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Texture and interlinked post-process microstructures determine the *in vitro* starch digestibility of Bambara groundnuts with distinct hard-to-cook levels

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Abstract

Particular storage conditions are described to promote the development of the hard-to-cook (HTC) phenomenon for most legumes. However, it is not clearly established whether the HTC phenomenon influences starch digestion kinetics. Therefore, this study explored how the HTC phenomenon influences *in vitro* starch digestion of Bambara groundnuts, taking into account three distinct HTC levels. Stored Bambara groundnuts required prolonged cooking times. Increasing storage time led to a decrease in the rate constant of texture degradation, signifying the development of the HTC phenomenon. For cooking times of 60 min and 120 min, high HTC level samples exhibited higher rate constants and extents of starch digestion compared to the fresh sample. The higher rate of digestion was attributed to the high hardness that resulted in greater cell rupture and faster access of amylase to starch. Adapting cooking times of Bambara groundnuts with distinct HTC levels to obtain equivalent hardness values and microstructures resulted in comparable starch digestion kinetics. Spectrophotometric analysis overestimated the amount of digested starch, in contrast to the more accurate HPLC analysis, which further provided more insight by quantifying multiple digestion products. This work demonstrates that it is the hardness and interlinked pattern of cell failure (microstructure) that determines starch digestion of Bambara groundnuts with distinct HTC levels.

1. Introduction

Bambara groundnut (*Vigna subterranean* (L.) Verdc.) is an indigenous pulse crop grown mostly in sub-Saharan Africa but also in Indonesia, Malaysia, Philippines and Thailand mostly for subsistence farming (Duke, 1981). Most indigenous crops possess several benefits to the communities where they are grown (Mabhaudhi, Chibarabada, Chimonyo, & Modi, 2018). Bambara groundnuts have immense endurance for abiotic stresses, and in particular, they possess remarkable drought tolerance capabilities compared to other legumes (Chai, Massawe, & Mayes, 2016; Collinson, Clawson, Azam-Ali, & Black, 1997; Jørgensen et al., 2010). Additionally, they have a high nutrient density owing to their composition consisting of approximately 63% carbohydrates, 19% protein and 7% lipids and are a good source of some micronutrients (Hillocks, Bennett, Mponda, & Maritime, 2012; Olaleye, Adeyeye, & Adesina, 2013). In light of these benefits, Bambara groundnuts may be a nutritious and reliable famine ready crop in these regions since they can be stored for extended periods after harvest.

Extended storage of particular legumes under conditions of high temperatures (>25 °C) and humidity (>65%) results in prolonged cooking times to attain a palatable texture; a condition termed the hard-to-cook (HTC) (Reyes-Moreno, Okamura-Esparza, Armienta-Rodelo, Gómez-Garza, & Milán-Carrillo, 2000). Besides the inconvenience of the extended cooking time, it becomes costly and unsustainable in cases where firewood is used for fuel (FAO, 1988). Various mechanisms have been proposed to explain the causes of the defect (Hincks & Stanley, 1986; Reyes-Moreno, Paredes-López, & Gonzalez, 1993). In the pectin-cation-phytate hypothesis, phytase dephosphorylates phytate releasing cations, while pectin methylesterase is thought to demethylesterify pectin, which is abundant in the middle lamella region of the cell walls (Reyes-Moreno et al., 1993). The demethylesterified pectin binds the released cations thereby increasing the strength of adhesion in the middle lamella (Garcia-Vela, del Valle, & Stanley, 1991). Also, deposition of lignin in the cell wall may occur through the crosslinking of free amino acids with phenols and condensed tannins (Garcia, Filisetti, Udaeta, & Lajolo, 1998). Besides these mechanisms, starch and protein functionality have been observed to change during storage and are possibly involved in HTC development (Hincks & Stanley, 1986; Liu & Bourne, 1995).

The cell wall-middle lamella complex seems to be the common structural feature being influenced the most by HTC mechanisms. Alteration of the cell wall structure during storage may have cascading effects on technological or nutrition-related functionality of legumes. In fact, cell walls of fruit, vegetables and legumes have long been recognised as key determinants of food quality as they can influence texture, flavour release and nutrient bioaccessibility (Waldron, Parker, & Smith, 2003). Targeted processing techniques have proven potential to modify the state of the cell walls. For instance, techniques that ruptured the cell wall caused an increase in carotenoid bioaccessibility of raw carrots (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010). In addition, carrots with tailored textures could be generated after high-pressure procesing and the addition of calcium salts (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009).

Textural properties of cell walls in plant matrices are known to influence the mode of cell failure upon mastication or application of a force (Waldron et al., 2003). Roughly, the pattern of tissue failure might be divided into two cases; (i) cell rupture, which opens up the cell or (ii) cell separation, which retains cellular integrity. Cellular integrity is considered a critical microstructural feature that determines the starch digestibility of legumes, e.g., common beans (Berg, Singh, Hardacre, & Boland, 2012). Indeed, the slower starch digestibility of legumes compared to other starchy food has been attributed to the encapsulation of starch in the cell wall which limits the access of enzymes into the cell (Berg et al., 2012; Mishra, Hardacre, & Monro, 2012). Other factors affecting starch digestion may include the protein matrix (Rovalino-Córdova, Fogliano, & Capuano, 2018).

The current study was designed to explore the influence of HTC levels on *in vitro* starch digestibility of Bambara groundnuts. This task was approached by assessing texture, microstructure and gelatinisation parameters of Bambara groundnuts with distinct HTC levels, with the aim of evaluating if these factors play a role in starch digestibility. To the best of our knowledge, HTC development in Bambara groundnuts has not been proven in scientific literature before. To this end, Bambara groundnuts stored at predetermined storage conditions were assessed for development of the HTC phenomenon by determining hardness as a function of cooking time. Hardness was evaluated as it is an essential textural attribute of legumes that determines their palatability and consequent acceptance by consumers (Sasikala, Ravi, & Narasimha, 2011). The corresponding microstructures after cooking and mechanical disintegration were evaluated by microscopic analysis.

While the HTC phenomenon per definition influences texture kinetics during the cooking of legumes, its effect on starch digestion is not established. Accordingly, in this work, the starch digestion kinetics of Bambara groundnuts with distinct HTC levels but processed for a particular cooking time were evaluated after simulated *in vitro* digestion. To date, the few studies focusing on the influence of the HTC phenomenon on starch digestion of common beans and cowpeas, respectively, considered only a single endpoint evaluation of digestion (Nyakuni et al., 2008; Tuan & Phillips, 1991). However, knowledge of rates of starch digestion is vital as sudden spikes in blood glucose *in vivo* may increase the risk for some chronic diseases (Temelkova-Kurktschiev et al., 2000).

Furthermore, the use of more advanced quantitative techniques to analyse starch digestion products has been recommended (Dona, Pages, Gilbert, & Kuchel, 2010). In this context, an HPLC method to quantify multiple starch digestion species was implemented. The estimated kinetic parameters from the data obtained were compared to those obtained from the commonly used dinitrosalicylic spectrophotometric method. The dinitrosalicylic assay is a fast and cheap analysis, but, at the same time being a black box type of assay as it measures total reducing power from any species present. The HPLC method quantifies at the molecular level increasing mechanistic understanding of starch digestion.

2. Materials and methods

Dry Bambara groundnuts were obtained from communal farmers in Masvingo Province in Zimbabwe after the harvesting season of 2016. On arrival at the laboratory, sorting was done to remove foreign matter and any damaged seeds. The cleaned Bambara groundnuts were then either kept at -40 °C or stored at conditions aimed to induce biophysical changes as will be described in Section 2.2.1. The Bambara groundnuts kept at -40 °C would represent the fresh sample (low HTC level) and hence act

as a control. All chemicals and reagents were from Sigma Aldrich, except for NaCl, HCl, and ethanol (from VWR), $CaCl_2.2H_2O$, and $MgCl_2$ (from Merck), Nitric acid and acetone (from Chem Lab), and $NaHCO_3$ (from Fisher Scientific) and KCl (from MP Biomedicals). Chemicals and reagents used were of analytical grade. The total starch kit was obtained from Megazyme Inc. (Bray, Ireland).

2.1 Characterisation of the Bambara groundnut batch

2.1.1 Moisture content determination

The moisture content of the different samples was determined using an oven drying method (Ahn, Kil, Kong, & Kim, 2014). Crucibles were first dried in an oven set at 105 °C for 1 h after which they were cooled in a desiccator for 30 min and weighed. One gram of sample was weighed in triplicate into the previously weighed crucibles and placed in an oven set at 105 °C. After 18 h, the samples were taken out of the oven and immediately put in a desiccator for 30 min to cool. Samples were weighed after cooling, and moisture content was determined as the change in weight after drying divided by the initial weight.

2.1.2 Total starch determination

The Megazyme total starch kit (AA/AMG) assay, following the potassium hydroxide format was used to determine the total starch content of all the created samples in duplicate according to the instructions from the supplier. First, endogenous glucose and maltodextrins were removed by washing with ethanol to avoid overestimation of the glucose after enzyme treatment. Next, the samples were hydrolysed with cold KOH to dissolve any resistant starch. After that, amylase and amyloglucosidase were used to hydrolyse the starch to glucose completely. A factor of 0.9 was used to convert the glucose to total starch (%) present in the sample.

