioca exhibited no significant changes, consistent with the maintained peak position and concentration distribution noted in Fig. 4D and G, as well as the similar density distribution across the entire molecular mass spectrum (Fig. 5D and G).

In MWT-mediated hydrolysis, more compact samples are expected to exhibit a smaller percentage reduction in the mass-to-size ratio (see Section 3.5). However, larger molecules undergo more pronounced absolute changes. This pattern aligns with the wheat starch sample, which, despite its high compaction, shows a significant decrease ($p < 0.05$) in its mass-to-radius ratio due to its initially high molecular mass. Notably, even after treatment, this sample retains higher compaction values compared to the other treated samples.

The reduction in the mass-to-radius ratio in zone 3 of the rice samples is consistent with their short amylopectin chain structure in the crystalline region, which, as explained in Section 3.2.2, facilitates hydration, loss of crystallinity and increased susceptibility to hydrolysis. In normal rice, the high compactness of the structure may explain why the reduction in the ratio was much lower than in its low-amylose counterpart.

The stability of the mass-to-radius ratio in zone 3 of waxy maize is notable, as the absence of amylose would suggest a potential reduction. This does not necessarily mean hydrolysis did not occur, but rather that it may have been accompanied by a decrease in branching degree [35]. Combined with the presence of longer amylopectin chains in the crystalline region, this could enhance the formation of double helices after treatment, preserving a compaction level like the untreated sample. The reduced peak concentration in zone 3 (Fig. 4D) suggests that hydrolysis occurred while maintaining molecular parameters such as the mass-to-radius ratio.

In tapioca starch, the amylopectin chain distribution may explain the stability of the mass-to-radius ratio after treatment. This starch has a higher proportion of long chains (25–36 glucose units) and fewer short chains (6–12 glucose units) compared to maize starch [36]. The high long-to-short chain ratio reduces compactness in the untreated sample, making it more prone to disaggregation. Additionally, its lower number of hydrolysis-susceptible branching points and high aggregation capacity upon treatment could account for the preserved apparent density profile and mass-to-radius ratio, along with an increased concentration of aggregated particles, in contrast to waxy maize.

In the case of potato, the significant increase ($p < 0.05$) in the mass-to-size ratio in zone 3 after MWT is evident in the higher molecular mass portion of the apparent density profile, above 10^8 Da (Fig. 5F). This region contains the highest concentration of molecules in the treated sample, showing density values comparable to the untreated sample (Fig. 2F). The substantial increase in radius observed for potato in zone 3, relative to other samples, supports the hypothesis that MWT promotes molecular aggregation in this material. This is consistent with previous findings that B-type starches tend to adopt more ordered arrangements upon heat treatment compared to A-type starches [45,46].

MWT also caused a significant ($p < 0.05$) increase in the mass-to-size ratio in zone 1 for waxy rice, wheat and tapioca starches, while a non-significant increase was observed for normal rice, normal maize and potato. The increase in the first samples is due to the appearance of branched fragments, which are more compact than the original linear molecules of the untreated sample, as well as the possible aggregation of amylose molecules into more compact structures. On the other hand, waxy maize exhibited a more than four-fold reduction in this ratio in zone 1 (Table 2). It has been mentioned that these large mass and size structures present in this region underwent a significant change due to the treatment, showing a reduction in average masses of the order of 80 % and a reduction in radii of about 30 %, which explains the decrease in the mass-to-radius ratio. This can also be seen in the apparent density (Fig. 5D), where all molecules of the treated sample in the range between 5×10^5 Da and 3×10^6 Da had lower densities than the untreated samples.

3.5. Correlation study. Impact of MWT depending on starch molecular characteristics

A correlation study was proposed to examine the molecular changes induced by MWT in relation to the initial molecular properties of the treated starches. Fig. 6 presents a correlation matrix comparing the values of untreated starch with the absolute value of their changes after MWT (|Treated-Untreated|). This analysis allowed for the integration of all samples to determine the reasons for the observed changes based on their initial molecular properties, rather than solely on their botanical origin.

