

Effect of processing on the microstructure and composition of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) seeds, flour and protein isolates

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ABSTRACT

Pre-processing treatments are generally employed to circumvent negating effects, such as the hard-to-cook and hard-to-mill properties, associated with legume seeds. Several studies have investigated the effects of soaking and roasting on the macroscopic qualities of Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] seeds, yet knowledge is lacking on the effects of these treatments on the microstructural and molecular properties of the seeds and resulting fractions (i.e. flour and protein isolates). Size-exclusion chromatography coupled with light scattering results have shown that roasting induces thermal aggregation in BGN proteins, resulting in the formation of insoluble aggregates. To that end, the molecular composition of the roasted and soaked-roasted samples were comparable, yet different to the control (untreated seeds), whereas the samples which were subjected to soaking compared favourably to the control. The morphology of the seeds were also well characterized by microscopic techniques, including a confocal imaging technique which appears novel for legume seeds, revealing cotyledon cells with several starch granules embedded in a matrix of protein bodies. The findings of this study provide valuable insights on the microstructural and molecular compositional changes occurring in BGN seeds and fractions when subjected to soaking and/or roasting, which could be linked to some macroscopic properties as previously reported.

1. Introduction

Nowadays, there is a renewed and growing interest in the consumption of plant proteins. This stems from the necessity to (partially) transition from animal-based protein diets, as a means of creating a more sustainable, nutritious and secure food supply (Aiking, 2011; Day, 2013). Next to cereal grains, legumes are one of the most important plant groups for human protein nutrition, with specifically pulses (dry seeds) forming an important part of the traditional diets in many developing countries (Calles, Xipsiti, & del Castillo, 2019; Day, 2013; Tiwari, Gowen, & Mckenna, 2011). Yet, despite their nutritional importance, many (indigenous) pulses remain to be cultivated by small scale farmers and are yet to be exploited commercially as protein sources (Calles et al., 2019).

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) [BGN] is such a pulse crop, which despite its high nutritional value are still

characterized as an underutilized subsistence crop. Widely grown in sub-Saharan Africa and Southeast Asia, BGN is considered as a high-quality protein food, with a reported protein content up to 27% and higher methionine levels compared to other grain legumes (Arise, Amonsou, & Ijabadeniyi, 2015; Feldman, Ho, Massawe, & Mayes, 2019; National Research Council, 2006). Also known to withstand harsh environmental conditions, BGN can be considered as a valuable crop in the current context of sustainability and climate change (Calles et al., 2019; Feldman et al., 2019), thus making it an important crop to be valorised beyond its local boundaries. As with other pulses, however, there are several factors associated with the limiting use of BGN; amongst others the hard-to-cook (HTC) and hard-to-mill (HTM) properties of the seeds which also have an influence on the nutritional quality (Gwala et al., 2019; Martín-Cabrejas, Esteban, Perez, Maina, & Waldron, 1997; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017; National Research Council, 2006). These factors are especially limiting in the local areas of

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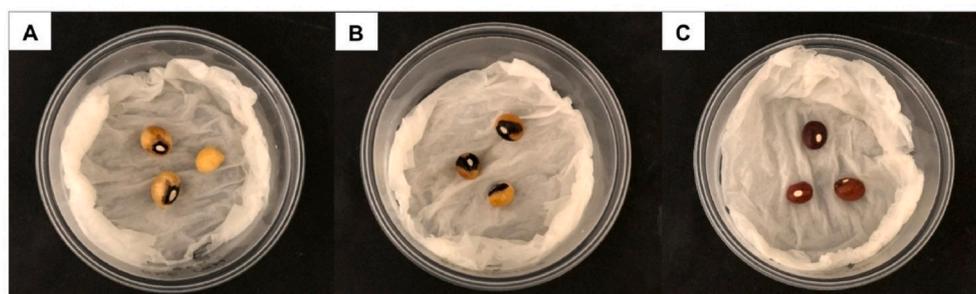


Fig. 1. Imbibition of Bambara groundnut seeds for 48 h at room temperature (20 °C). (A) Black-eye control; (B) Black-eye roasted; (C) Red control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

consumption, considering that extensive cooking times in comparison to other legumes are required to soften BGN seeds, with firewood being the predominant source of energy (Mubaiwa et al., 2017).

To circumvent these negating factors, the seeds are subjected to various pre-treatments before cooking or processing into flour and resulting products. These include amongst others soaking, roasting, germination and fermentation, or a combination of these treatments. The effect of these treatments on the functionality, nutritional composition and physical properties of BGN seeds have been the subject of several studies (Adegunwa, Adebawale, Bakare, & Kalejaiye, 2014; Barimalaa & Anoghalu, 1997; Ijarotimi & Esho, 2009; Mazahib, Nuha, Salawa, & Babiker, 2013; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2018; A. T.; Oyeyinka, Pillay, Tesfay, & Siwela, 2017; Yusuf, Ayedun, & Sanni, 2008); of which the outcomes provided valuable knowledge in terms of pre-processing geared towards specific applications and the influence of varietal differences. However, to gain further insight on the effect of these pre-processing treatments on the seed qualities, it is important to also investigate the resulting microstructural changes.

The aim of this study was therefore to gain insight into the microstructural changes of BGN seeds, upon various pre-processing treatments. In order to link the microstructural characteristics to the reported macroscopic properties of the BGN seeds and resulting flour, we have obtained seeds of the same varieties (red and black-eye) and subjected to the same treatments (soaking and/or roasting) as used by Mubaiwa et al. (2018). The microstructure of the seeds were well characterized with microscopic techniques, which included a novel means of imaging legume seeds based on the inherent autofluorescence of the protein bodies present in BGN seeds. In addition to the flour, we have also investigated the effect of these treatments on the protein isolates extracted from the flour, by means of size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). This is a well-known technique used amongst others for monitoring and characterizing the (thermal) aggregation behaviour of proteins, as it allows for the accurate determination of absolute molecular weight (M_w) (Choi & Ma, 2006; Ye, 2006; Zhao, Mine, & Ma, 2004). Through this approach we have demonstrated for the first time, as per our knowledge, the molecular changes occurring upon roasting and/or soaking of BGN seeds, as such providing further insights into their previously reported macroscopic properties.

2. Materials and methods

2.1. Materials

Bambara groundnut [BGN] seeds were obtained from the Director of Research and Specialist Services (red variety) and Dee Spice Private Company (black-eye variety) in Zimbabwe. The seeds were subjected to three different treatments (soaking, roasting and combined soaking and roasting) as described by Mubaiwa et al. (2018), with the raw untreated seeds used as the control. Briefly, seeds were soaked by immersion in deionized water for 24 h at room temperature followed by oven drying

at 50 °C for 48 h. Roasted seeds were prepared by dry-roasting in a coffee roaster (KN-8828-2K, Pullman Espresso Accessories, Australia) at various temperature intervals ranging from 70 to 179 °C. A third batch of seeds were subjected to soaking and drying, followed by dry-roasting.