2.1.3 Determination of the glass transition temperature

Prior to selection of storage conditions likely to induce biophysical changes that would lead to the HTC defect, the glass transition temperature for the batch of Bambara groundnuts was determined using differential scanning calorimeter (DSC) (Leprince & Walters-Vertucci, 1995). A V24.11 Build, 124 Q2000 heat flux DSC, designed with Advanced T_{zero} technology (TA Instruments, ™ Delaware, US) and equipped with a refrigerated cooling system (RCS 90) was utilised. Indium of known weight was used to perform cell constant and temperature calibrations in a T_{zero} aluminium pan. About 10 mg of Bambara groundnuts powder was weighed into a pre-weighed T_{zero} aluminium pan, and the

pan was hermetically sealed. A similar empty pan was used as a reference. Thermal scanning involved equilibration at 60 °C for 1 min followed by a first heating ramp to 90 °C at 10 °C/min. This first ramp ensured that irreversible changes and molecular relaxation do not influence the T_g determination. The sample was quench cooled back to 60 °C and was kept there for 1 min. A second heating ramp to 90 °C at 10 °C/min was performed. On this second heating ramp, the limits of the glass transition were determined using TA Universal Analysis software (Delaware, US) and the midpoint of the step was considered as the T_g.

2.2 Storage conditions to induce the HTC phenomenon

To induce ageing the legumes, part of the batch was incubated at 35 °C and 78% RH. These conditions are typical of the environmental conditions in most areas where Bambara groundnuts are grown. A saturated slushy solution of potassium chloride was prepared at 35 °C, to attain the desired relative humidity. This solution was then placed in an acrylic lab desiccator equipped with a hygrometer and left to equilibrate (Chigwedere, Olaoye, et al., 2018). Bambara groundnuts in perforated tins were later introduced into the desiccator and placed in the incubator set at 35 °C. Monitoring of the evolution of hardness with cooking time was done every four weeks to determine the level of HTC development **(Section 2.4).** At 16 and 32 weeks of storage, samples were withdrawn and were kept at -40 °C until further processing. The storage experiment was stopped at 32 weeks because beyond that period no further HTC development was observed.

2.3 Thermal treatment of fresh and stored Bambara groundnuts

Bambara groundnuts (fresh and stored) were soaked in distilled water (1:5 w/v) for 16 h. After that, the soaking water was discarded, and the seeds that did not sufficiently imbibe water during soaking were also discarded. To thermally process the legumes, water (1:5 w/v), was put in a Duran flask which was placed in an oil bath at 100 °C. When the water was at 95 °C, the soaked legumes were added and then cooked for different cooking time moments. An evolution of hardness with cooking time was ultimately required, and therefore an individual Duran flask was dedicated for each cooking time moment. At the end of each predetermined processing time, Duran bottles were placed in an ice bath for 5 min to allow immediate cooling (Pallares Pallares et al., 2018). Before *in vitro* digestion, the cooked sample was mechanically disintegrated using a mortar and pestle. This step was included as solid food does not enter the gastrointestinal tract as a whole, but rather a form of disintegration either by mastication or by other processing techniques occurs. The obtained

'puree' was freeze-dried in a Christ Alpha Plus 2–4 lyophilizer (Osterode, Germany). The effect of freeze-drying on starch digestion was evaluated, and no significant influence was observed.

2.4 Determination of hardness

Following thermal treatment of the stored and fresh samples and a cooling step of 5 min, the hardness of cotyledons was determined by measuring the compression force using a TA-X2i texture analyser (Stable Microsystems, Goldaming, England). It was equipped with a 25 kg force cell and a cylindrical flat-head aluminium probe of diameter 25 mm which was utilised to compress a cotyledon to 75% strain at a speed of 1 mm/s. The maximum peak force (gram-force, g) recorded from compression of a half a cotyledon was defined as the hardness. At least 20 cotyledons of different seeds from the different cooking times were measured, to obtain an evolution of hardness with storage (Chigwedere, Olaoye, et al., 2018).

2.5 Evaluation of microstructural changes after thermal processing and mechanical disintegration

Microscopy analysis was conducted to observe the changes in microstructure in relation to ageing and processing. In particular, the objective was to visualise the mode by which cells fail after the mechanical disintegration after processing. The lyophilised powder was diluted with demineralised water (approx. 1:12 w/v), and 20 µL of 0.1 N iodine was added to stain starch. From the suspension, approximately 20 µL were taken and placed on a glass slide and observed under an Olympus BX-41 light microscope (Olympus, Optical Co. Ltd, Tokyo, Japan). The microscope was equipped with Cell Image analysis software, (Soft Imaging System, Munster, Germany) which was used to capture the observed micrographs (Chigwedere, Olaoye, et al., 2018). Differential interference contrast (DIC) was used to view the microstructures of the samples with an objective lens of 40× magnification.

2.6 Thermal analysis of (un-)processed Bambara groundnuts with distinct HTC levels

The DSC equipment described in (**Section 2.1.3**) was used to quantify gelatinisation parameters as influenced by HTC levels and cooking. Flattened indium of known weight was encapsulated in a high volume stainless steel pan and was used for enthalpy and temperature calibration. The pan contained in it a weighed thin layer of aluminium oxide, which was added to improve calibration of enthalpy in the DSC. Another high-volume pan containing a layer of aluminium oxide was used as a reference pan. Preparation of the samples involved weighing approximately 10 mg of sample in a

high volume stainless steel pan and adding 30 µL of water. The pan was then closed with a lid secured with an o-ring to make it hermetic. The weighed samples were then left to equilibrate at room temperature for 24 h before running on the DSC. The analysis was conducted by allowing a temperature rise from 20 °C up to 120 °C at a heating rate of 5 °C/min (Chigwedere, Olaoye, et al., 2018). TA Universal analysis software version 4.5A was used to integrate the thermograms obtained to determine the thermal transitions of starch gelatinisation.

2.7 *In vitro* digestion procedure

Simulation of the gastric and small intestinal phases of the gastrointestinal tract was performed to investigate how the development of the HTC phenomenon influences starch digestion. The simulation was done following the international consensus method (Minekus et al., 2014) with modification. A preliminary experiment where the sample was treated with salivary amylase as explained in the original protocol (Minekus et al., 2014) and without salivary amylase was done. The preliminary data showed that at 5 min into the small intestinal phase, the difference in digested starch between the two treatments was less than 5%. This difference completely disappeared at 30 min into the small intestinal digestion phase. Moreover, the exclusion of the salivary amylase did not significantly alter the kinetics of starch digestion in the small intestine (data not shown). Accordingly, we excluded salivary amylase in the oral phase but included the simulated salivary fluids (SSF).

Oral phase: Since all samples were freeze-dried after processing they were reconstituted with the equivalent amount of water lost during freeze-drying to make up to a mass of 1.25 g for all samples. To the 1.25 g wet sample, 1 mL of SSF electrolyte stock solution was added and the mixture mixed well. Next, 0.125 mL of 0.015 M $CaCl_2$ solution and 0.125 mL of demineralised water was then added.

Gastric phase: To the 2.5 mL of the oral bolus, 1.6 mL of simulated gastric fluid (SGF) was added followed by the addition of 0.025 mL of $CaCl_2$ (0.015 M). The pH of the mixture was then adjusted by using 2 M HCl to attain a pH of 3. A volume of demineralised water was added depending on the amount of acid added during the pH adjustment. After that, 0.4 mL of porcine pepsin previously dissolved in SGF solution was added to achieve 2000 U/ml in the final digestion mixture. The digestion mixture was then incubated at 37 °C for 2 h in an end-over-end rotation at a speed of 40 rpm.

Intestinal phase: To the 5 mL of the gastric chime, 2.75 mL of simulated intestinal fluid (SIF) was added followed by 0.2 mL $CaCl_2$ (0.015 M). Next, 0.625 mL of fresh bile (160 mM), prepared in SIF

electrolyte stock solution) was added to give a final concentration of 10 mM. The pH of the mixture was adjusted to 7 by using 1 M NaOH. Demineralised water was later added depending on the volume of NaOH solution added. Next, 1.25 mL of pancreatic enzymes dissolved in SIF were added. The added volume resulted in 200 U/mL of pancreatic amylase standardised to result in 9 U/mg starch, 100 U/mL of trypsin, 25 U/mL of chymotrypsin and 200 U/mL of pancreatic lipase in the final digest. Samples were incubated at 37°C for a total time of 2 h with an end-over-end rotation of 40 rpm. Digestion was performed using a kinetic approach by taking predetermined time moments of 0, 10, 20, 30, 60, 90 and 120 min. For each time moment, digestion was performed in a separate individual tube. The 7 consecutive time moments, which were a function of digestion time in the small intestine, characterise a single 'digestive' system. Accordingly, they can be seen as repetitions of the same system and are used together for the determination of kinetic parameters (Verkempinck et al., 2018) as explained in **Section 2.9**. Enzymes were inactivated by performing a heat shock at 100 °C for 5 min. The incubation was stopped at 120 min since this is considered as the duration of small intestinal digestion (Minekus et al., 2014). The digests were centrifuged at 3 000 g for 5 min to sediment solid particles. The supernatants were transferred to plastic microfuge tubes and kept at -40 °C until further use.

2.8 Determination of starch digestion products

After the *in vitro* procedure, the amount of starch digested was first determined by measuring total reducing sugars using a spectrophotometric method. Next, an analytical technique quantifying multiple digestion products was used as a comparison.

