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Ageing, dehulling and cooking of Bambara groundnuts: consequences for mineral retention and *in vitro* bioaccessibility†

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Postharvest storage of legumes is a strategy to ensure food security. However, the hard-to-cook phenomenon, partly explained by pectin–cation–phytate theory, may develop during storage. Based on this theory, minerals could be redistributed within the matrix, affecting the concentration of free/bound minerals and hence their bioaccessibility. Therefore, this study investigated the influence of storage-induced ageing and subsequent dehulling, soaking and cooking treatments on the concentration and *in vitro* bioaccessibility of Ca, Mg, Fe and Zn in Bambara groundnuts. ICP-OES was performed to determine Ca, Mg, Fe and Zn concentrations after applying different treatments and after simulated digestion in the context of bioaccessibility determination. This study illustrates that while the seed coat is rich in Ca (51%), it contains only less than 15% of Mg, Fe or Zn. Hence, dehulling negatively influenced Ca, but not Mg, Fe or Zn concentration. During soaking, up to 10% or 56% of the initial Mg concentration leaches into whole or dehulled seeds, respectively. Prolonging the cooking time (300 min) of whole aged Bambara groundnuts (32 weeks), necessary for obtaining palatable textures, decreased the overall Mg, Fe and Zn concentrations by 72%, 57% and 48%, respectively. Storage-induced ageing significantly decreased Ca solubility and bioaccessibility. Bioaccessibility of trace minerals in Bambara groundnuts is low, especially for Fe, and cannot be improved by dehulling or cooking treatments. Ageing did not influence Fe and Zn bioaccessibility. Populations relying on both fresh and aged Bambara groundnuts may still be at risk for mineral deficiencies.

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1. Introduction

Dietary minerals play a crucial role in sustaining human life as they are involved in numerous biochemical pathways.^{1,2} As such, a diet rich in minerals is vital, given that the body cannot synthesise them on its own. After meal ingestion, minerals must be released from the food matrix to enable their absorption across the epithelial cells that line the gastrointestinal (GI) tract.³ However, minerals in food do not always exist in a bioaccessible state and can be bound to several antinutrients, for instance, phytates, polyphenols, oxalates and pectin present in most plant-based food. The presence of these indigestible mineral–antinutrient complexes potentially limits mineral bioaccessibility.^{4–6}

Several studies have investigated the bioaccessibility of minerals in legumes (*e.g.*, common beans, cowpeas, chickpeas and lentils) after processing.^{7–9} However, little attention has been paid to the influence of postharvest storage-induced changes. In reality, the storage of legumes in most developing countries is largely uncontrolled, yet the climate there is hot and humid. Besides, the storage period between two harvesting seasons can easily reach 6–12 months or longer. Under such storage conditions (>25 °C and 65% RH), the hard-to-cook phenomenon (HTC) is most likely to develop.¹⁰ Of the widely accepted hypotheses explaining the HTC phenomenon, pectin–cation–phytate theory has received much attention. In this theory, phytase dephosphorylates the potent mineral chelator phytic acid (IP₆) to lower inositol phosphates (IP_{5,4,3,2}). These possess less mineral chelating ability compared to IP₆.^{11,12} When dephosphorylation of phytic acid occurs, some minerals are released. Alongside this reaction, pectin is demethylesterified by pectin methylesterase as ageing continues, enhancing its ability to chelate cations.¹³ The demethylesterified pectin could bind the released ions, potentially leading to the formation of crosslinks that strengthen the pectin-rich middle lamella of plant cell walls.¹⁴ This mechanism implies that

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there is a redistribution of minerals within the cotyledon matrix where this phenomenon occurs.

Following postharvest storage of legumes, there is always some level of processing, which can alter the matrix on different scales. Milling, for example, can cause a redistribution of minerals and antinutrients compartmentalised in different substructures of the seed.⁴ Dehulling is a simple pre-processing step that may decrease the content of antinutrients, for instance, tannins.¹⁵ The cooking of whole or dehulled legume seeds, after a soaking step, is the most common household processing step. A general belief is that minerals leach into the soaking or cooking water,^{8,16} yet it is not clear to what extent this happens and whether this is inferable to all minerals. Moreover, the influence of other factors such as storage or dehulling on the degree of leaching is still an open question.

Most studies concerning mineral bioaccessibility of legumes focus on globally marketed sources such as common beans, chickpeas and lentils.^{7,9,17} Nonetheless, it is crucial to gain this knowledge for other legume types. These legumes may not necessarily have a global market share but are of key importance in the regions where they are grown, chiefly in the context of food security. Bambara groundnuts (*Vigna subterranea*) were selected for this study, first because they are relied on to provide nutrition to famine prone rural populations, due to their drought-tolerance and high nutrient-density.^{15,18} Bambara groundnuts are reported to be a rich source of minerals. A serving of 100 g is calculated to potentially provide more than 50% of the recommended daily allowance of K, Mg and Zn.¹⁹ However, initial mineral concentrations do not always provide a good indication of the true bioaccessible amounts.^{9,20} Second, several antinutrients, for instance, polyphenols, phytates and pectin are present in Bambara groundnuts.²¹ Notably, these antinutrients, *e.g.*, phytates and pectin, which can limit mineral bioaccessibility, also participate in the described mechanism of the pectin-cation-phytate hypothesis of the HTC phenomenon.^{22,23} As such, there is a need to investigate the consequences of storage-induced ageing on mineral bioaccessibility.