The control (raw) and treated seeds were dehulled (automatic and manual dehulling where necessary) and coarse-milled with a pin mill (Condux-Werk LV 15M, Wolfgang bei Hanau, Germany) into grits, followed by fine-milling with a 0.5 mm mesh sieve ring fitted on a rotor mill (Fritsch GmbH Pulverisette 14, Idar-Oberstein, Germany) to obtain flour. The flour was defatted with n-Hexane (1:3 w/v) under continuous stirring for 1 h at room temperature, the hexane decanted and the procedure repeated twice. In the final step, vacuum filtration was used to remove the hexane and the flour collected and air-dried overnight. The defatted flour was sieved (315 µm mesh sieve) with an air jet sieve (Hosokawa Alpine E200 LS, Augsburg, Germany) and stored at -20 °C in airtight containers. All chemicals used were of analytical grade.

2.2. Extraction of Bambara groundnut protein isolates

The commonly utilised isoelectric precipitation method (Boye, Zare, & Pletch, 2010) was used to extract protein isolates from the defatted flour prepared from the control and treated BGN seeds. Briefly, defatted flour dispersed in deionized water (1:10 w/v) was adjusted to pH 9.5 with 1 M NaOH to solubilize the proteins, before isoelectric precipitation at pH 4 (adjusted with 1 M HCl), followed by centrifugation (4000 g, 30 min) with an Avanti J-26 XP centrifuge (Beckman Coulter, USA) to obtain the protein-rich residue. This residue was adjusted to pH 7 before being freeze-dried as the protein isolate (BGN-PI).

The total yield was expressed as the dry weight of BGN-PI per weight defatted flour, whereas the total protein content as determined with the Dumas nitrogen combustion method (FlashEA 1112 series, Thermo Scientific, The Netherlands) was used to express the protein yield of the final freeze-dried material (nitrogen-to-protein conversion factor N x 5.7).

2.3. Microstructure of Bambara groundnut seeds

2.3.1. Cryo-planing, cryo-scanning electron microscopy (cryo-SEM) and energy-dispersive x-ray spectroscopy (EDS)

The control (untreated) red and black-eye seeds, as well as the roasted black-eye seeds, were prepared for cryo-SEM imaging by imbibing the seeds between sheets of wet tissue paper in a closed container for 48 h at room temperature (see Fig. 1). After the imbibition process the seeds were sampled at a specific location from a longitudinal section, the samples mounted to the sample holder and frozen in melting propane or ethane. The frozen samples were transferred to a cryo-ultramicrotome (Leica MZ6 or Leica EM UC7; Leica Microsystems, Amsterdam, The Netherlands) where the initial planing were carried out with a glass knife, followed by final planing with a diamond knife. Known as cryo-planing, this technique is advantageous as it creates flat internal surfaces which are largely devoid of artefacts due to the low

Table 1

Protein content of Bambara groundnut flour and protein isolates, and dry weight yield of protein isolates.^a

Variety	Sample	Protein content flour	Protein content BGN-PI	Yield (dw)
		(g/100 g)		BGN-PI
Black-eye	Control	14.8 ± 0.1 ^a	68.3 ± 0.8 ^{ab}	11.5
	Soaked	15.8 ± 0.3 ^b	66.2 ± 0.7 ^{ac}	12.1
	Roasted	15.6 ± 0.3 ^b	61.6 ± 1.0 ^d	4.4
	Soaked-roasted	10.8 ± 0.1 ^c	61.8 ± 3.6 ^d	3.2
Red	Control	14.7 ± 0.3 ^a	72.0 ± 0.9 ^c	14.7
	Soaked	14.8 ± 0.1 ^a	70.5 ± 1.0 ^{bc}	12.5
	Roasted	13.0 ± 0.2 ^d	63.3 ± 2.1 ^{bd}	5.5
	Soaked-roasted	12.7 ± 0.2 ^d	61.4 ± 0.3 ^d	3.4

^a Protein content values of flour and BGN-PI (Bambara groundnut protein isolate) presented as means ± standard deviations; means within a column followed by the same superscript letter are not significantly different ($p > 0.05$). Dw refers to the dry weight yield of BGN-PI per 100 g defatted flour.

operational temperatures (Nijse & Van Aelst, 1999). Once a smooth area was obtained, the samples were transferred to a SEM preparation chamber (Gatan Alto 2500; Gatan, Abingdon, UK) for brief sublimation (freeze-etching) followed by sputter coating with a thin layer of platinum. The samples were then transferred into the SEM analysis chamber for observation at $-125\text{ }^{\circ}\text{C}$ at an acceleration voltage of 5 kV (JSM-6490LA SEM; Jeol, Tokyo, Japan) or 3 kV (Auriga field emission SEM; Zeiss, Jena, Germany). In addition, energy dispersive X-ray spectroscopy (Aztec X-Max 80 mm²; Oxford Instruments, Abingdon, UK) was

performed to identify the elemental composition of the samples.

2.3.2. Confocal laser scanning microscopy (CLSM)

CLSM was performed to visualize the morphology of a dry BGN (black-eye) seed. The seed was sampled with a razor blade on the outer edge of the cotyledon, the cut surface placed on a cover slip with immersion oil and observed with a Leica TCS SP5 confocal microscope equipped with a Leica DMI6000B-CS inverted microscope. Three objectives were used, i.e. 10x dry objective (0.4 NA), 40x (1.25 NA) and 63x (1.4 NA) oil immersion objectives. After a signal hunt, the protein bodies could be detected in fluorescence mode and the oil bodies in reflection mode, both at an excitation wavelength of 476 nm (Argon laser) and emission wavelengths of 481–795 nm and 472–481 nm, respectively. In addition, the starch granules and cell walls were visualized in the “dark” areas where no fluorescence or reflection were detected.

2.4. High performance size-exclusion chromatography coupled with multi-angle laser light scattering (HPSEC-MALLS) analysis of BGN flours and protein isolates

HPSEC-MALLS was performed as previously described (Diedericks, de Koning, Jideani, Venema, & van der Linden, 2019). The HPSEC system consisted of four TSK gel analytical columns—PW_{XL} guard, G6000 PW_{XL}, G4000 PW_{XL} and G3000 PW_{XL} (Tosoh Bioscience LLC, USA)—which were thermostated at 35 °C and connected in series, a vacuum degasser (1200 series degasser, Agilent Technologies, USA) and a pump (1200 series binary pump, Agilent Technologies). The filtered mobile phase (100 mM NaNO₃ + 0.02% NaN₃) were pumped through