2.8.1 Evaluation of reducing sugars using the dinitrosalicylic method

The concentration of total reducing sugars released from starch digestion at various time moments was measured by the dinitrosalicylic (DNS) method (Miller, 1959). Briefly, 2.75 g of 3,5-dinitrosalicylic acid was dissolved in 125 mL of Milli-Q water and the solution heated to temperatures between 50 °C and 70 °C. After that, 75 g of potassium tartrate tetrahydrate was added slowly to the acid while mixing. Next, 50 mL of NaOH (2 M) was added to the mixture which was then diluted to 250 mL with Milli-Q water. For the sample assay, 100 µL of the supernatant was pipetted into Pyrex test tubes. Exactly 1 mL of the DNS solution was added to each test tube and boiled for 15 min in an oil bath (Memmert, Germany) set at 100 °C. After that, the test tubes were then cooled in an ice bath. Next, 9 mL of Milli-Q water was then added and the test tubes were mixed by inversion. Standards containing known concentrations of maltose (0.5 – 2 mg/mL) were

included. An aliquot of the diluted solutions was transferred to macro cuvettes and absorbance was read at 540 nm in a spectrophotometer (Shimadzu UV-1800, Shimadzu, Japan). The absorbance evaluated at time 0 of digestion was taken as a blank. Total reducing sugars were then expressed as maltose equivalents based on the standard curve. The obtained maltose equivalent was converted to digested starch by a conversion factor of 0.947. Digested starch (%) (reducing sugars) was then calculated as starch equivalents of reducing sugars in the digests divided by the initial amount of starch present in the sample that was digested, expressed as a percentage.

2.8.2 Determination of multiple starch digestion products by HPAEC-PAD

HPAEC-PAD was used to quantify maltooligosaccharides resulting from the starch digestion. The method described by Morales, Corzo, & Sanz (2008) who quantified maltooligosaccharides in honey, was followed with some modifications. Appropriate dilutions of the samples with Milli-Q water were made prior to injection on to a Dionex HPLC DX600 equipped with a GP 50 gradient pump, an eluent degas module, a CarboPac™ column PA100 (4 × 250 mm), a CarboPac™ guard column (4 × 50 mm) and an ED 50 electrochemical detector (Dionex, Wommelgem Belgium). The system was equilibrated with previously degassed 3% 1 M sodium acetate, 10% 1 M of NaOH and 87% Milli-Q water at a flow rate of 0.7 mL/min. Exactly 20 µL of diluted sample was injected, and a first linear gradient eluted maltooligosaccharides from time 0 to 30 min where sodium acetate increased from 3 to 12.5% and water decreased from 87% to 80% while the NaOH was kept constant at 10% throughout the whole run. A second linear gradient was introduced from 30 min to 40 min by increasing the sodium acetate from 12.5 to 20% while the water was reduced to 70%. For detection and quantification of the sugars, an electrochemical detector that measures the electric current generated by oxidised sugar at the surface of a gold electrode was used (AgCl as a reference). For this, potentials of E_1 = +0.15 V (t_1 = 400 ms), E_2 = +0.75 V (t_2 = 200 ms) and E_3 = −0.8 V (t_3 = 200 ms) and sampling time (t_s) of 20 ms were employed. Before this, a mix of commercial standards of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, maltoocatose, maltonanoose and maltodecaose at varying concentrations of 5-40 ppm, were injected on each run to obtain a calibration curve on which the quantification was based. Conversion factors to account for the loss of a water molecule were employed to express the sugar as starch: glucose (0.9), maltose (0.947), maltotriose (0.964). Digested starch (%) from multiple digestion species was calculated by summing the starch equivalents of the individual sugars, all divided by the initial starch content in the sample that was digested, expressed as a percentage.

2.9 Data analysis

Experimental data obtained from texture analysis were modelled by a fractional conversion model (Equation 1) using the SAS Proc Model procedure (SAS version 9.3, SAS Institute, Inc., Cary, NC, USA). Two parameters, i.e., final hardness and the rate constant of texture degradation, k_s (min $^{-1}$), were estimated.

$$\text{Hardness}_{(t)} = \text{final hardness} + (\text{initial hardness} - \text{final hardness}) \times e^{(-k_s \cdot t)} \qquad \text{Equation 1}$$

One way ANOVA was used to compare means of the gelatinisation enthalpy data. Differences between the means were computed at $\alpha = 0.05$.

Starch digestion kinetics during the small intestinal phase was modelled by a fractional conversion model (Equation 2) using the same SAS software. Two parameters were estimated namely, the rate constant of digestion k_d (min $^{-1}$) and the estimated digested starch at (C_f).

$$C_{(t)} = C_f + (C_0 - C_f) \times e^{(-k_d \cdot t)} \qquad \text{Equation 2}$$

Where:

$C_{(t)}$ is the digested starch (%) at digestion time t (min);

C_0 is the initial parameter value at time 0 (start of the intestinal phase), i.e., the initial concentration of sugars (converted to starch %) present at time 0;

C_f is the asymptotic value (digested starch (%)) at the end of the small intestinal digestion.

All modelled curves were assessed for the fit of the model by calculating adjusted R^2 and visually analysing the residual and parity plots. The differences between the estimated parameters were determined by the 95% confidence intervals given in the output.

3. Results and discussion

3.1 Storage conditions to induce hard-to-cook characteristics

The T_g range of the fresh batch (moisture content 7.7%, dry basis) was detected to be between 12.8 - 15.2 °C with a midpoint temperature of 14.1 °C as determined by DSC measurements (**Supplementary Figure A1**). It was essential to create storage conditions that would allow the system to transition from a glassy (metastable) state to a rubbery state where molecular mobility is increased (Slade, Levine, Ievolella, & Wang, 1993). This transition is associated with changes in the textural, enzymatic or chemical properties of amorphous food (Ross, Arntfield, & Cenkowski, 2013). Next to the information provided by this analysis, we considered the environmental conditions in areas where the crop is grown, which are typically high temperature and humidity conditions.

Conditions of storage comparable to the ones used in this experiment have been used elsewhere to cause the development of the HTC defect in other legumes (Kinyanjui et al., 2015; Reyes-Moreno, Rouzaud-Sandez, Milán-Carrillo, Garzón-Tiznado, & Camacho-Hernández, 2001).

3.2 Texture evolution changes of (non-)stored Bambara groundnuts

Bambara groundnuts stored at 35 °C and 78% RH for 0, 16 and 32 weeks showed a decrease in hardness as cooking progressed **(Figure 1)**. The longer stored (16w and 32w) Bambara groundnuts however had consistently higher hardness values compared to the fresh sample (0w). This trend has been previously observed in faba beans (Nasar-Abbas et al., 2008) and common beans (Kinyanjui et al., 2015). The 32w Bambara groundnuts required 5 h to soften completely; approximately 2.5 times longer than the 0w sample. Such long cooking times are undesirable as they increase fuel costs, are unsustainable and greatly increase the inconvenience associated with cooking legumes.

Kinetic modelling of hardness values as a function of cooking time using a fractional conversion model enabled the quantitative description of the differences in rates of texture degradation and final hardness values in relation to the variable storage periods (**Figure 2**). The rate constant of texture degradation, k_s, for 32w Bambara groundnuts was significantly lower (0.007 ± 0.001 min^{-1}) in comparison to the 0w sample (0.015 ± 0.001 min^{-1}) (**Figure 2A**). A higher k_s value implies that a shorter time is required to reach the final residual hardness (plateau), while a lower k_s suggests that a longer time is required to reach the final residual hardness. The prolonged cooking times and the lower k_s values for stored Bambara groundnuts compared to the fresh, indicate that the HTC development occurred.

Texture degradation during cooking of legumes mostly involves alterations of the pectin-rich middle lamella and starch gelatinisation (Bernal-Lugo, Parra, Portilla, Peña-Valdivia, & Moreno, 1997). Since pectin can be extracted using hot water (85 °C) (De Vries, Voragen, Rombouts, & Pilnik, 1981; Srivastava & Malviya, 2011), the cooking treatment applied potentially solubilised water extractable pectin in Bambara groundnuts. Thermally induced pectin solubilisation in fresh legumes could differ from that of stored legumes due to changes in the physicochemical state of pectin in the middle lamella. Indeed, Chigwedere, Nkonkola, et al. (2018) and Njoroge et al. (2015) showed a substantial decrease in water extractable pectin fraction in HTC common beans compared to the fresh ones. These authors observed an increase in the amount of Na_2CO_3 extractable pectin in HTC common beans.

Characteristics of pectin from common bean cotyledon cell walls show a medium degree of methylesterification (about 55%), which does not markedly change during storage, and is of low linearity (Chigwedere, Nkonkola, et al., 2018; Njoroge et al., 2016). Given these properties, β

elimination, another possible pectin degradation reaction, would likely not occur because it requires pectin with a high degree of methylesterification and linearity (Fraeye et al., 2007). Although these properties have not been determined in Bambara groundnuts, it is highly probable that they are not remarkably different from those of common beans. Differences in pectin solubility are hypothesised to be the primary cause of the prolonged cooking time and lower k_s value of Bambara groundnuts stored under the given conditions. In summary, storage (time) under the given conditions, induced distinct HTC levels in Bambara groundnuts as evaluated by cooking time and the rate constant of texture degradation.