The goal of this study was, first, to evaluate the evolution of the concentration of the selected minerals during storage (distribution between the seed coat and the cotyledon) and after subsequent dehulling, soaking and cooking treatments. Second, this work aimed to provide insight into the effects of these treatments on the *in vitro* bioaccessibility of the selected minerals using Bambara groundnuts as a model legume. Ca and Mg were selected as macro-elements which are essential in the diet and at the same time interact with the antinutrients pectin and phytates to different degrees.²⁴ The trace elements Fe and Zn were selected as theirs are the most widespread deficiencies²⁵ and they may be highly chelated by phytates and pectin as well.²⁶

2. Materials and methods

2.1 Description of the plant material and prepared samples

Fresh (dry) Bambara groundnuts were obtained from communal farmers in the Masvingo Province in Zimbabwe after the

harvesting season of 2016. On arrival at the laboratory, the groundnuts were sorted to remove foreign matter and any damaged seeds. A part of the cleaned Bambara groundnuts was then kept at -40°C and this represented the fresh sample (denoted 0 weeks). Another part of this batch was stored for 16 and 32 weeks, under the conditions of 35°C and 78% RH aimed at inducing the hard-to-cook phenomenon (ageing) as established in our previous study which used the same batch of Bambara groundnuts.²⁷

A part of the fresh and aged samples was soaked and cooked whole. The other part was manually dehulled with a knife before soaking and cooking. Dehulling was included because it may favourably reduce the concentrations of some antinutrients, including those which are mineral chelating.¹⁵ Soaking was done by immersing the seeds in distilled water (1:5 ratio w/v) for 16 h at 25°C . The soaking water was decanted and evaporated to account for solute loss.

The soaked samples were placed in individual Duran bottles with fresh preheated (95°C) distilled water (1:5 w/v) and were cooked for 60 or 120 min. Cooking times of 60 and 120 min were selected as they represent the upper and lower palatable hardness levels of the fresh sample (0 weeks). Since aged Bambara groundnuts need longer cooking time to sufficiently soften, the cooking time for the aged samples was also adapted (180 min for 16 weeks and 300 min for 32 weeks) to prepare samples with a palatability level equivalent to that of the fresh sample (0 weeks–120 min). Palatability levels were already known for this batch from our previous study,²⁷ where the texture of the same Bambara groundnuts batch prepared under the same conditions was determined.

The cooked samples were mashed with a mortar and pestle to disintegrate the sample before digestion. Raw samples were milled with a bench mill (IKA Labortechnik – A10, Germany). All prepared samples were freeze-dried and kept in a desiccator until further use. Freeze-drying was assumed to not affect the minerals or the structure of the samples. All chemicals and reagents used were of analytical grade.

2.2 Determination of the degree of methylesterification

Fourier transform infrared spectroscopy (FT-IR) was used to determine the degree of methylesterification (DM) of pectin of the fresh and aged Bambara groundnuts. Transverse sections, 40 μm thick, of 4 seeds of the fresh and aged cotyledons (soaked), were cut using a manual sliding microtome after freezing them on a cryo-stage. The sections were cleaned several times with water using a brush to eliminate starch and protein from within the cell walls. The sections were then stored in 70% ethanol until use.

For FT-IR measurements, each section (3 sections for each seed) was placed on parafilm and allowed to dry. The dry section was placed on the sample holder of the FT-IR, and the transmittance of the sample was measured in the range of 4000 cm^{-1} to 400 cm^{-1} . Integrated spectral mean values of 100 scans were collected per sample at a resolution of 4 cm^{-1} . After pre-processing of the spectra, the absorption intensity of the bands located at around 1740 cm^{-1} (due to ester carbonyl

group (C–O) stretching) and 1630–1600 cm^{-1} (due to carboxylate group (COO^-) stretching) were obtained. The DM was calculated as $136.86x + 3.987$, where x is the ratio of the intensity of the ester carbonyl band to the sum of the intensities of the ester carbonyl band and the carboxylate group.²⁸

2.3 Determination of mineral concentrations

Before mineral concentration determination, the whole, dehulled seeds and the seed coats were subjected to ashing to incinerate all the organic matter.²⁹ Ten mg of powder was ashed in a muffle furnace at 550 °C for 20 h. This was performed in triplicate for each sample. After cooling, the obtained ash was dissolved in 9.9 mL of Milli-Q water, and 0.1 mL of 65% nitric acid was added. The solution was left to stand at room temperature overnight. The next day, the samples were filtered using a 0.45 μm membrane filter (Chromaphil® A-45/25, Macherey-Nagel, Germany) and were analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES). The Mg, Ca, Fe and Zn contents in the ashed samples or the aliquots of the soluble or bioaccessible fraction (sections 2.4 and 2.6, respectively) were measured using an iCAP 7400 ICP-OES Duo spectrometer (USA). Mg and Ca were measured radially at 285 nm and 318 nm, respectively, while Fe and Zn were measured axially at 238 nm and 206 nm. Two certified reference materials BCR-129 and BCR-679 (Joint Research Centre, European Commission) were ashed and analysed as described above and good recoveries were obtained (Table S1†). Calibration was performed using SPS-SW2, before each run and after measuring 20 samples. The mineral content was expressed as mg per 100 g dry weight (dw).

2.4 Determination of soluble minerals

To determine the content of soluble minerals 500 mg of milled and freeze-dried sample of the raw fresh or aged Bambara groundnut flour was dispersed in 10 mL of Milli-Q water (pH 7).⁹ After 2 h of continuous stirring, the samples were centrifuged, and the supernatant was assessed for the soluble Ca, Mg, Fe and Zn contents as described in section 2.3. The solubility was calculated as follows:

$$\text{Soluble mineral}(\%) = \frac{\text{concentration in the supernatant}}{\text{concentration in the sample}} \times 100\% \quad (1)$$