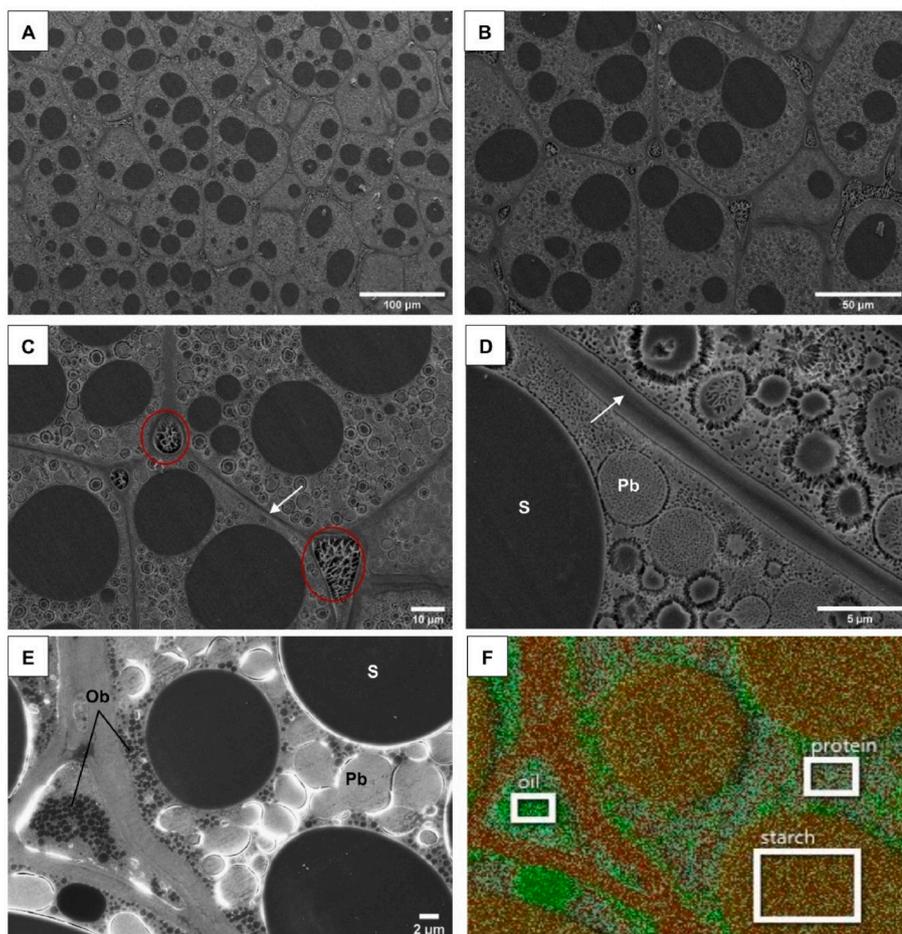


Fig. 2. Morphology of BGN red control seeds after imbibition for 48 h, as imaged with cryo-SEM at different magnifications. (A)–(B) overview of the cotyledon cells; (C)–(D) at higher magnifications the protein bodies appear more distinct, arrows indicate cell walls and the circled areas indicate intercellular spaces; (E) oil bodies visible as dark spots (<0.5 μm) mostly concentrated at the cell walls; and the corresponding EDS elemental map (F) with carbon indicated in green, oxygen in red and nitrogen in blue. S: starch granules, Pb: protein bodies, Ob: oil bodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

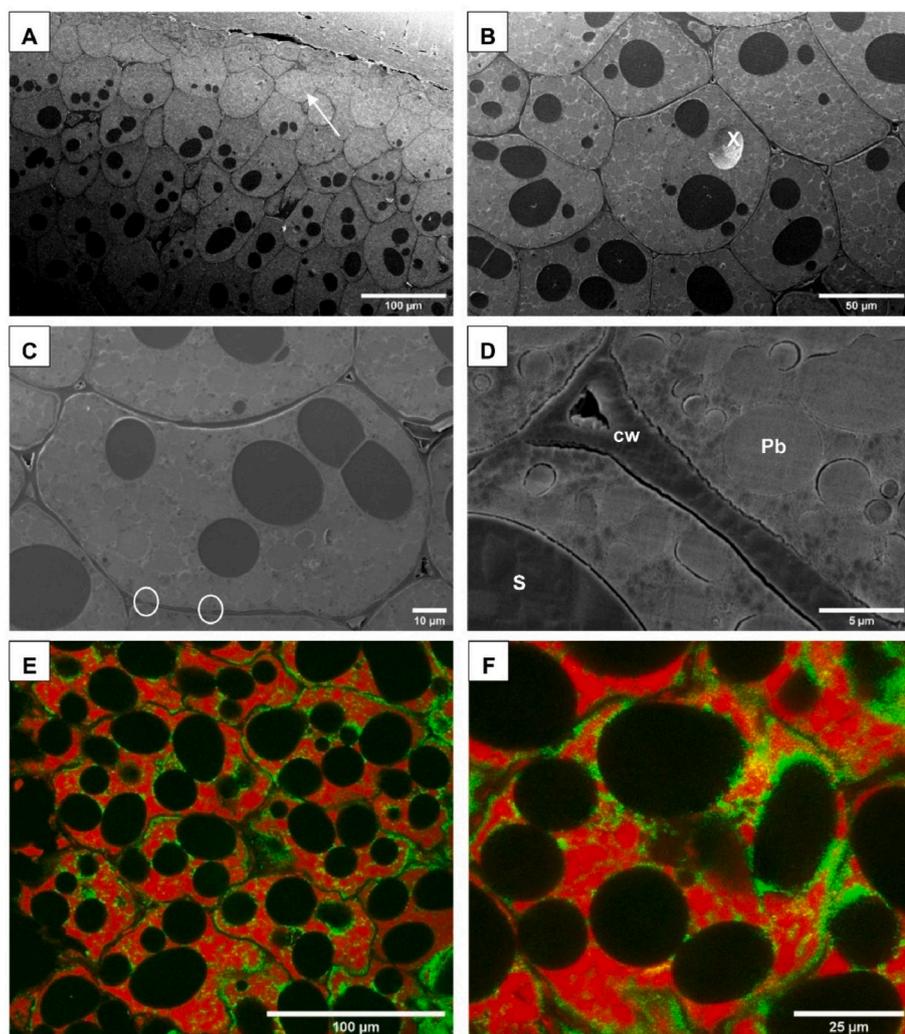


Fig. 3. Morphology of BGN black-eye control seeds imbibed for 48 h as imaged with cryo-SEM and dry seeds as imaged with CLSM, at different magnifications. (A)–(B) overview of the cotyledon cells, arrow indicates cells close to the hull devoid of starch granules and X indicates an empty starch cell; (C)–(D) at higher magnifications the plasmodesmata (circled areas) and oil bodies along the cell wall are visible; (E–F) CLSM micrographs showing protein bodies in red, oil bodies in green and starch in black. S: starch granules, Pb: protein bodies, cw: cell wall. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