3.3 Microscopy observations of Bambara groundnuts with distinct HTC levels

Micrographs of milled uncooked Bambara groundnuts with distinct HTC levels show free starch granules (approx. < 60 µm) that are stained purple or blue-black with iodine solution (**Figure 3 a**, **b**, **c**). With uncooked samples, cell rupture occurred during milling because of their general hard nature (approx. 21 000 g). As cooking progressed to 60 min, the fresh sample (0w) **(d)** whose average hardness was 8 326 g, showed mostly separated intact cells (~ 100 – 150 µm) and a few starch granules upon mechanical disintegration. By contrast, the 16w **(e)** and 32w **(f)** sample, cooked for 60 min (13 222 g and 15 190 g respectively), showed an abundance of free starch granules from ruptured cells and a few separated intact cells. At 120 min cooking time, where the hardness values of these samples had decreased, the number of free starch granules observed in the 16w and 32w samples **(h and I, respectively)** decreased and more and more separated intact cells could be observed.

Different patterns of cell failure, i.e., cell rupture or cell separation (creating intact cells) can concomitantly occur depending on the strength of cell to cell adhesion provided by the pectin-rich middle lamella (Van Buggenhout, Sila, Duvetter, Van Loey, & Hendrickx, 2009a). As suggested by the pectin-phytate-theory development, an increase in strength of the middle lamella is expected with HTC development. The hypothesised lower pectin solubilisation in stored Bambara groundnuts is supported by their higher hardness values and is consistent with the corresponding cell rupture and delayed separation (**Supplementary Figure A3**). Similar observations of delayed cell separation were observed after common beans were stored under comparable conditions (Brummer, Kaviani, & Tosh, 2015). In summary, HTC Bambara groundnuts have consistently greater cell rupture and delayed cell separation compared to the fresh sample.

3.4 Gelatinisation parameters

Some functional properties of starch may change during storage. Therefore, we assessed the gelatinisation parameters of the raw and cooked Bambara groundnuts. The starch gelatinisation enthalpy for raw samples with distinct HTC levels ranged between 2.8 – 3.0 J/g sample and the differences in the means of enthalpy or raw samples were not significant (**Table 1**). The onset (T_o) and peak (T_p) temperatures for gelatinisation were highly comparable for the raw samples despite storage history. Other authors have similarly observed no relationship between gelatinisation parameters and HTC development (Bernal-Lugo et al., 1997). Upon processing, two peaks were observed (**Supplementary Figure A2**), of which the first peak was attributed to possible retrogradation, with enthalpies of about 0.25 J/g for most samples. The second peak was assumed to be the residual gelatinisation peak for the processed samples. The means of gelatinisation enthalpy of cooked samples were significantly lower than those of the raw as expected. However, the sample is a complex system with protein and lipids. As such, other transitions which are not necessarily related to starch gelatinisation could be in the same endotherm as highlighted by Chigwedere, Olaoye, et al. (2018).

The residual gelatinisation enthalpy was very low (0.23 – 0.35 J/g) for all the cooked samples signifying that starch is easily gelatinised during thermal processing despite storage history. Previous studies have shown that starch isolated from HTC beans often possesses a more significant swelling and water absorption capacities as compared to fresh beans (Reyes-Moreno et al., 1993). However, this seems not to influence the gelatinisation behaviour of Bambara groundnuts. In fact, pectin solubilisation, and not starch gelatinisation, was shown to be the rate-limiting step of softening of common beans (Chigwedere, Olaoye, et al., 2018). The little differences observed in enthalpies, in contrast to the remarkable differences in the cooking time and rate constant of texture degradation as well as microscopic observations, support those findings.

3.5 *In vitro* starch digestion kinetics of Bambara groundnuts in relation to HTC

The influence of the HTC phenomenon on starch digestion kinetics in Bambara groundnuts was evaluated from the generated (non-)stored cooked samples of distinct HTC levels. The first part of this section considered samples cooked for particular cooking times, i.e., 60 min and 120 min. With these samples, the influence of distinct HTC levels after cooking for similar times on *in vitro* starch digestion could be studied. The choice for 60 min cooking time was based on the observation that at this time, much cell separation (a result of pectin solubilisation) had occurred for fresh Bambara groundnuts. A cooking time of 120 min was further included, as a comparison to 60 min as longer

processing time would probably influence (possible) further pectin solubility. The second part of this section considered samples with distinct HTC levels but possessing comparable hardness (approx. 5 000 g), considered palatable. Therefore, the fresh sample processed for 120 min, 16w sample cooked for 180 min and the 32w sample cooked for 300 min were included. The final part of this section considered all digestion samples and gives a comparison of the data from the implemented HPAEC-PAD to that from the conventional DNS assay which measured total reducing sugars.

3.5.1 Influence of distinct HTC levels after cooking for similar times

The time dependency of starch digestion of Bambara groundnuts with distinct HTC levels at similar cooking times was monitored during 2 h of static small intestinal digestion. Starch digestion products were determined by measuring reducing sugars (**Figure 4A**) or by HPAEC-PAD (**Figure 4B**). For both analyses, an increase of starch digestion products with digestion time is observed at all HTC levels. However, high HTC level samples cooked for 60 min reached a plateau in the early phases of digestion (**Figure 4**). Estimation of kinetic parameters after modelling the data with a fractional conversion model, quantify these observations (**Table 2**). The high HTC level sample processed for 60 min (32w-60min) exhibited a significantly higher rate constant, k_d, and extent of starch digestion compared to the fresh sample (0w-60min). The previously determined textural and microstructural attributes of the samples can be used to explain the differences in digestion. Microscopy images show that the 0w-60min sample, represented in **Figure 3 d,** had predominantly separated intact cells (hardness ~ 8 000 g). By contrast, a heterogeneous mixture of separated cells and ruptured cells (which released starch granules) is observed for 16w-60min and 32w-60min (**Figure 3 e** and **f,** respectively). The starch granules released from ruptured cells potentially resulted in immediate access of the amylase to its substrate during digestion. Accordingly, high HTC Bambara groundnuts cooked for 60 min, possessing many free starch granules after mashing, had the highest rate constant of starch digestion.

When the cooking time was increased to 120 min (**Figure 4**), k_d significantly decreased for the 16w-120min and 32w-120min samples but not significantly for the 0w-120min sample (**Table 2**). The decrease in k_d is logical because, with increased thermal treatment, potentially more pectin could be solubilised. Even more cell separation due to potentially greater pectin solubilisation meant more intact cells were present. Indeed, with intact cells, starch is encapsulated within cell walls and the number of free starch granules that could be easily accessed by the amylase was reduced. This is

reflected in microscopy images of the samples stored for 16w and 32w that show that more cell breakage occurred at 60 min compared to 120 min cooking time (**Figure 3 e** compared to **h**).

We could not find any relationship of starch digestion to gelatinisation enthalpies of these samples, as no striking differences were observed between the residual starch gelatinisation enthalpies of cooked samples (~ 0.3 J/g) (**Table 1**). Furthermore, the residual enthalpy values reflect that more than 80% (c.a.) of the starch molecular order had been lost at 60 min cooking time. A previous study on wheat flour possessing various degrees of gelatinisation showed that beyond a degree of gelatinisation of just 6% there was no significant influence of gelatinisation level on the rates and extent of starch hydrolysis (Wang, Wang, Liu, Wang, & Copeland, 2017). Furthermore, starch structural features in cooked garbanzo beans and peas which include crystallinity, melting temperatures and enthalpy change, did not influence their starch digestion kinetics (Xiong et al., 2018). These authors concluded that starch digestion kinetics of pulses is limited by the cell wall and protein matrix, rather than the molecular ordering of starch.

3.5.2 Influence of distinct HTC levels and cooking to obtain similar hardness

Low (0w) and medium (16w) and high HTC level (32w) Bambara groundnuts were processed for different periods (120 min, 180 min and 300 min respectively) to attain a similar hardness (~ 5 000 g). The digestion curves of Bambara groundnuts with distinct HTC levels, but similar hardness values are nearly superimposable (**Figure 5**). No significant differences in the estimated kinetic parameters k_d and final digested starch (%) were observed amongst these samples (**Table 2**). Microstructurally, these samples were also comparable (**Supplementary Figure A3 m**, **q** and **r**), showing mostly separated intact cells. By extending the processing time accordingly, the 16w and 32w Bambara groundnuts obtained similar hardness values that had comparable microstructures to the 0w sample, resulting in similar digestion kinetics.

Bambara groundnuts systems with mostly intact cells (e.g., 0w-120min, 16w-180 min, 32w-300min) showed digestion trends similar to that of intact sieved cells from red kidney beans (Rovalino-Córdova et al., 2018) and navy beans (Berg et al., 2012). In addition, the extent of starch digestion (80%) exhibited by high HTC level Bambara groundnuts with high hardness (ruptured cells), was similarly observed for the kidney beans with broken cells (Rovalino-Córdova et al., 2018). Lower starch digestibility values in legume systems (<5%) have been reported by other authors (Bhattarai,

Dhital, Wu, Chen, & Gidley, 2017; Dhital et al., 2016). The lower values could be due to either lower enzyme concentrations or different mixing conditions used in the *in vitro* digestion model considered.

The relationship between the estimated rate of digestion, k_d, and the hardness of all the samples that were considered for digestion was linear and strongly positive (R^2=0.97) (**Figure 6**). This trend implies that faster starch digestion would occur in Bambara groundnuts that have higher hardness values. Overall, the starch digestion kinetics results show that the hardness and interlinked microstructures of the samples after cooking influenced starch digestion.

