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Detailed analysis of seed coat and cotyledon reveals molecular understanding of the hard-to-cook defect of common beans (*Phaseolus vulgaris* L.)

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ABSTRACT

The hard-to-cook (HTC) defect in legumes is characterized by the inability of cotyledons to soften during the cooking process. Changes in the non-starch polysaccharides of common bean seed coat and cotyledon were studied before and after development of the HTC defect induced by storage at 35 °C and 75% humidity for 8 months. Distinct differences in the yields of alcohol insoluble residues, degree of methoxylation (DM), sugar composition, and molar mass distribution of non-starch polysaccharides were found between the seeds coat and cotyledons. The non-starch polysaccharide profiles, both for seed coats and cotyledons, significantly differed when comparing HTC and easy-to-cook (ETC) beans. In conclusion, differences in the structure, composition and extractability of non-starch polysaccharides between the ETC and HTC beans confirmed the significant role of pectin polysaccharides in interaction with divalent ions in the HTC development, which consequently affect their cooking behaviors.

1. Introduction

Common beans (*Phaseolus vulgaris* L.) are one of the most important sources of nutrients to people in many developing countries. However, leguminous seeds submitted to long periods of storage at high temperature (>25 °C) and relative humidity (>65%) undergo gradual loss of nutritional and textural qualities (Kinyanjui et al., 2014; Njoroge et al., 2015). This phenomenon, known as the hard-to-cook (HTC) defect is characterized by extended cooking times of the seeds to allow softening to the desired texture. The HTC defect results in reduced palatability and higher cooking costs (because of the higher energy consumption), thus reducing consumer acceptability of beans.

To date, several hypotheses have been formulated to explain the development of the HTC defect in common beans. One of the mechanisms, known as the pectin-cation-phytate-phytase theory, suggests that the formation of insoluble pectates in the cell wall-middle lamella would render the tissue more resistant to cell separation during cooking (Liu & Bourne, 1995). In detail, when phytase hydrolyzes phytate, the released divalent cations diffuse to the middle lamella and combine with the pectates (due to pectin methyl esterase catalyzed pectin demethoxylation), resulting in a decreased solubilization of pectates during cooking (Galiotou Panayotou, Kyriakidis, & Margaris, 2008; Kruger, Minnis-Ndimba, Mtshali, & Minnaar, 2015). Another mechanism implies that lignification may be involved in the development of the HTC defect (Hincks & Stanley, 1987). Varriano Marston and Jackson (1981) found that the degradation of cell membranes is also related with the HTC phenomenon. In addition, the gelatinization temperature of the bean starch and the degree of gelatinization after cooking was influenced by storage, however, it is believed that the changes in starch properties are due to, and not the cause of, the HTC defect (El Tabey Shehata, 1992). Besides, an alternative mechanism based on protein solubilization was established by Del Valle, Cottrell, Jackman, and Stanley (1992), who found that bean softening is correlated with increased protein denaturation of isolated protein bodies, but not generally associated with pectin solubilization during cooking. Based on these hypotheses, combinations of mechanisms have also been suggested (Hincks & Stanley, 1986).

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The plant cell wall, which plays a major role in tissue soften during cooking, is made up of complex polysaccharides, phenolic compounds and proteins stabilized by covalent and non-covalent (e.g. ionic) linkages. The cell wall of leguminous seeds is generally depicted as a pectin-rich structure, containing high amounts of arabinans (El Tabey Shehata, 1992; Gooneratne, Needs, Ryden, & Selvendran, 1994; Shiga & Lajolo, 2006). Pectin is a complex polysaccharide which generally consists of three domains, i.e. homogalacturonan (HG) (smooth region), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II) (hairy regions) (Sila et al., 2009). Previous studies showed that bean cell wall non-starch polysaccharides were mainly composed of arabinose-rich pectins, arabinans, xylan, XG, galactans and β-glucans (Shiga, Cordenunsi, & Lajolo, 2011; Shiga & Lajolo, 2006), rather than predominant HG. Xylogalacturonan (XGA) is another class of pectin that has been confirmed to be present in *P. vulgaris* bean (Shiga & Lajolo, 2006), mung bean (Gooneratne, Needs, et al., 1994) and soybean (Huisman et al., 2001) cell walls, which is composed of a backbone of galacturonan substituted at O-3 by terminal Xyl residues or 1,2-linked Xyl residue.