2.5 Fluorescence microscopy of labelled Ca–pectin crosslinks

Fresh and aged cotyledon sections (40 μm) were treated with the monoclonal antibody 2F4 (PlantProbes, Leeds, United Kingdom), which is specific for Ca crosslinked regions of pectin.³⁰ Exactly 30 μL of a ten-fold dilution of 2F4 in 3% milk–saline phosphate buffer was added to the sections in a Petri dish, and incubated at 28 °C for 1.5 h. Later, the sections were washed for 5 min in phosphate buffer (3 times) to remove the excess unbound antibody. For visualisation, anti-mouse immunoglobulin G secondary antibody coupled with fluorescein (diluted 50 times) in 3% milk phosphate buffer was

added to the sections. The sections were incubated for 1 h at 28 °C in a dark environment.³¹ After this, the sections were washed 3 times in phosphate buffer for 5 min each cycle. The samples were then viewed under epifluorescence light (excitation filter between 460 and 490 nm) using an Olympus BX-51 microscope equipped with epifluorescence illumination (X-Cite® 120Q, X-Cite® Fluorescence Illumination, EXFO Europe, UK). Images were viewed at $\times 40$ magnification using a photo-analysing software (CellF®) after ensuring that there was no background autofluorescence.

2.6 *In vitro* digestion protocol

Simulated *in vitro* digestion of whole and dehulled Bambara groundnuts was performed using the INFOGEST protocol.³² Briefly, for the oral phase, freeze-dried samples were first reconstituted to their original moisture content after cooking with distilled water to obtain 1.25 g as the starting weight. After this, 1 mL of simulated salivary fluid (SSF) electrolyte stock solution, 0.125 mL of 0.015 M CaCl_2 solution and 0.125 mL of demineralised water were added and mixed. Next, precisely 1.6 mL of simulated gastric fluid (SGF) and 0.025 mL of CaCl_2 (0.015 M) were pipetted into the 2.5 mL oral bolus. The mixture was adjusted to pH 3 using HCl, and then 0.4 mL of porcine pepsin (Sigma P7012) dissolved in SGF solution was added to achieve 2000 U mL^{-1} in the final digestion mixture. The mixture was incubated at 37 °C for 2 h (with end-over-end rotation at 40 rpm) to simulate the gastric digestion phase.

For the small intestinal phase, the following solutions were added: 2.75 mL of simulated intestinal fluid (SIF), 0.2 mL CaCl_2 (0.015 M) and 0.625 mL of bile freshly dissolved in water (final concentration of 10 mM). The pH of the mixture was adjusted to 7, and next, 1.25 mL of pancreatic enzymes (pancreatin – Sigma P1750; chymotrypsin – Sigma C4129; trypsin – Sigma T2011) dissolved in SIF was added. The added enzyme volume resulted in 200 U mL^{-1} of pancreatic amylase, 100 U mL^{-1} of trypsin, and 25 U mL^{-1} of chymotrypsin. The samples were immediately incubated at 37 °C with end-over-end rotation at 40 rpm. Preliminary data showed that during the small intestinal phase, mineral bioaccessibility was independent of digestion time. Therefore, only the endpoint determinations at 2 h of the small intestinal digestion phase were considered. At least 2 cycles of digestion were performed for each sample. The samples were heat shocked (100 °C) to deactivate the enzymes and to stop digestion. The digests were centrifuged, and the supernatant was collected and frozen until use. The mineral concentrations were analysed, as described in section 2.3. Mineral bioaccessibility was then calculated as shown in eqn (2). For the blank, we replaced the sample weight with 1.25 mL distilled water, and all the other steps were followed as described above.

$$\text{Mineral bioaccessibility} = \frac{\text{mineral conc. in the digest} - \text{conc. in the blank}}{\text{mineral conc. in the sample}} \times 100\% \quad (2)$$

2.7 Estimation of contribution towards the recommended nutrient intake

The contribution of a 130 g serving (wet basis) of fresh and aged Bambara groundnuts towards the recommended nutrient intake (RNI) of Ca, Mg, Fe and Zn per day was calculated. Since a population has groups with different RNI requirements, the RNI of the group needing the most amount per day was considered. For Ca, the RNI of 1000 mg day⁻¹ as required by an adolescent was used and for Mg, the RNI of 260 mg day⁻¹ as required by an average adult male was used.³³ For Zn, the RNI of 12 mg day⁻¹ for pregnant women was used (assuming that they regularly consume a diet with low Zn bioavailability). For Fe, the RNI of 21.8 mg day⁻¹ as required by 11–14 year old females who regularly consume a diet with low Fe bioavailability was considered.³³

2.8 Data analysis

The effects of ageing and subsequent steps of dehulling, soaking and cooking time on Ca, Mg, Fe and Zn concentrations and bioaccessibility were assessed. Regression analysis (least squares approach) was used to evaluate the treatments that were significant in influencing the concentration or bioaccessibility of a particular mineral, through the Fit model platform of JMP® (version 14.0.0, SAS Institute, Inc., Cary, NC, USA). Non-significant variables were excluded from the final model or only included if their exclusion caused a lack of fit or reduced the R^2 adj. The model was assessed by the lack of fit test and by evaluating residuals and QQplots. ANOVA was used to analyse the solubility results.

3. Results and discussion

In this work, we investigated the effect of storage-induced ageing, dehulling, soaking and cooking on the concentration levels of Ca and Mg as macro-elements and Fe and Zn as trace elements (section 3.1). In plant-based materials, minerals are not necessarily free. We studied the complexation of the selected minerals as affected by storage-induced ageing, discussed in section 3.2. Finally, treatment effects of storage, dehulling and cooking on mineral bioaccessibility levels are evaluated (section 3.3).