These findings imply that different starch digestibility's could be obtained depending on the consumers' preference for hardness after processing given legumes that may possess different extents of the HTC. On the one hand, a consumer who tolerates harder legumes may shorten the cooking time. Upon mastication, some cells may rupture releasing starch granules, which might increase the rate of starch digestion. On the other hand, a consumer who prefers a softer texture may extend the cooking time. If cooking is done just long enough, it can potentially guarantee starch encapsulation within cell walls, consequently limiting starch digestion. Limited small intestinal starch digestion is preferred in the context of controlling postprandial blood glucose levels and in increasing the amount of "resistant" starch that can be fermented in the large intestines.

3.5.3 Comparison of DNS and HPAEC-PAD data

Starch digestion products can be determined using various techniques e.g., spectrophotometric methods such as the DNS assay to quantify the level of reducing sugars (Edwards et al., 2015; Hardacre, Lentle, Yap, & Monro, 2016). In this work, we implemented an HPAEC-DAD method to quantify maltooligosaccharides. We further used the DNS method, which is relatively cheap and simple and compared the data obtained to that from the more advanced HPAEC-PAD analysis encouraged in the literature (Dona et al. 2010).

A representative chromatogram of digestion fluid is shown in **Figure 7**. For all samples, maltose was the most abundant sugar, followed by maltotriose and little amounts of glucose. The higher maltooligosaccharides from maltotetraose up to maltodecaose (the highest standard used in this analysis) were barely detected in the samples. Information from *in vivo* studies shows that maltose and maltotriose are the reaction products mostly released in luminal starch digestion (Quezada-Calvillo et al., 2007).

The DNS and HPAEC-PAD data show comparable kinetic behaviour (**Figure 4 A with B and Figure 5 A with B**). However, the DNS assay mostly overestimated the level of digested starch by about 10 % in most cases, although these differences were not significant at 95 % confidence interval **Table 2**. The overestimation could be due to the inherent principle of the DNS assay in evaluating overall reducing potential. Only a single standard is taken into account for quantification (in our case: maltose). Possibly, other reducing sugars (e.g. maltotriose and glucose) produce varying colour intensities with the dinitrosalicylic acid reagent (Saqib & Whitney, 2011). The DNS assay may be used for routine analysis of a large number of samples. Nevertheless, it is a black box type of method that will not provide mechanistic insight into the multiple responses of starch digestion as offered by the HPAEC-PAD analysis.

4. Conclusions

This is the first study that demonstrates that storage high temperature and humidity induce the HTC phenomenon in Bambara groundnuts. The longer the storage time, the more prolonged the cooking time, accompanied by a lower rate constant of texture degradation. Higher hardness values as a function of cooking time were associated with greater cell rupture releasing free starch granules upon mechanical disintegration. It was hypothesised that cooking-induced pectin solubilisation, was greater in fresh compared to HTC Bambara groundnuts. By adjusting the cooking time, Bambara groundnuts from different storage levels can be cooked to attain a comparable hardness and microstructure.

This work proves that starch digestibility of Bambara groundnuts with distinct HTC levels is strongly related to the hardness values and their corresponding microstructures after processing. At fixed cooking times, samples with high hardness values showed more cell rupture and resulted in an increased rate constant and extent of starch digestion. Bambara groundnuts with distinct HTC levels but processed to attain similar palatable hardness values had similar starch digestion kinetics. Consequently, this means that by adaptation of the cooking time, Bambara groundnuts of different HTC levels do have similar starch digestion behaviour. Finally, HPAEC-PAD proved to be slightly more accurate compared to the DNS assay. Additionally, HPAEC-PAD analysis can provide mechanistic understanding of starch digestion, for instance, through multi-response modelling of the multiple digestion species data. Future work could study mineral bioaccessibility in legumes with different HTC levels since it is known that cation reorganisation occurs during storage as is described in the phytate-pectin-cation mechanism of the HTC phenomenon.

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Tables

Table 1: Onset (T_o), peak (T_p), conclusion (T_c) temperatures for gelatinisation and associated enthalpy (J/g sample) of raw and processed Bambara groundnuts obtained from DSC analysis. Values are averages ± standard deviations from two runs.

Table 2: Comparison of estimated rate constants of digestion, k_d, and final digested starch (%) of data obtained by measuring reducing sugars or by summation of multiple starch digestion species after quantification by HPAEC-PAD. All data was modelled by a fractional conversion model. Values are estimates of parameters ± standard error, while different superscript letters within columns indicate significantly different values at 95% confidence intervals.

Figure Captions

Figure 1: Cooking profiles for 0w (**+**), 16w **(+**) and 32w (**+**) stored Bambara groundnuts cooked in water at 95 °C. Markers represent an average hardness (g) value of 20 seeds. Continuous lines are predicted values of hardness from the fractional conversion model. The grey rectangles show the samples that were selected for the *in vitro* digestion study.

Figure 2: Estimated parameters obtained from the fractional conversion model: (A) Rate constant of texture degradation, k_s (min^{-1}) and (B) estimated final hardness (g), as a function of increasing storage time.

Figure 3: Micrographs of processed and mechanically disintegrated Bambara groundnuts stained with iodine solution to give a violet to blue-black colour of the iodine-starch complex. White arrows show free starch granules while green arrows show an individual separated cell. Scale bar = 100 µm.

Figure 4: Kinetics of *in vitro* small intestinal starch digestion for the fresh and stored Bambara groundnuts processed for 60 and 120 min: 0w-60min (●), 16w-60min (▲), 32w-60min (◆), 0w-120min (○), 16w-120min (△) and 32w-120min (◇). Starch digestion products were analysed as reducing sugars (A) or as summation of multiple digestion species (B). Experimental data are shown as markers while values as predicted by the fractional conversion model, are shown as lines.

Figure 5: Kinetics of *in vitro* small intestinal starch digestion for the fresh and stored Bambara processed to attain a similar hardness of 5 000 g**:** 0w-120min (○)**,** 16w**-**180min (×) and 32w-300min (■). Graph (A) is obtained from data from reducing sugars while (B) is based on the summation of multiple starch digestion products from HPAEC-PAD. Experimental data are shown as markers and values predicted by the fractional conversion model are shown as lines.

Figure 6: A correlation of hardness (g) to the estimated rate constant of digestion k_d (from HPAEC-PAD data), of 0w-60min (●), 16w-60min (▲), 32w-60min (◆), 0w-120min (○), 16w-120min (×), 16w**-**180min (△), 32w-120min (◇) and 32w-300min (■) Bambara groundnuts' samples.

Figure 7: A representative chromatogram of the digests at the end of the *in vitro* small intestinal digestion phase. Glucose, maltose and maltotriose were the oligosaccharides detected at all the time moments considered in the *in vitro* small intestinal phase.

Figure A1: A representative DSC curve of the second heating ramp from -60 °C to 90 °C at 10 °C/min. The peak is integrated and shows the T_g range of Bambara groundnuts with a midpoint of 14.26 °C.

Figure A2: Representative DSC thermographs of unprocessed (**A**) and processed (**B** and **C**) Bambara groundnuts with distinct storage levels.

Figure A3: Micrographs of processed and mechanically disintegrated Bambara groundnuts stained with iodine solution to give a violet to blue-black colour of the iodine-starch complex. Scale Bar = 100 μm.

Table 3: Gelatinisation parameters for raw and processed Bambara groundnuts with distinct HTC levels. The onset (T_o), peak (T_p), conclusion (T_c) temperatures and the enthalpy (J/g sample) for gelatinisation are defined. Values are means ± standard deviation of 2 separate runs. Gelatinization enthalpy values with different superscript letters are significantly different.

	T_o (°C) Gelatinisation	**T_p (°C)**	**T_c (°C)**	**enthalpy (Jg^{-1} sample)**
0-weeks-raw	75.64 ± 0.18	80.20 ± 0.37	98.46 ± 1.97	2.85 ± 0.02^{A}
16-weeks-raw	75.78 ± 0.51	80.03 ± 0.18	96.67 ± 0.54	3.06 ± 0.13 ^{A}
32-weeks-raw	75.74 ± 0.15	80.08 ± 0.18	99.71 ± 0.57	2.81 ± 0.16 ^{A}
0-weeks-60 min	85.55 ± 0.38	94.52 ± 0.24	105.00 ± 0.45	0.31 ± 0.06^{B}
16-weeks-60 min	85.54 ± 0.65	94.33 ± 0.67	105.35 ± 0.45	0.35 ± 0.05 ^{B}
32-weeks-60 min	85.71 ± 0.07	92.00 ± 0.07	107.96 ± 2.33	0.25 ± 0.02 ^{B}
0-weeks-120 min	93.94 ± 0.29	98.48 ± 0.01	107.42 ± 0.01	0.34 ± 0.04 ^{B}
16-weeks-120 min	86.62 ± 0.62	98.31 ± 1.07	105.73 ± 0.67	0.34 ± 0.00 ^{B}
32-weeks-120 min	83.35 ± 0.39	97.99 ± 0.02	108.50 ± 2.82	0.24 ± 0.03 ^{B}
16-weeks-180 min	92.65 ± 0.25	99.08 ± 0.01	107.40 ± 1.24	0.23 ± 0.05 ^{B}
32-weeks-300 min	93.02 ± 1.41	98.19 ± 0.20	106.40 ± 0.20	0.28 ± 0.04 ^{B}

Table 2: Comparison of the estimated rate constant of digestion, k_d, and final digested starch (%) of data obtained by measuring reducing sugars or by summation of multiple starch digestion species after quantification by HPAEC-PAD. All data were modelled by a fractional conversion model. Values are estimates of parameters ± standard error, while different superscript letters within columns indicate significantly different values at 95% confidence intervals.