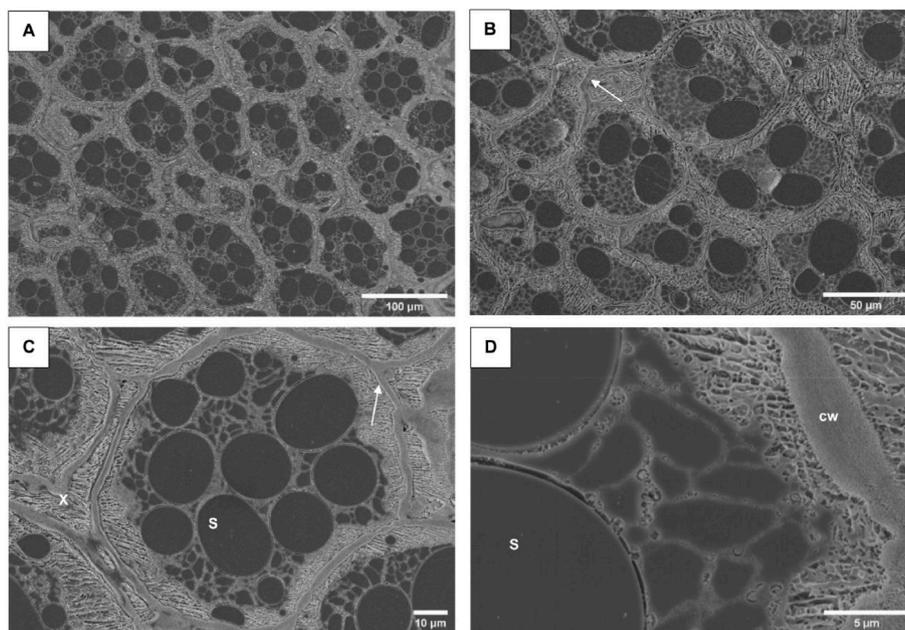


Fig. 4. (A)–(D) Morphology of BGN black-eye roasted seeds imbibed for 48 h, as imaged with cryo-SEM at different magnifications; arrows indicate cell walls and the area marked with an X indicates an intercellular space. S: starch granules, cw: cell wall.

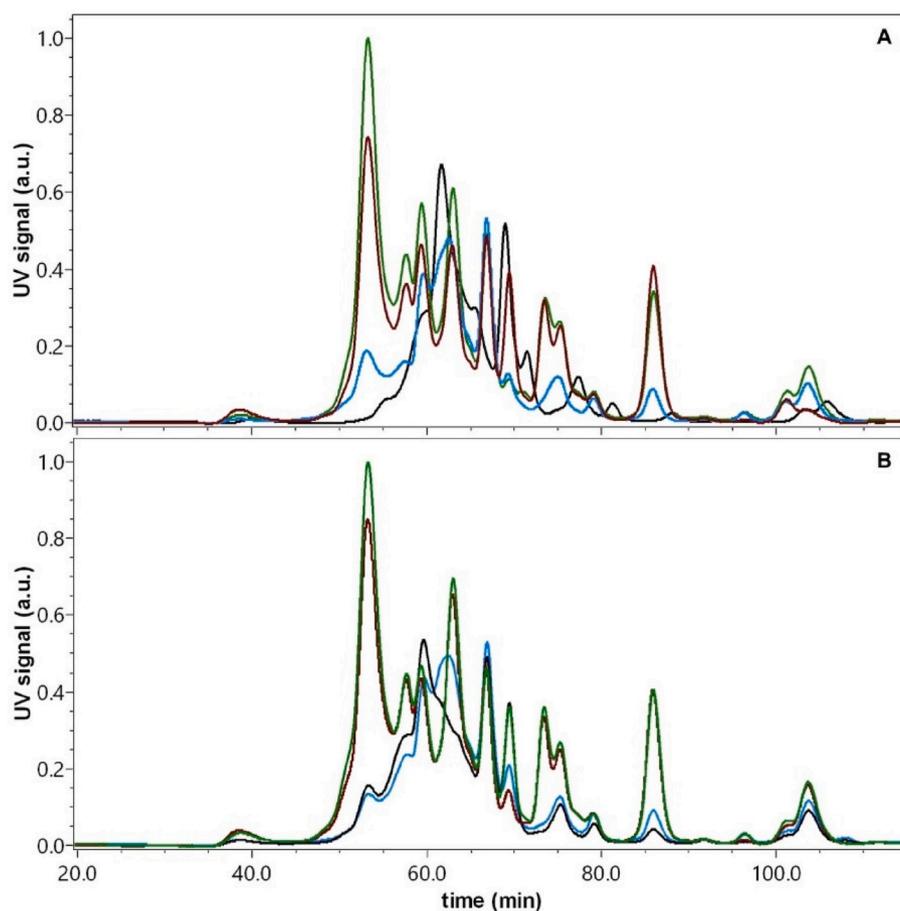


Fig. 5. SEC-UV elution profiles of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the system at a flow rate of 0.5 ml/min, and 200 μ l of the BGN flour dispersions and protein solutions (0.5% w/w) were injected onto the columns with an autosampler (Agilent Technologies).

Three detectors measuring static light scattering, UV absorption and refractive index (RI) were used for characterization of the samples. ASTRA 6 software (Wyatt Technologies) was used for collection and processing of data. The reference material bovine serum albumin (BSA, M_w 67 kDa) was used for normalization, alignment and band broadening.

2.5. Statistical analysis

Analysis of variance was performed on the total protein data, obtained from at least triplicate measurements, and Duncan's multiple range test was used to determine significant differences ($p \leq 0.05$) among the means (IBM SPSS Statistics 25, Chicago, USA).

3. Results and discussion

3.1. Protein composition and yield

The yield and protein content of BGN flours and protein isolates are shown in Table 1. Compared to the control flour, the protein contents of the soaked and roasted black-eye flour were significantly higher, whereas in the red variety the protein contents of the roasted and soaked-roasted flour were significantly lower. Overall these values are lower in comparison to that reported by Mubaiwa et al. (2018), which could be attributed to the extra fine milling step and lower sieve mesh size employed in their study. The protein content of the protein isolates

from both varieties was in a similar range, with that of the soaked BGN-PIs being comparable to the control samples. In contrast, the protein content of the roasted and soaked-roasted BGN-PIs decreased significantly compared to that of the control. During roasting, samples are exposed to high temperatures for short times, which can cause denaturation of proteins to varying extents depending on their thermal stability (Meng & Ma, 2001; Tiwari et al., 2011). Roasting also had the largest effect (in reference to the control samples) on protein yield in both varieties, with the roasted and soaked-roasted BGN-PIs characterized with the lowest yields in a range of 3.2–5.5 g protein per 100 g defatted flour. The lower protein yield of the roasted and soaked-roasted samples is attributed to the protein aggregate formation, where the larger protein aggregates are removed during centrifugation before the isoelectric precipitation step. In applications where the protein yield is of importance, soaking would be better suited as a pre-processing treatment. On the basis of protein content, soaking and roasting are comparable. However, soaking would still be preferable as opposed to roasting, since it is a milder and less laborious process.