3.1 Concentration of Ca, Mg, Fe and Zn as affected by treatments

3.1.1 Influence of storage-induced ageing. There was limited variability in the concentration of the minerals in raw seeds over the ageing time as explored in this study: Ca (47.7–49.9 mg per 100 g dw), Mg (166.9–169.6 mg per 100 g dw), Fe (4.6–4.9 mg per 100 g dw) and Zn (3.1–3.8 mg per 100 g dw) (Table 1). Since there was no dry matter loss with storage time, the result is not surprising. Similarly, little variation in the concentration of these minerals in the seed coat was observed with ageing (Table 2), implying that there was limited migration of minerals between the seed coat and the cotyledon during storage. In fact, in mature seeds, the vascular

Table 1 Concentration of Ca, Mg, Fe and Zn in the 0, 16 and 32 weeks stored whole and dehulled Bambara groundnuts, when raw and after soaking and cooking treatments. Values are means ($n = 3$) $\pm$ standard deviation

Sample	Raw	Soaking	60 min cooking	120 min cooking
Ca (mg per 100 g dw)				
0 week-whole	49.9 $\pm$ 3.2	48.8 $\pm$ 1.1	49.8 $\pm$ 6.1	48.3 $\pm$ 5.8
0 week-dehulled	32.2 $\pm$ 2.4	31.8 $\pm$ 0.7	24.2 $\pm$ 5.2	23.4 $\pm$ 3.9
16 week-whole	47.3 $\pm$ 1.4	46.7 $\pm$ 0.4	45.0 $\pm$ 6.1	51.0 $\pm$ 3.7
16 week-dehulled	32.8 $\pm$ 3.0	31.3 $\pm$ 1.0	22.2 $\pm$ 6.8	19.5 $\pm$ 2.2
32 week-whole	47.7 $\pm$ 5.8	49.1 $\pm$ 2.9	50.3 $\pm$ 3.7	49.6 $\pm$ 7.1
32 week-dehulled	31.2 $\pm$ 1.9	30.1 $\pm$ 0.7	21.4 $\pm$ 2.2	20.7 $\pm$ 1.3
Mg (mg per 100 g dw)				
0 week-whole	166.9 $\pm$ 5.1	164.2 $\pm$ 1.9	139.3 $\pm$ 2.8	122.9 $\pm$ 7.3
0 week-dehulled	188.8 $\pm$ 14.7	119.4 $\pm$ 2.7	87.7 $\pm$ 5.9	62.9 $\pm$ 7.8
16 week-whole	167.8 $\pm$ 1.9	150.7 $\pm$ 1.1	104.9 $\pm$ 4.7	108.8 $\pm$ 10.3
16 week-dehulled	182.0 $\pm$ 7.0	116.5 $\pm$ 7.2	66.4 $\pm$ 1.4	58.9 $\pm$ 6.0
32 week-whole	169.6 $\pm$ 8.5	152.1 $\pm$ 11.5	95.5 $\pm$ 1.3	94.5 $\pm$ 6.7
32 week-dehulled	187.9 $\pm$ 2.4	82.3 $\pm$ 6.9	56.0 $\pm$ 1.2	49.8 $\pm$ 0.9
Fe (mg per 100 g dw)				
0 week-whole	4.9 $\pm$ 0.1	3.8 $\pm$ 0.3	3.6 $\pm$ 0.1	3.4 $\pm$ 0.1
0 week-dehulled	4.3 $\pm$ 0.1	3.7 $\pm$ 0.2	3.5 $\pm$ 0.1	3.1 $\pm$ 0.2
16 week-whole	4.6 $\pm$ 0.1	3.6 $\pm$ 0.1	3.3 $\pm$ 0.4	3.2 $\pm$ 0.1
16 week-dehulled	4.9 $\pm$ 0.2	3.5 $\pm$ 0.1	3.4 $\pm$ 0.1	2.7 $\pm$ 0.3
32 week-whole	4.7 $\pm$ 0.2	3.4 $\pm$ 0.1	3.2 $\pm$ 0.5	3.3 $\pm$ 0.5
32 week-dehulled	4.4 $\pm$ 0.3	3.5 $\pm$ 0.1	3.3 $\pm$ 0.1	2.7 $\pm$ 0.4
Zn (mg per 100 g dw)				
0 week-whole	3.1 $\pm$ 0.1	2.8 $\pm$ 0.1	3.0 $\pm$ 0.1	2.2 $\pm$ 0.1
0 week-dehulled	3.2 $\pm$ 0.2	2.9 $\pm$ 0.1	2.8 $\pm$ 0.1	2.6 $\pm$ 0.1
16 week-whole	3.2 $\pm$ 0.6	3.0 $\pm$ 0.1	2.8 $\pm$ 0.5	2.5 $\pm$ 0.1
16 week-dehulled	3.2 $\pm$ 0.2	2.9 $\pm$ 0.1	2.5 $\pm$ 0.1	2.2 $\pm$ 0.2
32 week-whole	3.8 $\pm$ 0.7	2.9 $\pm$ 0.1	2.5 $\pm$ 0.2	2.3 $\pm$ 0.3
32 week-dehulled	3.3 $\pm$ 0.2	2.9 $\pm$ 0.1	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1

Table 2 Concentration of Ca, Mg, Fe and Zn in the seed coat of Bambara groundnuts. Values are means ($n = 3$) $\pm$ standard deviation

Storage time (weeks)	Mineral content of the seed coat (mg per 100 g dw)			
	Ca	Mg	Fe	Zn
0	302.0 $\pm$ 22.4	311.6 $\pm$ 1.7	6.8 $\pm$ 0.7	3.8 $\pm$ 0.1
16	315.8 $\pm$ 13.1	298.7 $\pm$ 4.2	7.5 $\pm$ 1.0	3.3 $\pm$ 0.1
32	303.7 $\pm$ 20.0	330.6 $\pm$ 6.4	7.1 $\pm$ 0.1	3.1 $\pm$ 1.2
Relative contribution (%) of the seedcoat to the Ca, Mg, Fe or Zn concentration	51%	14%	12%	7%

connections between the seed coat and filial tissues (embryo and endosperm) cease to exist.³⁴