Sample	k_d (min^{-1})	Final digested starch (%)
based on reducing sugars		
0-weeks-60 min	0.04±0.01^{a,b}	61.7±3.6^{x}
16-weeks-60 min	0.11±0.01^{d}	75.5±1.3^{y}
32-weeks-60 min	0.11±0.01^{d}	83.9±1.5^{4y,z}
0-weeks-120 min	0.02±0.01^{a}	67.7±3.2^{x}
16-weeks-120 min	0.05±0.01^{c}	62.7±2.3^{x}
32-weeks-120 min	0.08±0.01^{d}	79.7±3.5^{y}
16-weeks-180 min	0.03±0.01^{a}	59.8±6.1^{x}
32-weeks-300 min	0.03±0.01^{a}	64.4±6.1^{x}
based on HPAEC-PAD data		
0-weeks-60 min	0.06±0.01^{c}	52.7±1.4^{x}
16-weeks-60 min	0.08±0.01^{c,d}	76.5±1.9^{y}
32-weeks-60 min	0.13±0.01^{d}	76.79±3.1^{y}

0-weeks-120 min	0.03±0.01[a]	59.6±3.6[x]
16-weeks-120 min	0.06±0.01[c]	54.7±1.8[x]
32-weeks-120 min	0.09±0.02[c,d]	62.71±1.7[x]
16-weeks-180 min	0.03±0.01[a]	61.9±6.4[x]
32-weeks-300 min	0.03±0.01[a]	60.4±3.0[x]

Highlights

- Adverse storage decreased the rate constant of texture degradation during cooking.
- HTC development alters the dynamics of cell failure on application of a force.
- Harder cotyledons (> 9 000 g) result in higher rates of starch digestion.
- Despite HTC effects, similar textures result in similar starch digestion kinetics.
- HPAEC-PAD analysis implemented to analyse starch digestion products.

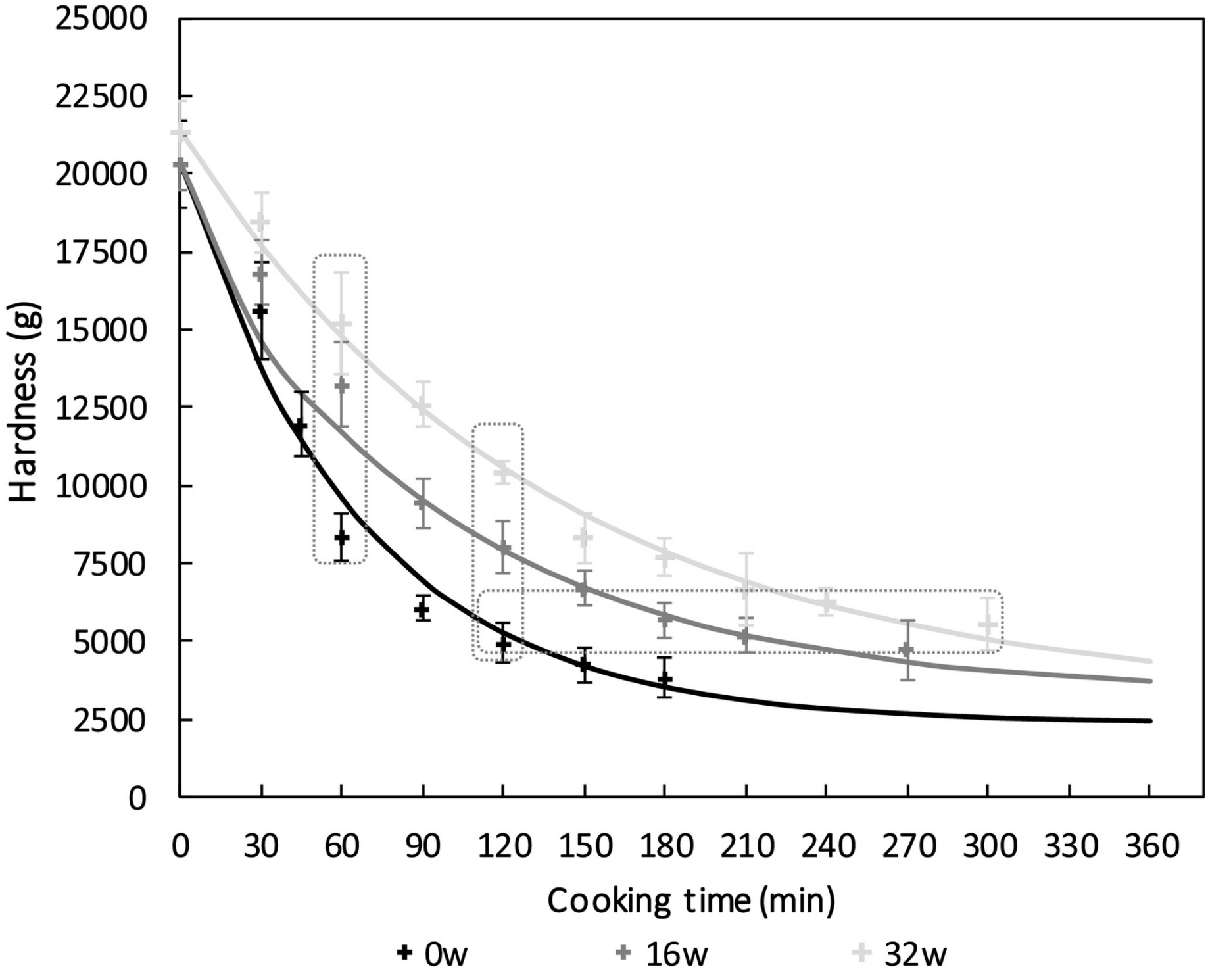
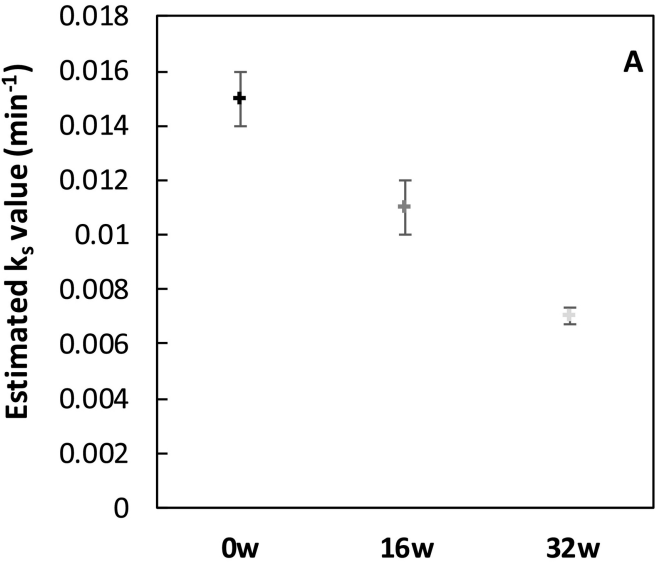

25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
Hardness (g)
0
30
60
90
120
150
180
210
240
270
300
330
360
Cooking time (min)
0w
16w
32w

Figure 1


A
Estimated k_s value (min^{-1})
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0w
16w
32w

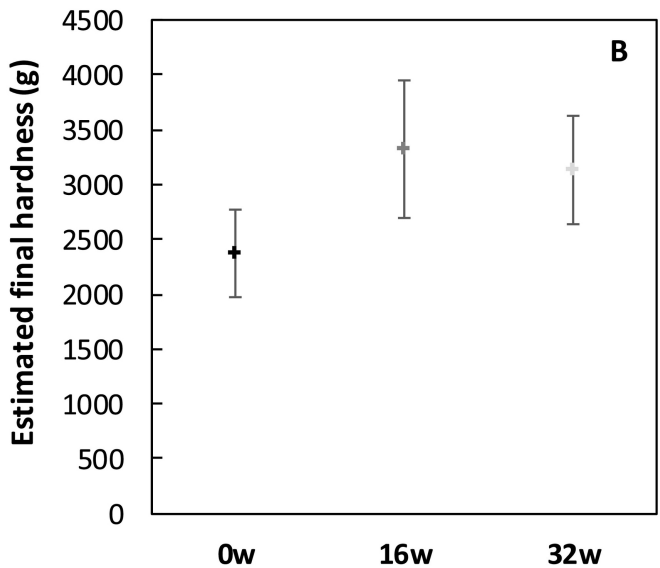

B
Estimated final hardness (g)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0w
16w
32w