3.2. Microstructural characteristics of control and processed BGN seeds

The microstructures of BGN control and roasted seeds were well characterized with the microscopic techniques employed. An overview of the cotyledon cells in the control red BGN seed, as obtained with cryo-SEM and shown in Fig. 2A and B, reveals the presence of several oval-shaped starch granules surrounded by numerous protein bodies. This morphological structure of cotyledon cells has also been reported for other pulse seeds such as cowpea and faba bean, whilst the starch granules have the same shape as previously reported for BGN starches

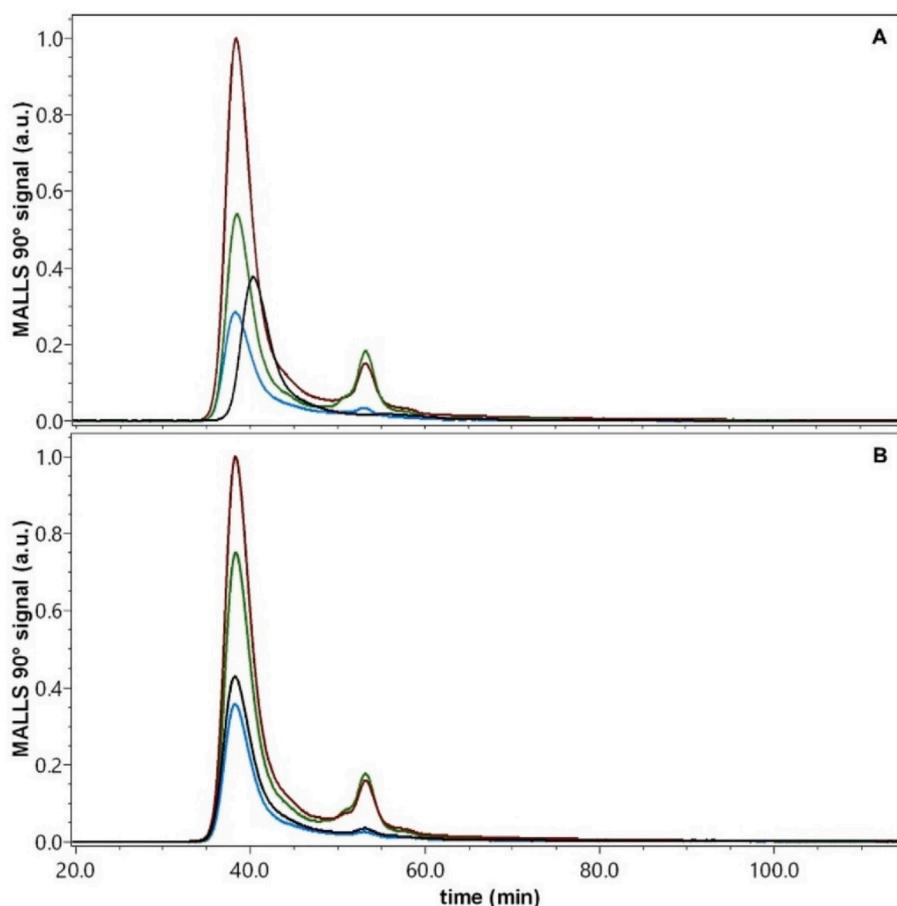


Fig. 6. SEC-MALLS elution profiles of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Oyeyinka, Singh, & Amonsou, 2017; Swanson, Hughes, & Rasmussen, 1985). In general, starch granules appear darker than protein bodies due to a lower amount of electrons reflected from their surface. At higher magnifications (Fig. 2C and D), the protein bodies appeared more distinct whilst ranging in colour/intensity from grey to black. This colour/intensity difference was also observed for protein bodies of yellow pea, which was attributed to differences in electron density caused by the orientation of the protein bodies, the planing depth or the formation of ice-crystals during cryofixation (Kornet et al., 2019). The size of starch granules and protein bodies ranged from 7–45 μm and 1–5 μm , respectively, which corresponds to that previously reported for these cell components from BGN and other legume seeds (Amonsou, Taylor, & Minnaar, 2011; Do & Singh, 2019; Kaptso et al., 2015; Kornet et al., 2019; Swanson et al., 1985; Wolf, 1970). Oil bodies (<0.5 μm) were also present in the cells, with most of them accumulating at the cell walls as shown in Fig. 2E. Furthermore, the corresponding EDS elemental map (Fig. 2F) provided further evidence of the composition of the structures—the carbon signal corresponded to the oil bodies, whilst the oxygen and nitrogen signals corresponded to the starch granules and the protein bodies, respectively.

Similar to the red BGN seeds, the cryo-SEM images of the control black-eye seeds (Fig. 3A–D) revealed cotyledon cells rich in starch granules and protein bodies, both in a similar size range as found for the red variety. However, the protein bodies appeared more uniform in colour, whilst the number of starch granules per cell were lower in the black-eye seeds with no granules visible in the cells close to the hull (see area indicated in Fig. 3A). In addition, CLSM imaging of the dry seeds (Fig. 3E and F) provided a structural overview which was in good agreement to the cryo-SEM images of the imbibed seeds. The principle of

this microscopic technique is based on the detection of fluorescing (or reflected) light following illumination of a sample with a scanning laser light. The structures of interest are generally stained with fluorescent dyes, although intrinsic fluorescence (autofluorescence) found in biological samples such as aromatic amino acids, phenolics and structural proteins are often advantageously used, as minimal sample preparation is required (Holopainen-Mantila & Raulio, 2016; Monici, 2005). In our samples, autofluorescence was found for the protein bodies, whilst the oil bodies were visualized through reflectance and the non-illuminated areas corresponded to the starch granules. Here it should be noted that this can be considered as a novel means of CLSM imaging for legume seeds, which holds true for these specific samples imaged under the specified conditions.

The microstructure of the roasted black-eye BGN seeds is distinctly different compared to the control seeds, as shown in Fig. 4A–D. The protein bodies could not be distinguished, as they became aggregated with other intracellular components and the intact starch granules. Whilst the cell walls remained swollen, no water was absorbed by the aggregated cell components as seen by the large ice-crystal layer between the cell components and the cell walls. The aggregated cell material is indicative of the dry state, leaving an imprint which can be linked to that in the neighbouring cells; whilst the large ice-crystal layer would likely facilitate a higher fluid entrapment as seen by the higher water absorption capacities for roasted (and soaked-roasted) BGN seeds reported by Mubaiwa et al. (2018). Similar morphological characteristics were reported for navy beans—SEM images revealed the destruction of outer structures of the cotyledon upon roasting, whilst starch granules and other intracellular materials became partly aggregated (Aguilera, Lusas, Uebersax, & Zabik, 1982). The morphology of the soaked and