3.1.2 Influence of dehulling. Dehulling decreased the concentration of Ca by 33%, while the concentration of Mg, Fe or Zn was only marginally changed (Table 1). This observation was statistically proven by the integrated data analysis performed to establish the effect of the different treatments considered (*i.e.* storage time, dehulling, soaking and cooking) per mineral type (Table 3). In this table, the higher the value of a coefficient (positive or negative), the greater the effect of the

Table 3 Regression coefficients ($\pm$ error) of the variables (storage time, dehulling, soaking and cooking treatments) influencing Ca, Mg, Zn and Fe concentration. Significance was tested at 95% confidence intervals

Mineral type	Intercept	Storage time	Dehulling	Soaking	Cooking time	Interaction term	R^2 adj
Ca	40.0 $\pm$ 0.8	n.s	-10.1 $\pm$ 0.6	n.s	-0.1 $\pm$ 0.0	Dehulling $\times$ cooking time -0.1 $\pm$ 0.0	0.95
Mg	157.8 $\pm$ 4.2	n.s	-7.0 $\pm$ 2.5	26.0 $\pm$ 2.9	-0.4 $\pm$ 0.1	Dehulling $\times$ soaking 16.5 $\pm$ 2.5 Storage $\times$ soaking 0.4 $\pm$ 0.2	0.96
Fe	4.0 $\pm$ 0.1	n.s	n.s	0.4 $\pm$ 0.1	n.s		0.27
Zn	3.1 $\pm$ 0.1	n.s	n.s	0.2 $\pm$ 0.1	-0.1 $\pm$ 0.0		0.54

n.s (not significant).

treatment. Intercept values represent average concentrations determined for all types of raw samples (Table 1). For Ca, dehulling was the most significant main effect influencing the Ca concentration (Table 3). The seed coat was only 8.2% (dry weight) of a whole Bambara groundnut seed but contributed about 51% to the total Ca concentration (Table 2). According to the literature, the accumulation of Ca in the seed coat is attributed to its restricted movement from the seed coat to the cotyledon during seed development due to the formation of insoluble Ca oxalates in the seed coat.^{35,36} The same trend was reported in white and mottled common bean varieties, where 70% of total Ca was associated with the seed coat.³⁷

In contrast, dehulling was not a significant main effect influencing the concentration of Mg, Fe or Zn (Table 3). Although these minerals were also present in the seed coat, their relative contribution to the total concentration was lower (<15%) compared to that of Ca (51%) (Table 2). From these results, it could be deduced that most of the Mg, Fe and Zn present in Bambara groundnuts is associated with the cotyledon. These results agree with a study that illustrated that the seed coat of common beans (black) contributed to the lower relative percentages of Mg, Zn and Fe (16%, 7% and 14%, respectively).³⁴

A clear advantage of dehulling is the concomitant reduction of the concentration of antinutrients.^{15,16} However, the fate of the minerals after dehulling is not always reported in most studies involving legumes. In Bambara groundnuts, dehulling could reduce the concentrations of antinutrients concentrated in the seed coat¹⁵ without remarkably influencing the Mg, Fe and Zn concentrations but reducing that of Ca.

3.1.3 Influence of soaking and cooking. A soaking treatment of 16 h at room temperature was performed prior to the cooking treatment. Each mineral showed its own distinct behaviour due to the applied treatments (Table 1). Soaking of whole and dehulled seeds did not result in notable changes in the Ca concentration (1–2% decrease from the initial concentration) (Table 1). Regression analysis showed that soaking was not a significant factor affecting Ca concentration (Table 3). However, a decrease in the Ca concentration (22–29%) occurred upon cooking of the dehulled samples for 60 min

(compared with the concentration after soaking) (Table 1). Only limited Ca continues to leach out at 120 min. This was statistically confirmed as both dehulling and cooking treatments were significant and interacting factors (Table 3). The seed coat may protect against Ca leaching by binding Ca³⁸ or may act as a physical barrier to the loss of solids during cooking.

For Mg, the soaking treatment decreased the initial concentration by 2% and 10% for 0 week and 32 week whole samples, respectively. For the dehulled samples, soaking resulted in an even more pronounced degree of Mg leaching. The Mg concentration decreased by 36% and 56% after soaking 0 week-dehulled and 32 week-dehulled samples, respectively (calculated from Table 1). The seed coat probably protects against some electrolyte leakage during soaking as also observed in the case of Ca. Soaking alone and soaking after a dehulling treatment were the most significant treatments (Table 3). In the particular case of Mg, storage had an effect on the leaching potential during soaking, as a sharper decrease in the Mg concentration was observed for aged samples (16 weeks and 32 weeks). We noted a general increase in the loss of solids (solids potentially contain leached minerals) in the evaporated soaking water for the aged samples compared to the fresh samples. An increase from 0.23 to 1.6 mg solids per mL soaking water was noted in the soaking water of fresh and aged seeds, respectively. Higher solute and electrolyte leakage during soaking occurred in aged as compared to fresh Faba beans (18–36 mg g⁻¹ vs. 4–7 mg g⁻¹).³⁹

In both whole and dehulled Bambara groundnuts, the Mg concentration further decreased with cooking time (Table 3).

After cooking (2 h), between 26 and 43% of Mg leached out from the whole seeds, whereas in the dehulled samples, the Mg concentration decreased between 66 and 74%. Such considerable losses of Mg as a result of soaking and cooking have also been reported in whole common beans (40%).⁴⁰ The relatively easy leaching of Mg suggests that it might be loosely chelated to matrix constituents and thus readily solubilises into the soaking and cooking water.

The Fe concentration ranged between 3.4 and 4.9 mg per 100 g dw of whole seeds and was between 2.7 and 4.3 mg per

100 g dw for dehulled seeds, when considering all treatments. The initial Fe concentration decreased by 28% due to soaking, and this treatment was the only main effect. Non-ferritin Fe is known to be preferentially bound to phytic acid.⁴¹ The complexes formed are probably not readily soluble and this minimises the extent of leaching of Fe during soaking and cooking.