Figure 2

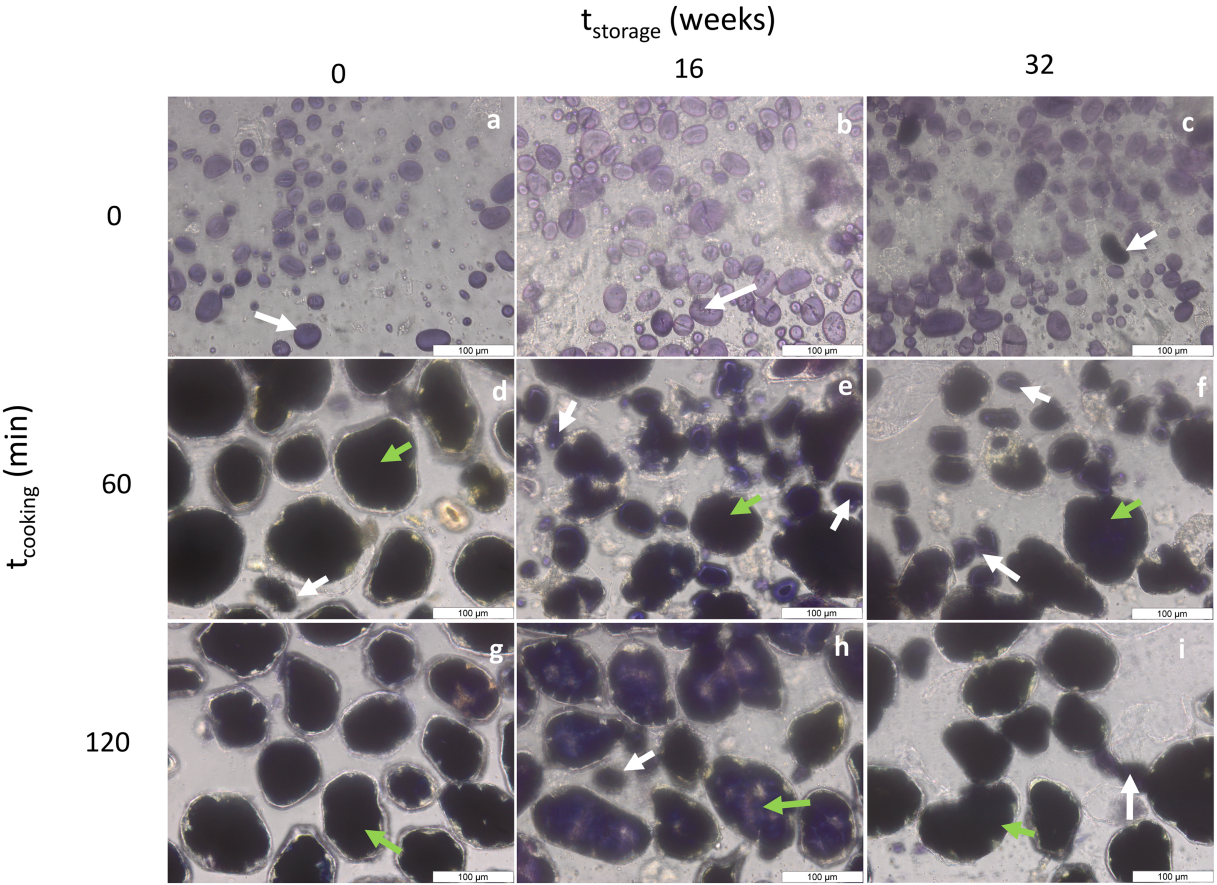


Figure 3

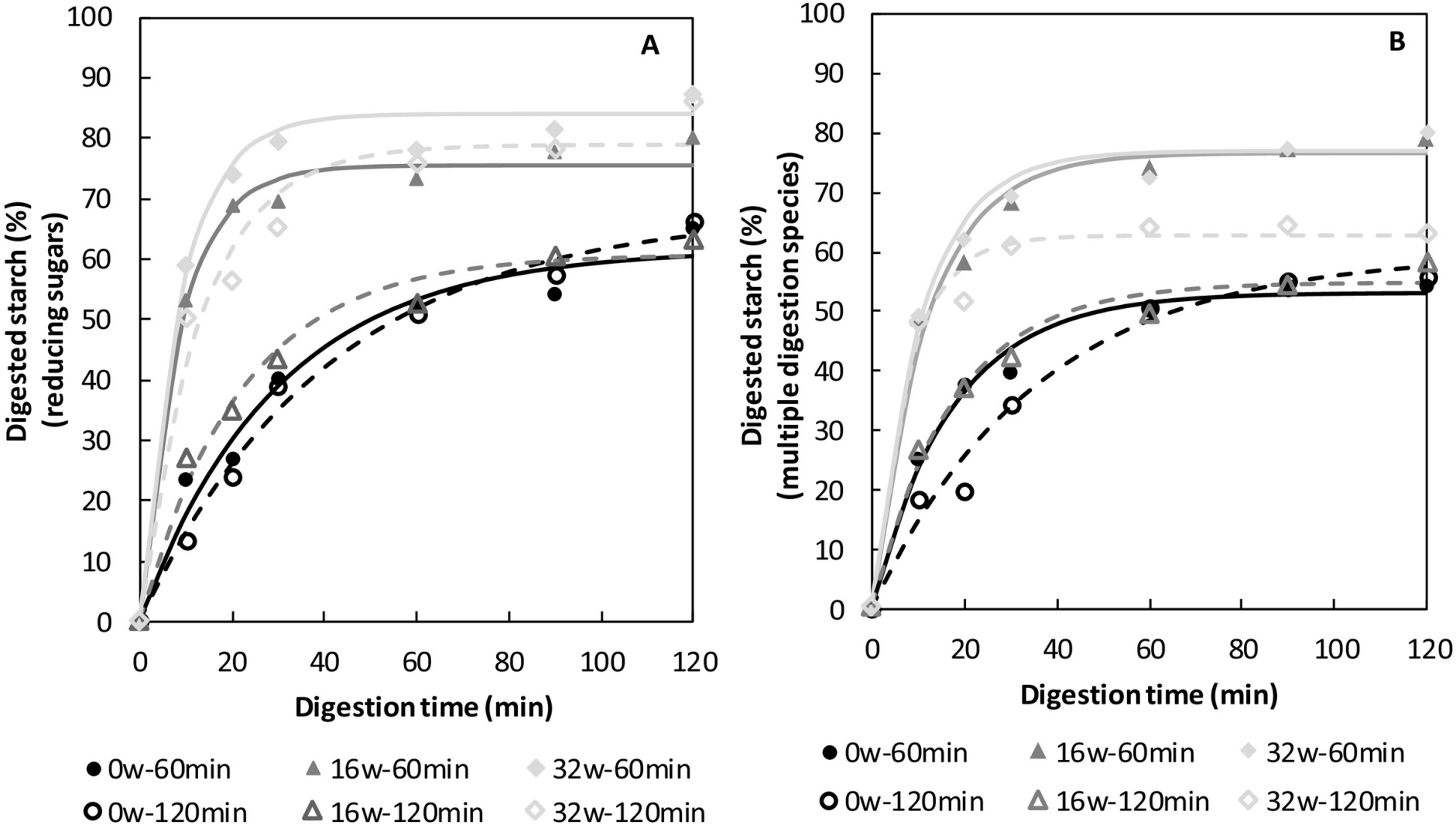

A
B
Digested starch (%)
(reducing sugars)
Digested starch (%)
(multiple digestion species)
Digestion time (min)
Digestion time (min)
0w-60min
16w-60min
32w-60min
0w-120min
16w-120min
32w-120min