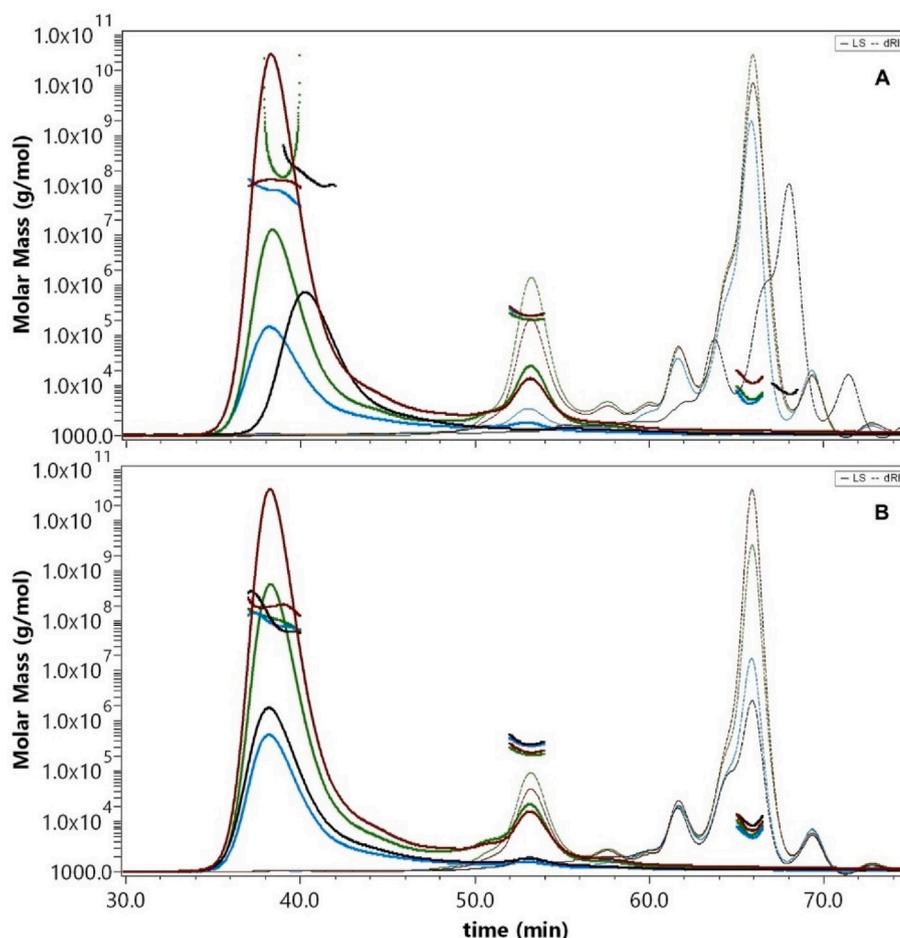


Fig. 7. Molar mass profiles (solid lines above/across peaks) as obtained from MALLS (continuous solid lines) and RI (dashed lines) responses of (A) black-eye and (B) red Bambara groundnut flours subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

soaked-roasted seeds were not microscopically examined. It is however expected that the soaked seeds will resemble that of the imbibed control seeds with swollen intercellular spaces due to the longer imbibition time and the imbibition method (immersion in water), whilst the morphology of the soaked-roasted seeds is expected to resemble that of the roasted seeds (Gowen et al., 2006; Mubaiwa et al., 2018; Swanson et al., 1985). These microstructural differences can also be linked to the higher dehulling and milling efficiencies of the roasted seeds as reported by Mubaiwa et al. (2018), which can be attributed to an increase in fracture points upon roasting, caused by increased mechanical stresses and vapor pressures (Aguilera et al., 1982; Köksel, Sivri, Scanlon, & Bushuk, 1998). Additional images of the cotyledon cells of the raw and roasted BGN seeds at various magnifications are provided as supplementary material (Appendix A).

3.3. Chromatographic profiles and molecular weight characterization of BGN flours

The chromatograms of BGN black-eye and red flours as detected by UV and MALLS detectors are shown in Figs. 5 and 6, respectively. The UV elution patterns were comparable for both BGN varieties, with several peaks observed for all treatments. In general, the elution patterns of the control and soaked flours were comparable to that reported for yellow pea flour, where the larger peaks eluting first were ascribed to the globulin proteins and the smaller peaks eluting later to the smaller proteins such as the albumins and the vicilin subunits (Kornet et al., 2019). The large peak eluting around 53 min for the control and soaked

BGN flours corresponds to that of the major storage protein fraction, vicilin, as reported in our previous study (Diedericks et al., 2019). In comparison, this peak almost disappeared in the roasted and soaked-roasted flours. This can be attributed to the denaturation of the proteins upon roasting and subsequent formation of larger, insoluble aggregates, which were mostly removed through centrifugation before size-exclusion analysis (Choi & Ma, 2006). This observation is also consistent in the MALLS elution patterns, where (small) peaks corresponding to the vicilin fraction were visible for the control and soaked flours, with almost no response detected for the samples subjected to roasting. In addition, a large peak appeared around 38 min for the control flours, and to a lesser extent for the processed flours, which are indicative of soluble aggregated protein fractions or high-molecular-weight Maillard reaction products (MRPs). The latter can be formed through the interaction of reducing sugars such as glucose and fructose, with proteins and amino acids. This is generally observed by a significant decrease in the free sugars upon roasting, indicating their thermal degradation and subsequent reactivity (Oracz & Nebesny, 2019). However, in the study by Mubaiwa et al. (2018), roasting had no significant influence on the fructose content of the seeds. In addition, considering that the large peak was also present in the control flours, it is more likely evident of soluble protein aggregates. These fractions are present in small amounts as deduced from the corresponding UV and RI responses, which were much smaller compared to the MALLS signal (Hoffmann, Sala, Olieman, & De Kruijff, 1997; Zhao et al., 2004).

The molar mass distribution profiles were determined from combined MALLS and RI signals. As shown in Fig. 7, the peaks eluting

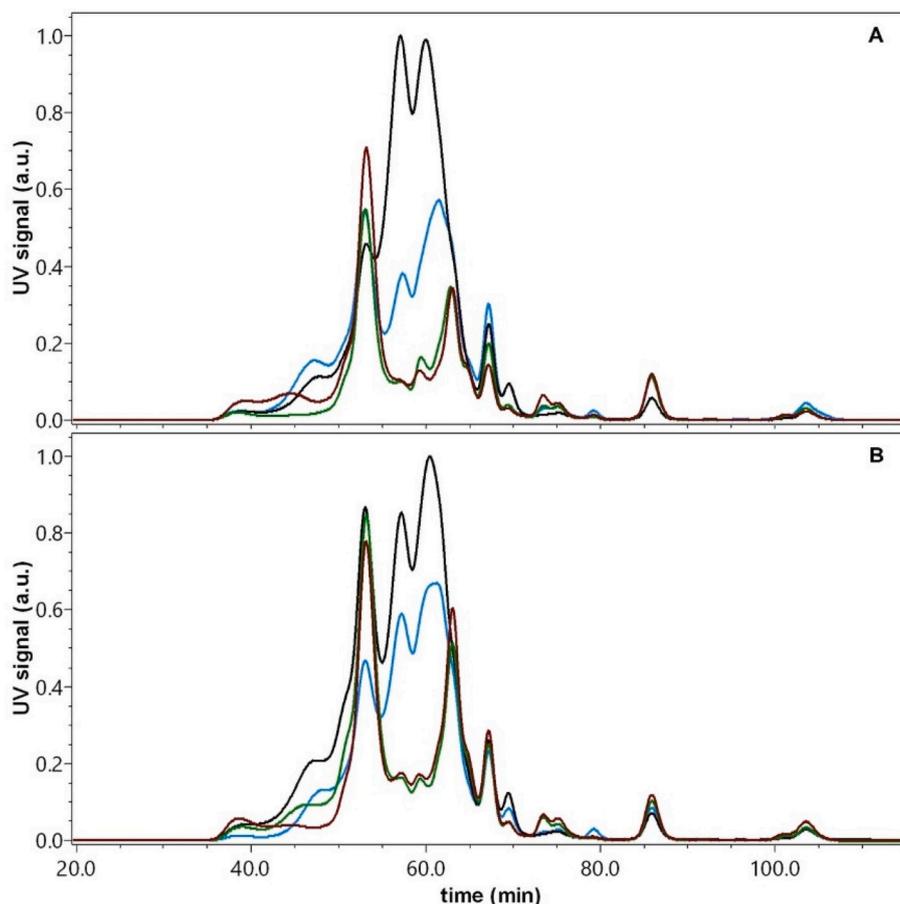


Fig. 8. SEC-UV elution profiles of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