The Zn concentration decreased during soaking (9%) and cooking (29%) in both whole and dehulled Bambara groundnuts, and both treatments were significant main effects (Table 3). However, for both Fe and Zn, the regression analysis could not sufficiently explain the variation in the data (low R^2 adj). This could have been due to the low variability of the experimental data of Fe and Zn concentrations as affected by the different treatments, as shown in Table 1.

3.1.4 Influence of prolonging the cooking time as a result of storage-induced ageing. Legume ageing has an effect on their cooking behaviour. In practice, it becomes essential to adapt the cooking time in order to achieve a certain desired palatable hardness, which can, of course, be consumer dependent.²⁷ We, therefore, analysed mineral concentrations after adapting cooking times of whole aged Bambara groundnuts to achieve similar palatability to the fresh sample. It can be questioned whether this adaptation of cooking time would affect the mineral concentrations in the legume, considering different postharvest storage levels.

Amongst the analysed minerals, Mg showed the most striking observation; its concentration decreased due to the necessary extension of the cooking time required for the aged samples (16 weeks and 32 weeks) (Fig. 1). For the 32 week sample, a further decrease of 50% in the Mg concentration occurred, when the cooking time was extended from 120 min to 300 min. This is consistent with what was already pointed

out in section 3.1.3, where soaking and cooking were significant factors determining the final concentration of Mg. Overall, soaking and then cooking of the aged whole seeds for 300 min (32 weeks–300 min) decreased the initial Mg concentration by 72%. In contrast, a fresh sample cooked for 120 min (0 weeks–120 min), but possessing comparable palatability, only lost 26% of the initial Mg concentration. For Fe and Zn, an overall decrease of 57% and 48%, respectively, occurred when the cooking time was extended to 300 min. Ca concentrations were only marginally changed by the prolonged cooking time (from 49.9 to 46.3 mg per 100 g dw as the cooking time extended from 120 min to 300 min).

In most food applications, the cooking water will indeed be discarded before consumption. If this is not the case, *e.g.* when included in a soup product, it would be possible to obtain higher concentrations of the minerals than when in the former case.

3.2 Insight into complexation of minerals as affected by storage

3.2.1 Effect of storage-induced ageing on the solubility of Ca, Mg, Fe and Zn. One of the questions in this study was whether storage-induced ageing could influence mineral chelation, as implied by pectin-cation-phytate HTC theory. Accordingly, the solubility of Ca, Mg, Fe or Zn was determined from the milled 0 week, 16 week and 32 week samples. It was revealed that the change in the percentage of soluble minerals with storage depends on the mineral in question.

The solubility of Ca in fresh whole samples (0 weeks) was 52%, and it decreased with ageing (32 weeks) to 30% (Fig. 2). The possible reason for this observation is discussed in more detail in section 3.2.2. Mg and Zn had the highest solubility

Fig. 1 Concentration of Ca, Mg, Fe and Zn in whole Bambara groundnuts after adapting cooking time for aged samples to obtain comparable palatability.

Fig. 2 Solubility of Ca, Mg, Fe and Zn in the supernatants of fresh and aged (0 weeks, 16 weeks and 32 weeks) Bambara groundnuts. Different letters (a–c) show differences due to the effect of storage time for each mineral.

(65%). Fe solubility was low (32%). In general, the solubility of Mg, Zn, and Fe seemed to be limitedly affected by ageing. Mineral solubility increased for dehulled samples (percentage increase was ~17% for Ca, ~21% for Mg, ~8% for Fe and ~9% for Zn). This increase can be attributed to the potential reduction in the antinutrients by seed coat removal. The seed coat of legumes is reported to be rich in antinutrients.^{4,5,8} At the pH used, we do not expect phytates or polyphenols to be solubilised as they require acidic conditions to be solubilised.⁴² Many antinutrients present in Bambara groundnuts, for instance, pectin, phytic acid and polyphenols, have different abilities to chelate minerals.⁴³ While these have different abilities for binding minerals, different minerals bind to varying extents to one antinutrient as well.⁴⁴ Typically, Fe is known to be strongly chelated to phytic acid in legumes and cereal seeds.¹² Based on our observations discussed

above, it was found that Mg and Zn seem to be relatively loosely bound to antinutrients in milled Bambara groundnuts, given their ease of solubility.

3.2.2 Fluorescence labelling of cotyledon sections to visualise Ca crosslinked pectin. The apparent reduction (36%) in the solubility of Ca with storage time (Fig. 2) suggests that more and more Ca was bound to matrix constituents as ageing proceeded as implied by the pectin–cation–phytate hypothesis. Therefore, an experiment was set up to visualise Ca crosslinked pectin in the cell walls of raw fresh and aged sampled after *in situ* immunolabelling with the 2F4 monoclonal antibody. Fluorescently labelled Ca crosslinked pectin could be clearly visualised in the intercellular spaces for both the fresh and aged samples (Fig. 3). However, in the aged samples (16 weeks and 32 weeks) much more fluorescence of the Ca–pectin crosslinks could be seen in the intercellular spaces and also along

Fig. 3 Representative micrographs showing fluorescence of Ca–pectin crosslinks (labelled with 2F4) in cotyledon sections of fresh (0 weeks) and aged (16 weeks and 32 weeks) Bambara groundnuts. Arrows highlight the intercellular spaces between the cells where much more Ca–pectin crosslinks were present in aged samples.

the cell wall. Storage-induced ageing seemed to promote pectin–Ca crosslinks both in the intercellular spaces and along the cell wall.