In zone 1 (Fig. 6, upper left quadrant), strong positive correlations were observed among the parameters related to mass (M_n, M_w), radii (r_w), $Đ_M$, $Đ_r$, and their respective changes after MWT. Pearson's coefficients ranged from 0.72 to 0.99 ($p < 0.01$). The only exception was r_n, which did not show a significant correlation with Δr_n. These robust correlations suggest that molecules with greater mass, size, and dispersity in amylose-related zone 1 experienced more substantial changes with MWT. Longer amylose molecules may have a higher propensity to aggregate either with each other or with amylopectin fragments from zone 3. This relationship between mass or radius and the magnitude of change is also applicable to molecules of considerable size, such as those found in zone 1 of waxy maize. In this instance, however, the modification involved disintegration or disaggregation rather than aggregation, but still a change of high magnitude related to the large molecular size.

In zone 3, the relationship between the parameters of the initial (untreated) samples and their changes after treatment demonstrated a high influence of the degree of compaction on the susceptibility to modification after MWT. The untreated mass-to-radius ratios (M_n/r_n and M_w/r_w) exhibited a negative correlation with Δr_n and Δr_w ($p < 0.01$, $r = -0.71$ to -0.75), indicating that more compact molecules demonstrated enhanced resistance to size alterations. In line with the latter, dispersion, both in terms of mass ($Đ_M$) and radius ($Đ_r$), correlated positively with their respective changes after treatment ($\Delta Đ_M$ and $\Delta Đ_r$, $p < 0.01$, $r = +0.88$ to $+0.90$), suggesting that more dispersed and less compact amylopectin molecules were more susceptible to greater changes in these parameters with MWT. This effect was particularly evident in samples with high molecular dispersion, where MWT tended to homogenise their molecular size and mass, reducing both $Đ_M$ and $Đ_r$ to values close to 1. In this context, average radii (r_n and r_w) demonstrated a negative correlation with their variations following MWT (Δr_n and Δr_w, $r = -0.87$ to -0.78, $p < 0.01$). This is logical given that the largest samples, such as wheat amylopectin, exhibited the highest mass-to-radius ratio and the least apparent density changes after MWT, while potato amylopectin had the smallest size and the lowest mass-to-radius ratio, undergoing the most significant change post-treatment. Conversely, there were no significant correlations between molecular masses and their variations with MWT (ΔM_n and ΔM_w), although a negative correlation was observed with the variation in radii (Δr_n and Δr_w). These results suggest that the treatment has a more pronounced impact on the molecular size than on its mass.

Correlations between untreated zone 3 values and changes in zone 1 (upper right quadrant) demonstrated a significant positive relationship between larger amylopectin-related molecules (higher r_n and r_w in zone 3) and the Δr_n and Δr_w of amylose-related molecules in zone 1 ($p < 0.01$, $r = +0.69$ to $+0.87$). This indicates that the hydrolysis of large amylopectin molecules during MWT resulted in particle fragments with retention times like those of amylose molecules, thereby contributing to the observed increases in mean radii in zone 1. It has been noted that