between 52 and 54 min which were attributed to the major storage protein fraction vicilin, were mostly characterized with constant molecular weights as indicated by the straight lines above the peaks—the slight upwards curving of the M_w slopes is however indicative of the presence of some larger oligomers (Pothecary, Ball, & Clarke, 2012). This is also reflected in the M_w values of the control black-eye (266 kDa) and red (257 kDa) BGN flours, which were slightly higher compared to that previously reported for the trimeric BGN vicilin fraction (196 kDa) (Diedericks et al., 2019). These differences are as expected, considering that vicilin is a purified protein fraction obtained from the flour which is inherently more heterogeneous. In comparison, the largest effect on the M_w for this fraction was seen in the red soaked-roasted (347 kDa) and roasted (378 kDa) flour, as well as the roasted black-eye flour where this elution peak disappeared completely. The effect of roasting was also evident on the size, i.e. radius of gyration (R_g), which increased from 34 nm for the red control flour to 51 nm and 55 nm for the soaked-roasted and roasted flours, respectively. An increased R_g is evident of aggregate formation, as was also observed for β -lactoglobulin solutions upon heating (Hoffmann et al., 1997). The sharp declining M_w slopes for the peaks eluting earlier is indicative of aggregates with heterogeneous sizes (Choi & Ma, 2006) and with M_w values > 1 MDa, these fractions are confirmed as large soluble aggregates eluting at the void volume. The formation of these large aggregates can be linked to the low foaming capacity and stability of roasted and soaked-roasted BGN flours as reported by Mubaiwa et al. (2018), as the molecular unfolding and subsequent aggregation of proteins are known to negatively affect their foaming properties (Pozani, Doxastakis, & Kiosseoglou, 2002). In addition, prominent RI peaks characterized with concave M_w slopes were identified at later elution times, which are indicative of lower

molecular weight fractions also with heterogeneous sizes. Similar to the fractions identified in yellow pea flour (Kornet et al., 2019), these later eluting fractions are comparable in M_w to the smaller albumins and vicilin subunits, with M_w values ranging from 5 to 13 kDa. Overall, the molecular composition of the roasted and soaked-roasted flours were comparable, whereas the soaked flour closely resembled that of the control.

3.4. Chromatographic profiles and molecular weight characterization of BGN protein isolates

To further elucidate on the effect of soaking and roasting on the composition of the BGN seeds, the extracted protein isolates were also subjected to HPSEC analysis. Fig. 8 shows the corresponding UV elution profiles of the black-eye and red BGN-PIs. Similar to what was found for the flour, the major peak which was attributed to the trimeric vicilin fraction eluting between 52 and 54 min, was observed in both varieties and for all treatments. In the red variety this peak appeared smaller for the soaked-roasted sample, whereas in the black-eye variety this peak was slightly smaller for all processed samples compared to the control. In addition, two large peaks which were partially resolved appeared directly after the major eluting fraction for the roasted and soaked-roasted samples in both varieties. A similar observation was made for buckwheat globulin upon heating at 100 °C and various time intervals, which was attributed to the dissociation of the major (hexameric) protein into monomers, as suggested by a decrease in the major peak and a simultaneous increase in a subsequently eluting peak (Choi & Ma, 2006). Similarities were also found in the elution patterns of dry beans and lentils, where after cooking the major peaks appeared at higher

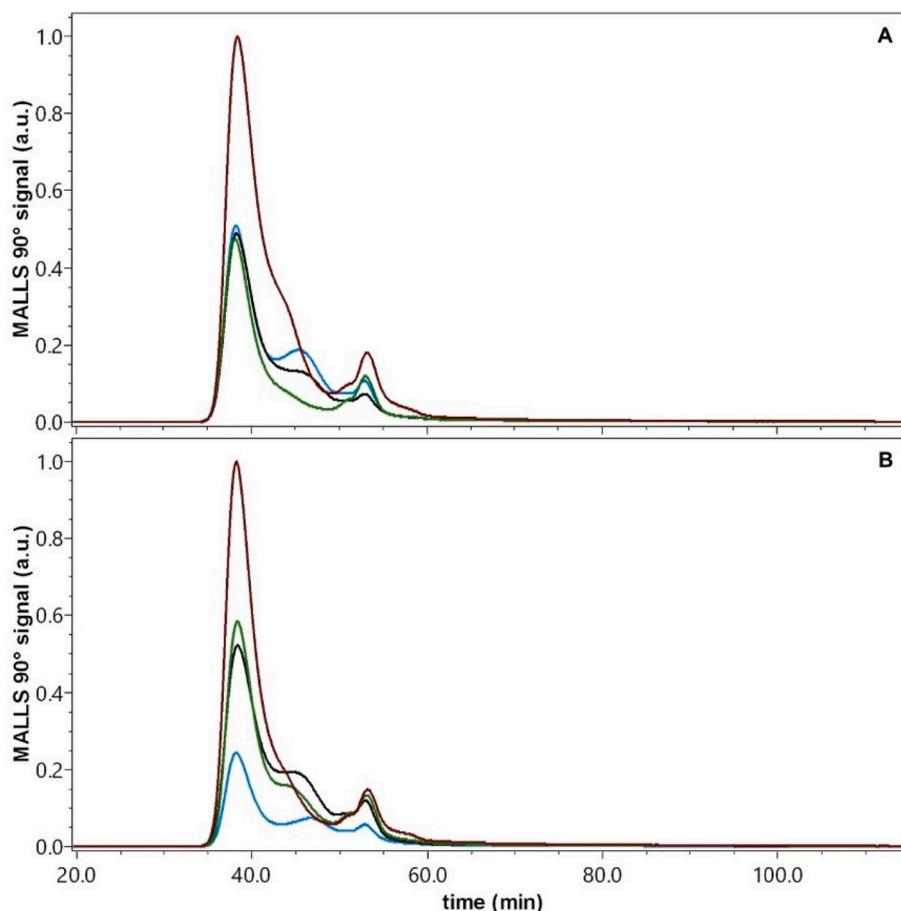


Fig. 9. SEC-MALLS elution profiles of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

retention times (Carbonaro, Cappelloni, Nicoli, Lucarini, & Carnovale, 1997). As seen in the MALLS chromatograms (Fig. 9), the large peak eluting around 38 min which was identified as large soluble aggregates in the flour, was also present in the BGN-PIs. In addition, a second (partially resolved) peak appeared as a shoulder to the large peak in all samples, but more prominently for the roasted and soaked-roasted BGN-PIs. Similar structural changes were observed for kidney bean, red bean and mung bean to varying extents, where after heating at 95 °C the formation of soluble aggregates or high molecular weight oligomers were evident at lower elution volumes (Tang, Chen, & Ma, 2009). In kidney bean, these aggregates were mostly characterized as vicilin dimers which were formed upon association of the unfolded monomers (Tang & Ma, 2009). Considering that this shoulder peak is also slightly present in the control samples, it is indicative that aggregate formation also occurs during the extraction of the protein isolates; possibly during freeze drying as reported for lupin protein isolates (Berghout, Venema, Boom, & van der Goot, 2015). Furthermore, the peaks eluting after the vicilin peak in the UV chromatograms are not present in the MALLS elution profiles; which are thus indicative of smaller-sized particles/aggregates.