Ca mediated pectin crosslinks, which promote cell adhesion and strength in plant cell walls, have been widely studied.⁴⁵ Ca infused low-temperature blanched broccoli cortex sections that were treated with 2F4 showed intense fluorescence in the intercellular spaces and along the cell wall, which was not present in blanched tissues without Ca infusion.³¹ These authors linked the higher fluorescence of Ca-pectin crosslinks noted in the Ca infused broccoli to higher texture stability during thermal processing. A previous study with the same batch of Bambara groundnuts aged under the same conditions²⁷ demonstrated that the aged seeds required prolonged cooking time (300 min) compared to the fresh seeds (120 min). In that work, it was hypothesised that the extended cooking time was necessary partly because of the increased formation of Ca crosslinks that strengthened the middle lamella, which can now be visualised here.

Other HTC hypotheses may also contribute to these observations. For instance, the membrane damage due to lipid oxidation may facilitate the migration of minerals to the cell wall and into the junction zones.⁴⁶ It is also possible that the preparatory soaking done before obtaining the sections may have allowed more efficient diffusion of minerals into the cell walls. Nonetheless, X-ray spectroscopic evidence of cowpeas (without any soaking) illustrated that more Ca is bound in the middle lamella of the cell walls of HTC cowpeas compared to fresh ones.²² The reduction of Ca solubility with storage time (Fig. 2) was observed in milled flours and the microscopic images depicted in Fig. 3 are from cotyledon sections. Nevertheless, we do not believe that the milling disturbed the electrostatic interactions between pectin and Ca.

3.2.3 Degree of methylesterification. The DM was determined from the cotyledon sections of raw (fresh and aged) Bambara groundnuts, to obtain information on one of the characteristics of the pectin that is closely related to the formation of Ca crosslinks. The DM of the fresh Bambara groundnuts was about 58%, but by 32 weeks of storage, it decreased to 47%. Similar ranges of DM have been reported for common beans.⁴⁰ Due to the humid storage conditions (78% RH), the moisture content of the Bambara groundnut samples increased from 9% (at 0 weeks) to 14% (at 32 weeks). It is possible that enzyme activities, including that of pectin methyl-esterase, increased under the experimental storage conditions and may have resulted in the slight demethylesterification that is noted. This decrease in DM may have caused an increase in the amount of Ca that crosslinked to pectin or promoted the strength of interaction during ageing and thereby reducing Ca solubility (Fig. 2). The DM quantified here shows that the pectin *in situ* still possesses the potential for its free carboxylic groups to participate in further crosslinking with Ca. We only measured the DM since it was not the focus of this work to characterise the nature of the pectin. However, the DM is not the only factor influencing ionic pectin crosslinks. The degree of blockiness and the presence of side chains are other essen-

tial factors that may affect the crosslinking of pectin.⁴⁷ Since the HTC involves multiple mechanisms, other complex crosslinks involving Ca, *e.g.*, pectin–Ca–ferulic acid crosslinks, could potentially exist.³⁸ Furthermore, membrane damage that occurs in the aged samples⁴⁶ could also promote the formation of ionic pectin crosslinks (more ions migrating to the cell wall without changes in the DM). Taken together, the results of solubility, microscopy and DM suggest that as storage-induced ageing takes place, Ca becomes much more strongly bound to pectin.

3.3 Influence of treatments on the bioaccessibility of Ca, Mg, Fe and Zn

It is essential to know how much of a mineral could be available for uptake (*i.e.*, bioaccessible), considering the changes in the concentration of minerals after the different processing steps. Thus the *in vitro* bioaccessibility (%) of Ca, Mg, Zn or Fe was analysed. Bioaccessibility of the minerals was never 100%, which shows that there is always some level of mineral chelation. The influence of the treatments on *in vitro* bioaccessibility was mineral dependent (Fig. 4). The impact of the different treatments considered on mineral bioaccessibility was evaluated by regression analysis (per mineral type) (Table 4). In this case, for treatments, we included storage time, dehulling and cooking time as these are relevant factors considered during the processing of legumes before consumption. People will not consume raw legumes; however, we included the results for raw samples in the analysis in order to assess the changes induced by the treatments. The results for each mineral are discussed below.

3.3.1 Ca bioaccessibility. Dehulling improved the bioaccessibility of Ca; dehulled cotyledons of Bambara groundnuts had a higher Ca bioaccessibility (17–29% increase) compared to the whole seeds (Fig. 4). In fact, dehulling had the most significant effect on Ca bioaccessibility, followed by storage time (Table 4). Removal of the seed coats may have reduced the level of antinutrients that chelate Ca, as shown in other studies.¹⁵

The bioaccessibility of Ca decreased from 55% (for fresh whole seeds) to 31% (for 32 weeks aged seeds), due to storage-induced ageing (Fig. 4). This decrease is consistent with the reduction of Ca solubility as a function of storage time (section 3.2.1). Pectin is likely a predominant factor in influencing Ca bioaccessibility in Bambara groundnuts, and possibly in legumes in general as more Ca–pectin crosslinks for the aged samples were clearly shown (Fig. 3). The link between Ca-mediated pectin interactions and bioaccessibility has been demonstrated for tomato-based suspensions, amongst other vegetable matrices, with Ca bioaccessibility decreasing with increasing electrostatic interactions between pectin and Ca.²⁴ Additionally, the cooking time (60 min and 120 min) significantly improved the bioaccessibility of Ca (increase between 14 and 18%). This is likely due to the reduction of the concentration of antinutrients (*e.g.*, oxalates, tannins and phytates) during cooking.^{16,21,48} A wide range of Ca bioaccessibility values are reported. In red kidney beans, peas and lentils, Ca

Fig. 4 Bioaccessible Ca, Mg, Fe and Zn in Bambara groundnuts stored for 0, 16 or 32 weeks, evaluated as a function of cooking time (raw) or 60 and 120 min of the whole (left) or dehulled (right).