Figure 4

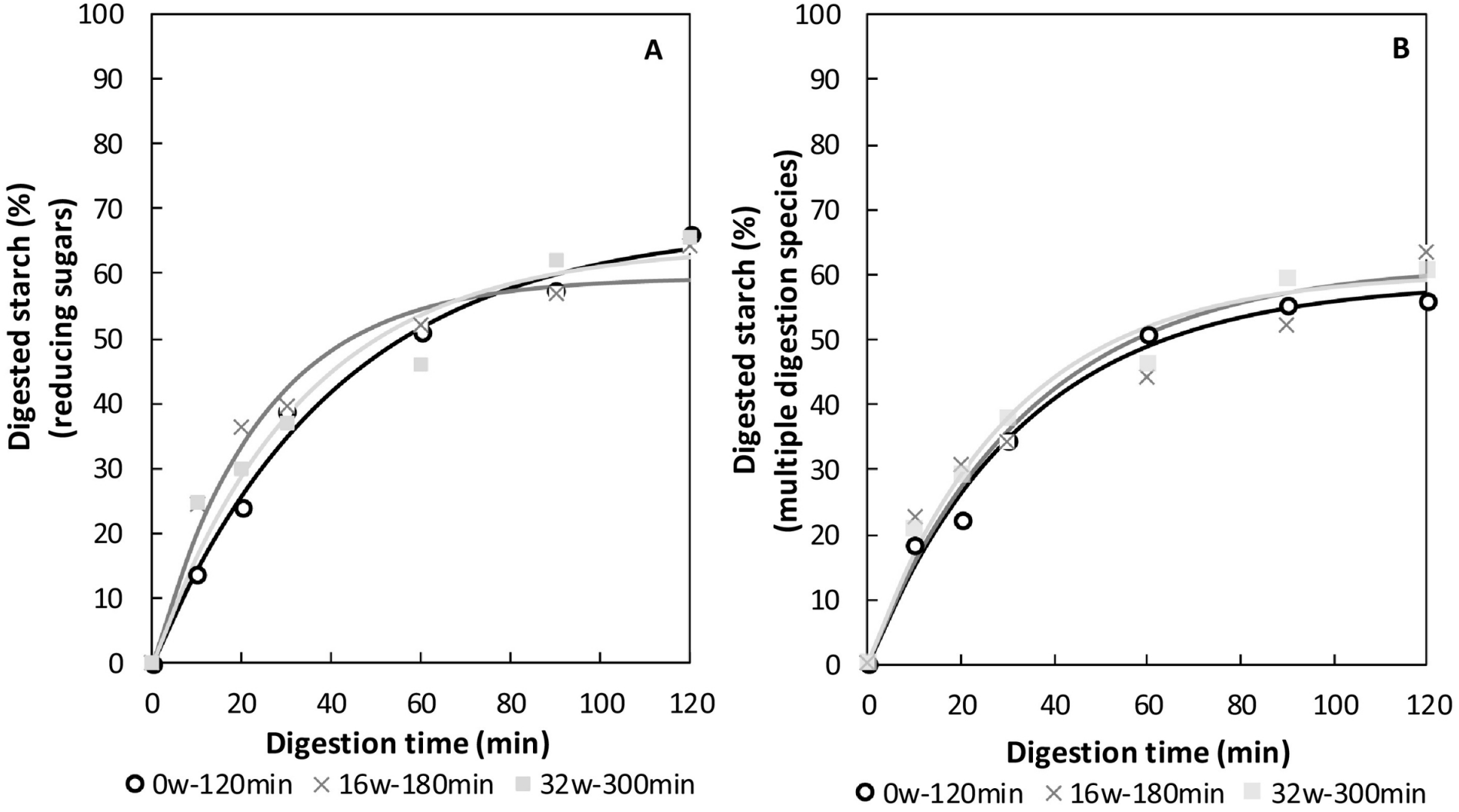

A
B
Digested starch (%)
(reducing sugars)
Digested starch (%)
(multiple digestion species)
Digestion time (min)
Digestion time (min)
0w-120min
16w-180min
32w-300min
0w-120min
16w-180min
32w-300min

Figure 5

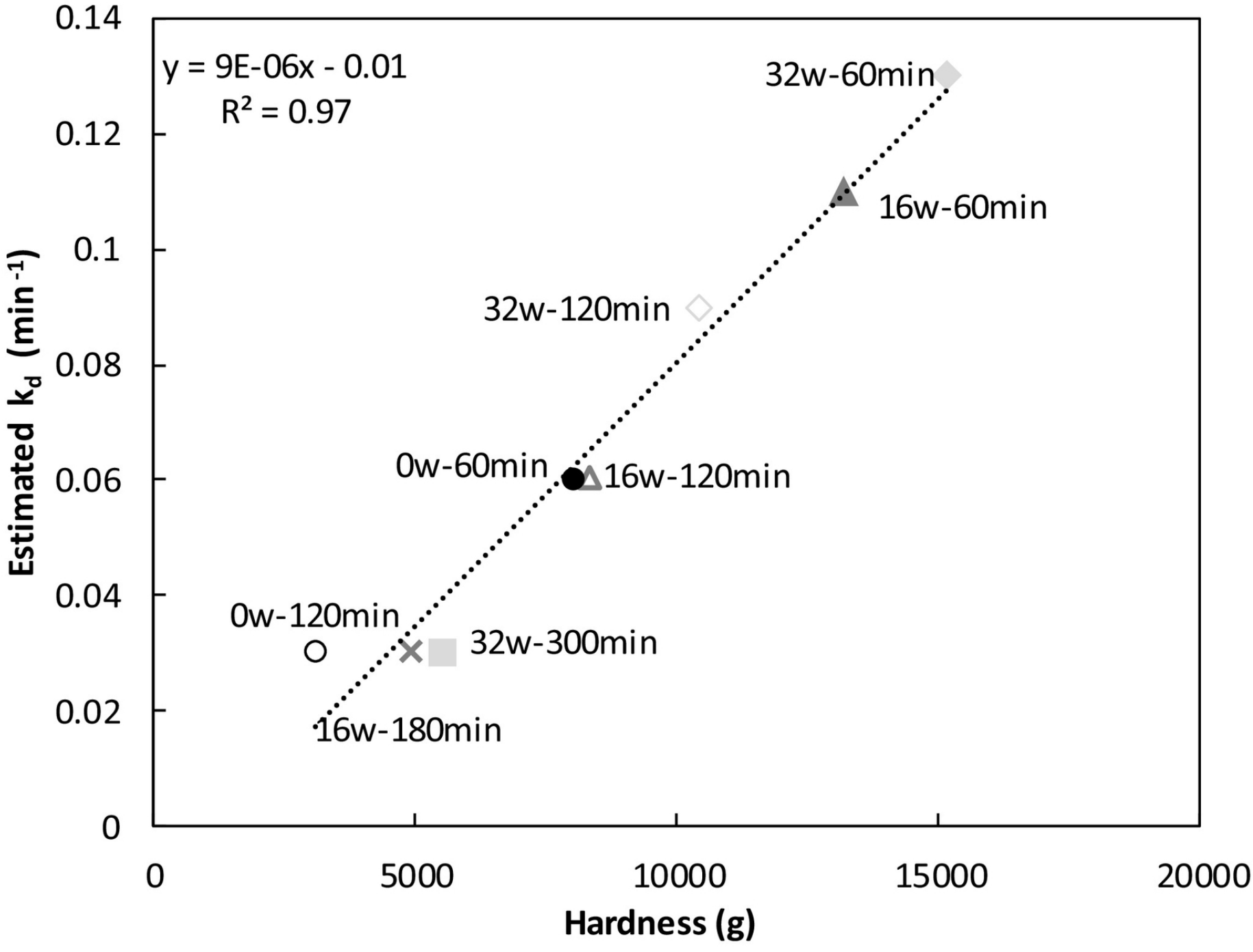

y = 9E-06x - 0.01
R² = 0.97
32w-60min
16w-60min
32w-120min
0w-60min
16w-120min
0w-120min
32w-300min
16w-180min
Estimated k_d (min^-1)
Hardness (g)
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0
5000
10000
15000
20000

Figure 6

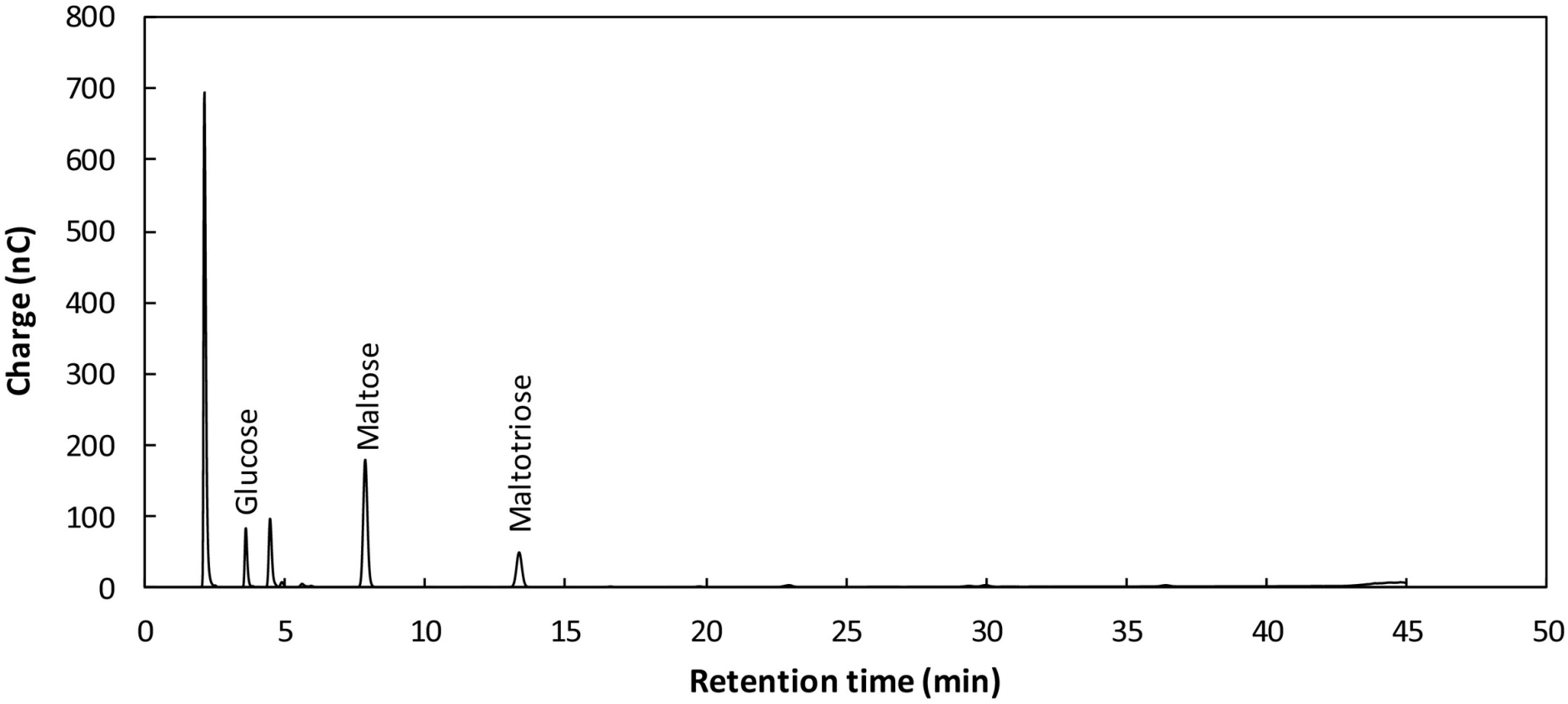

800
700
600
500
400
300
200
100
0
Charge (nC)
Glucose
Maltose
Maltotriose
0
5
10
15
20
25
30
35
40
45
50
Retention time (min)

Figure 7