The RI and MALLS signals were combined to obtain the molar mass distribution profiles of the BGN-PIs, as shown in Fig. 10. The M_w values for the vicilin fraction (eluting between 52 and 54 min) in the control samples were 279 kDa for the black-eye and 302 kDa for the red variety. As seen from the slight upwards curving of the M_w slopes, these fractions also contain some larger oligomers as were found in the corresponding flour samples. These M_w values are comparable to that found for BGN-PIs from different batches of black-eye seeds (Diedericks, Shek, Jideani, Venema, & van der Linden, submitted for publication) which

highlights the reproducibility of the protein extraction process. In contrast to the M_w values calculated for this peak in the roasted and soaked-roasted flours, lower values were reported for both BGN-PIs ranging from 207 to 210 kDa and 206–220 kDa, respectively. Similarly, the R_g for these samples were also lower compared to the control, ranging from 25 to 35 nm for the roasted BGN-PIs and an average of 31 nm for the soaked-roasted BGN-PIs. This can be attributed to the association-dissociation behaviour of the oligomers upon heating, similar to what was found for globulin proteins from soybean, oat and buckwheat (Choi & Ma, 2006; Mori, Nakamura, & Utsumi, 1982; Zhao et al., 2004). In these proteins it was found that heating caused the formation of soluble aggregates through association of the oligomers, followed by dissociation of these aggregates into monomers (upon further heating) and re-association into high molecular weight aggregates and finally insoluble aggregates. This thermal aggregation effect is seen in the M_w values of the large peaks at earlier retention times (around 38 min) and the subsequent shoulder peaks which appeared before the vicilin fractions. In these peaks, the M_w values were smaller (<2600 kDa) for the roasted and soaked-roasted samples compared to the control and soaked samples (>6100 kDa). This suggests that the aggregates of the roasted and soaked-roasted BGN-PIs became less soluble, to an extent where larger insoluble aggregates were formed, which would have been removed through centrifugation prior to HPSEC analysis (Choi & Ma, 2006). Compared to the flour, the molecular composition of the BGN-PIs from all processed seeds differed to some extent from the control samples. Overall it can be noted that BGN-PIs have a high thermal stability, despite aggregate formation, as most peaks which were found in the control BGN-PIs were also present in those subjected to heat treatment.

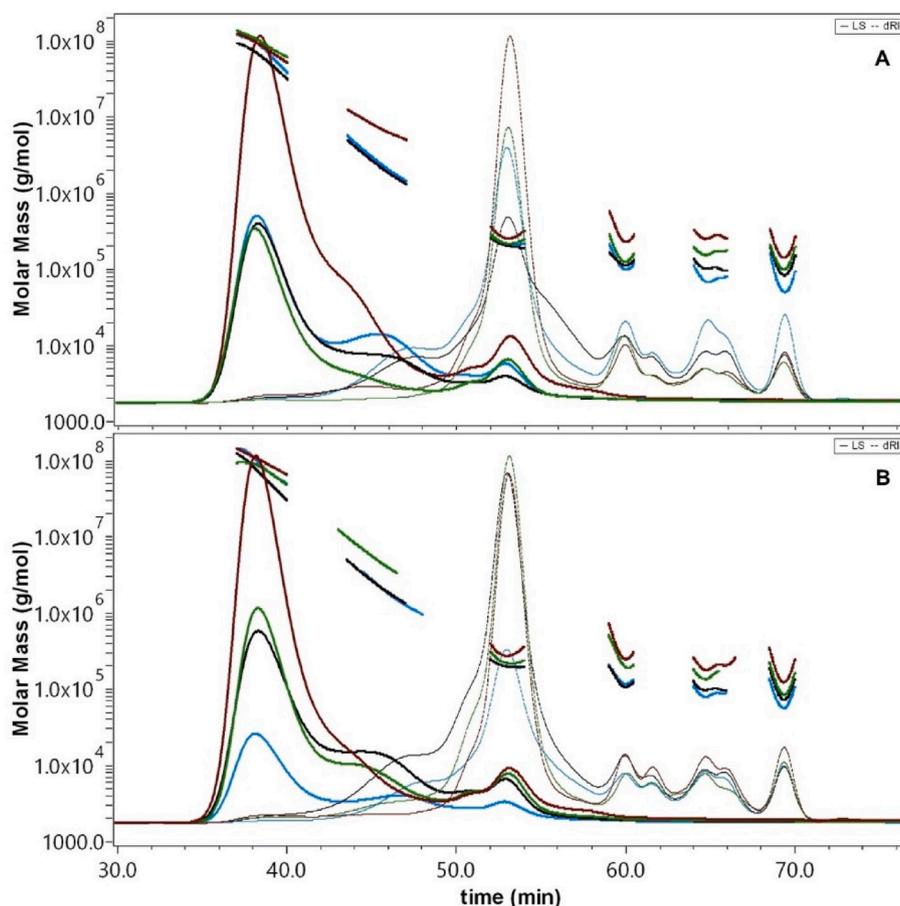


Fig. 10. Molar mass profiles (solid lines above/across peaks) as obtained from MALLS (continuous solid lines) and RI (dashed lines) responses of (A) black-eye and (B) red Bambara groundnut protein isolates subjected to different treatments. Red—control, green—soaked, blue—soaked-roasted, black—roasted. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

We have shown that roasting as a pre-treatment method of BGN seeds changes the molecular composition of the resulting flour and protein isolates, whereas soaking had a minimal effect with samples comparing closely to the control. Using SEC-MALLS as a tool to accurately determine the size and weight-averaged molar masses, it was found that the major storage protein fraction vicilin—as previously identified in BGN seeds—was present as the main fraction in the control and processed flours and resulting protein isolates of both varieties. Furthermore, the thermal aggregation behaviour of BGN proteins was also evident in the roasted and soaked-roasted samples, as observed by the formation of soluble and insoluble aggregates. Yet, BGN-PIs are recognized for their high thermal stability, considering that the same elution patterns found for the control samples were also present in the samples subjected to roasting. In addition, no marked differences were observed between the red and black-eye varieties, as was also evident from microstructural imaging. These findings can be linked to some macroscopic properties of BGN seeds as reported in literature, which is valuable in terms of establishing the best pre-treatment method for a given application from both a structural and functional perspective.

Declaration of competing interest

The authors declare that this study does not have any conflict of interest.

CRediT authorship contribution statement

Claudine F. Diedericks: Conceptualization, Investigation, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Paul Venema:** Conceptualization, Writing - review & editing, Supervision. **Juliet Mubaiwa:** Resources, Writing - review & editing. **Victoria A. Jideani:** Writing - review & editing, Supervision. **Erik van der Linden:** Conceptualization, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodhyd.2020.106031>.

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