Table 4 Regression coefficients ($\pm$ error) of the variables (storage time, dehulling and cooking treatments) influencing the bioaccessibility of Ca, Mg, Fe or Zn. Significance was tested within 95% confidence intervals

Mineral type	Intercept	Storage time	Dehulling	Cooking time	R^2 adj
Ca	62.0 $\pm$ 1.4	−0.8 $\pm$ 0.1	−6.33 $\pm$ 0.1	−0.09 $\pm$ 0.0	0.95
Mg	82.7 $\pm$ 2.0	−0.3 $\pm$ 0.1	n.s	−0.15 $\pm$ 0.1	0.80
Fe	21.1 $\pm$ 0.1	n.s	n.s	n.s	—
Zn	54.4 $\pm$ 0.1	n.s	n.s	−0.13 $\pm$ 0.1	0.92

n.s – not significant.

bioaccessibility values were 28%, 42% and 59%, respectively.⁴⁹ In particular cases, even lower Ca bioaccessibility values (<10%) have been reported for both fresh and aged cowpeas.²²

3.3.2 Mg bioaccessibility. Mg is the most bioaccessible mineral amongst the minerals (raw samples) studied (83%), and the bioaccessibility decreased to 66% by cooking for 2 h (Fig. 4). Mg bioaccessibility was also considerably influenced by ageing (Table 4). The concentration of Mg reduced after soaking and cooking time due to leaching, especially for aged and dehulled samples (section 3.1.3). The remaining fraction may be relatively more chelated than the fraction that leached out, thus the apparent decrease in bioaccessibility with storage and cooking time. Mg has similarly been found to be more bioaccessible compared to other minerals in citrus residues (94%)⁵⁰ as well in pectin model systems (87%),⁵¹ suggesting that it naturally exists in a readily bioaccessible state in various plant-based food. It could also be that the antinutrients present possess a lower affinity for Mg as compared to the other minerals studied here.⁵¹ Mg bioaccessibility was not significantly improved by dehulling.

3.3.3 Fe bioaccessibility. Fe bioaccessibility was low (18–25%) (Fig. 4), and none of the treatments reduced or improved the bioaccessibility significantly (Table 4). The low bioaccessibility was consistent with the solubility of Fe, which was also low (32%). Varying values of Fe bioaccessibility are reported depending on the type of legume. Bioaccessibility of Fe in cooked white beans was 43%,⁷ while the bioaccessibility of Fe in red kidney beans, green peas and lentils was 9%, 25% and 33%, respectively.⁴⁹ Even lower bioaccessibility values (<1%) have been reported for fresh and aged cowpeas.²² Although antinutrients were not characterised in this work, numerous studies have demonstrated that phytic acid largely inhibits the release of Fe from legume matrices.^{23,41} Differences in mineral bioaccessibility values may also be due to the type of separation technique used after digestion, *i.e.*, dialysis or centrifugation.

3.3.4 Zn bioaccessibility. Zn bioaccessibility decreased from 54% to 38% by cooking whole seeds for 2 h (Fig. 4). Cooking time was the only significant factor influencing Zn bioaccessibility (Table 4). The trend of Zn bioaccessibility decreasing with cooking time has been observed in some

legumes.⁵² For instance, the bioaccessibility of Zn in chickpeas and cowpeas decreased by 11% and 63%, respectively, after microwave cooking.⁵³ According to the study on chickpeas and cowpeas, the decrease in Zn bioaccessibility due to cooking time was attributed to the complexation of Zn with proteins. As whole legumes are cooked, both the starch and proteins become enclosed within the cell walls. Upon cooking, proteins denature, and this greatly reduces their solubility.⁵⁴ Perhaps due to the encapsulation of the aggregated protein and starch granules in the cotyledon cells, the release of Zn thus decreases with cooking time. In contrast, the raw milled sample, with opened up cells, where the proteins were not encapsulated by the cell walls, had higher bioaccessibility as well as solubility values (section 3.2.1).

3.4 Estimated contribution towards the daily RNI

The contribution towards the RNI was established based on a 130 g serving of cooked fresh or aged whole Bambara groundnuts. A serving of Bambara groundnuts could potentially provide 3% of Ca, 24% of Mg, 9% of Zn and 8% of Fe to the daily RNI. Based on the *in vitro* bioaccessibility results presented in section 3.3, the contribution to the RNI after factoring in bioaccessibility values will be much lower. As such, a serving of Bambara groundnuts (wet basis) seems to contribute very little to the daily RNI of these minerals.

4. Conclusions

The present study was designed to investigate the effects of storage-induced ageing, dehulling, soaking and cooking time on the concentration of (free/chelated) Ca, Mg, Fe and Zn and their *in vitro* bioaccessibility in Bambara groundnuts. Ageing has a profound effect on the solubility and bioaccessibility of some minerals (especially Ca) and is a relevant factor to consider when assessing the mineral bioaccessibility of legumes. Dehulling influences mineral concentrations based on the level of accumulation of that mineral in the seed coat. The extent to which minerals leach during soaking and cooking is hypothesised to be determined by the degree to which a mineral is bound in the matrix. While Bambara groundnuts are regarded as a rich source of minerals (on a dry weight basis), the bioaccessibility of these minerals is mostly low after processing. Besides, it seems that it is rather unfeasible to affect these values through treatments studied in this work. The contribution of a serving of Bambara groundnuts hardly meets the RNI requirements, and therefore populations relying on this legume may still be at risk for mineral deficiencies. This research opens the way to study the effect of ageing on the bioaccessibility of minerals in other legume matrices. It would be interesting to evaluate which antinutrients are mostly responsible for the chelation of specific minerals in the legume matrix. Finally, evaluation of the role of fermentation of the matrix by the gut microbiota on the mineral release in the colon could be another fruitful area of research.

Conflicts of interest

There are no conflicts to declare.

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