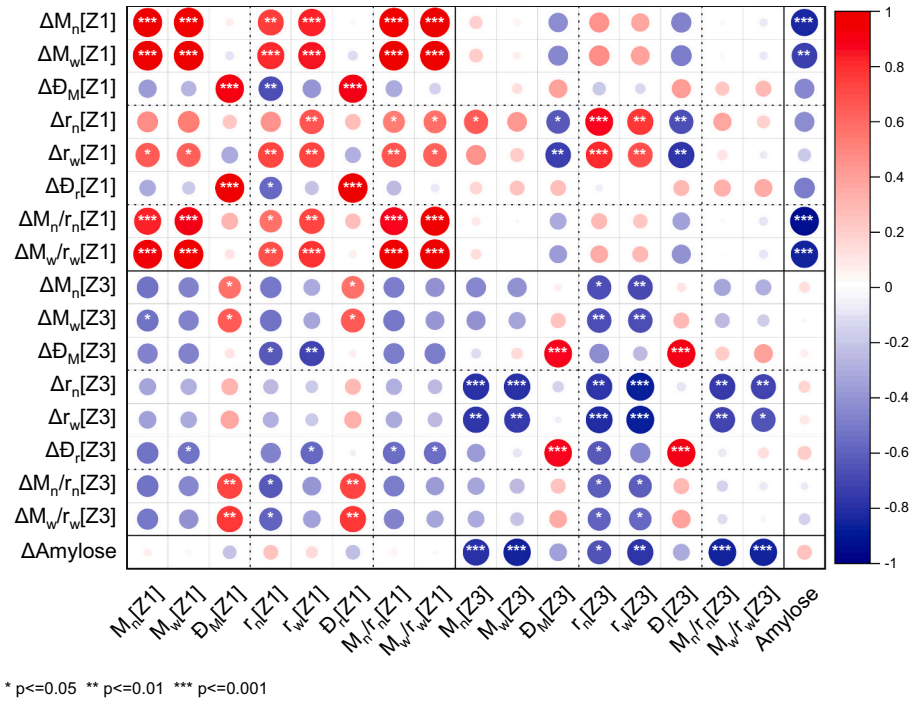

Fig. 6. Correlation matrix between molecular parameters of untreated samples (x-axis) and the increase in absolute value with microwave treatment (Δ = |Treated-Untreated|, y-axis). Z1 (zone 1, amylose-related molecules), Z3 (zone 3, amylopectin-related molecules), M_n (number-average molecular mass), M_w (weight-average molecular mass), $Đ_M$ (molecular mass dispersity, M_w/M_n), r_n (number-average molecular radius), r_w (weight-average molecular radius), $Đ_r$ = radius dispersity. Coloured sidebar: Pearson's coefficient.

larger and more compact untreated amylopectin molecules are more resistant to modification; however, the fragments produced post-treatment were likely larger than those derived from smaller amylopectins. Additionally, negative correlations were observed between zone 3 dispersity parameters ($Đ_M$ and $Đ_r$) and Δr_n and Δr_w in zone 1 ($p < 0.05$, $r = -0.63$ to -0.77). Amylopectin molecules with low $Đ_M$ and $Đ_r$ values, such as those from wheat, waxy maize, and tapioca, underwent the largest changes in zone 1 radii. Less dispersed amylopectin molecules in zone 3, with more homogeneous sizes, are less prone to modification. In addition, amylose-related molecules in zone 1 from these samples are likely less integrated into the compact amylopectin structure, making them more susceptible to modification by MWT.

Amylose concentration in untreated samples only correlated significantly with the changes observed in parameters of zone 1 (last column on the right, $p < 0.01$, $r = -0.73$ to -0.93). It correlated negatively with ΔM_n, ΔM_w, $\Delta M_n/r_n$ and $\Delta M_w/r_w$ in zone 1. This effect was particularly pronounced in waxy samples, which had very low amylose concentrations and exhibited the most prominent changes in zone 1 parameters (see Fig. 2A, B and C).

In Zone 3, significant negative correlations were found between untreated amylopectin parameters (M_n, M_w, r_w, M_n/r_n, M_w/r_w) and changes in amylose concentration (ΔAmylose) as result of MWT ($p < 0.001$, $r = -0.77$ to -0.85). This trend was particularly evident in potato, which had the lowest M_n and M_w values and the lowest compaction (mass-to-radius ratio) of all untreated samples and exhibited the greatest reduction in amylose concentration after MWT, a change attributed to a partial aggregation of amylose and amylopectin molecules after MWT as explained in Section 3.1.

This study has identified correlations that allow the prediction of structural changes associated with treatment. However, a more detailed relationship between the initial characteristics of the material, the extent of molecular changes and possible effects on technological and nutritional properties requires further studies. Nevertheless, the identified correlations can serve as an initial basis for inferring the degree of change in properties such as gelatinisation, retrogradation and crystalline structure, the rate of change of which would depend on the initial properties of the raw material [47].

4. Conclusions

This study highlights the innovative application of the AF4-MALS-dRI technique to provide a detailed and comprehensive understanding of how MWT modifies the molecular structure of starches from diverse botanical origins, each with a distinct initial molecular structure. In addition to evaluating the molecular mass, size, and dispersity, the integrated analysis of parameters such as radius, concentration, mass-to-radius ratio, and apparent density enabled an in-depth assessment of MWT-induced transformations in the starch samples. These findings underscore the significant influence of botanical origin on the response of starches to MWT, with clear distinctions observed between tuber and cereal starches.

In general, MWT has a hydrolytic effect on starches, with the degree of modification varying according to the starch type. Among cereals, non-waxy starches (normal maize, normal rice, and wheat) demonstrated greater resistance to hydrolysis than waxy starches (waxy maize and waxy rice). The higher resistance of non-waxy starches is likely due to the protective role of amylose and the higher mass-to-radius ratio in amylopectin-related zone 3, suggesting greater structural compaction. Non-waxy cereals also showed increased molecular mass dispersity in amylose-related zone 1 after MWT, indicating hydrolytic resistance and the possible formation of amylopectin fragments or aggregation. In contrast, waxy cereals underwent more pronounced hydrolytic changes after MWT, showing a homogenising effect on molecular radii and apparent densities, although with variations between samples. Waxy rice showed the largest reduction in amylopectin molecular mass, whereas its molecular radii and apparent density remained almost unchanged. Waxy maize, on the other hand, retained its amylopectin molecular mass and peak concentration but experienced significant hydrolysis in zone 1, which probably contained very low molecular mass amylopectin molecules or amylose aggregates.

The responses of tuber starches were markedly different. Tapioca starch demonstrated relative stability in amylopectin mass and radius following treatment, with its concentration peak and apparent density remaining unchanged. In zone 1, there was a relative increase in the average molecular mass and a reduction in the concentration of molecules with a molecular mass below 10^8 Da, suggesting that smaller molecules in tapioca starch aggregated to form larger structures. However, potato starch showed the most dramatic changes, which can be attributed to its B-type crystalline structure and relatively low average mass-to-radius ratio. Following the MWT, amylopectin in potato starch exhibited higher average mass and radius, indicating molecular aggregation and the formation of homogeneous structures characterised by low dispersity and a high concentration of large molecular mass particles.

This study demonstrates the potential of the AF4 technique as an innovative and powerful tool that improves existing methods for the analysis of starch molecular changes after MWT. In addition to assessing molecular size and mass changes, this work provides information on structural changes such as degradation, rearrangement and compaction. The results presented here pave the way for future research into how these structural changes influence the techno-functional and nutritional properties of starch.

CRediT authorship contribution statement

Raúl Ricardo Mauro: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ainhoa Vicente:** Writing – review & editing, Visualization, Formal analysis. **Felicidad Ronda:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

Acknowledgement

The authors thank the Spanish Ministerio de Ciencia e Innovación (EQC2021-006985-P), Ministerio de Ciencia, Innovación y Universidades (PID2023-153330OB-I00) and the Junta de Castilla y León/FEDER (CLU 2019-04 – BIOECOUVA Unit of Excellence of the University of Valladolid) for their financial support. Raúl R. Mauro and Ainhoa Vicente thank the Ministry of Science, Innovation and Universities for the FPI contract (RRM) and FPU contract (AV). The authors thank Dr. Lars Nilsson and Dr. Catalina Fuentes of the Department of Food Technology, Engineering, and Nutrition at Lund University (Sweden) for their training in the use of AF4 equipment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.144395.

Data availability

Data will be made available on request.

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