Changes in the cell wall polysaccharides and organization have been suggested as the main cause of the HTC defect (Liu & Bourne, 1995; Shiga, Cordenunsi, & Lajolo, 2009). However, the cell wall polysaccharides of *P. vulgaris* seeds, so far, have not been extensively studied in literature. O'Neill and Selvendran (1980) depicted the structure of xyloglucans (XG) in beans as a branched polymer containing domains with low degree of branching. Shiga et al. (2009) suggested that cell walls of bean cotyledons are mainly composed of arabinans, XG, galactans and RG-I, while those of bean seed coats contain xylans, xylogalacturonan (XGA), arabinans and XG (Shiga et al., 2011). Nevertheless, detailed data referring to changes in polysaccharides of bean cotyledon and seed coat after developing the HTC defect are still limited. Hincks and Stanley (1987) found that the HTC defect of legumes is associated mainly with alterations that occur in the cotyledon, while physical alterations in the cell structure of the seed coat cause the 'hard shell' defect, which is related to the water absorption capacity of the beans.

The aim of this study was to investigate possible changes in non-starch cell wall polysaccharides and their interactions with divalent cations of bean seed coat and cotyledon due to the development of the HTC defect, before and after cooking.

2. Materials and methods

2.1. Sample preparation

A batch of common beans (*P. vulgaris* c.v. Rose coco), harvested in March 2013, was obtained from the Kenya Agricultural Livestock Research Organization (KALRO). Part of the beans was frozen directly after harvest and stored in a Zip-lock polythene bag at −40 °C, and was defined and confirmed as easy-to-cook (ETC) based on its cooking time (1.0–1.5 h). The other part of the beans was stored at 35 °C and 75% relative humidity for 8 months to develop the HTC defect. These beans were defined and confirmed to be HTC based on its cooking time (3.0–3.5 h), and stored in a Zip-lock polythene bag at −40 °C until use. A schematic overview of the experimental set-up is shown in Fig. 1. For cell wall components analysis, bean seeds were either untreated (raw material) or subjected to thermal treatment at 96 °C in a thermostated water bath (Memmert WBU-45, Germany). Based on previous research (Kinyanjui et al., 2014), treatment times of 1.5 h and 3.5 h were used to obtain fully cooked Rose coco beans, for ETC and HTC samples, respectively. Prior to cooking, all samples were soaked in distilled water (beans/water = 1:5 w/w) at 25 °C for 16 h. The samples were dehulled manually to obtain seed coat and cotyledon fractions after soaking or thermal treatment. Hence, eight samples in total were obtained from ETC and HTC materials (Fig. 1). All samples were dried at 40 °C for 48 h, then ground using a blender mill (IKA Labotechnik-A10, Germany) and passed through a 500 μm mesh/sieve to obtain fine flour. The bean coat and cotyledon flour were used for the extraction of alcohol insoluble residue.

2.2. Extraction of alcohol insoluble residue (AIR)

AIR was extracted according to the method described by Njoroge et al. (2014). Bean seed coat or cotyledon flours (2.0 g) were treated at 96 °C for 10 min. Then, the suspensions were incubated with α-amylase (500 U/mL), protease (61.5 U/mL), and amyloglucosidase (300 U/mL) for 1 h, respectively. The suspension was centrifuged, and the pellet was washed with 80% ethanol (2 times) and acetone (1 time). The suspension was filtered and the residue was dried at 40 °C. The dry AIR was ground, weighed and stored in a desiccator over P_2O_5 until analysis. The extraction was performed in triplicate.

2.3. Cell wall polysaccharide fractionation

The cell wall polysaccharides from the AIR of the 8 samples were fractionated into water extractable polysaccharides (WEP), chelator extractable polysaccharides (CEP), Na_2CO_3 extractable polysaccharides (NEP) and hemicellulose polysaccharides (HP) according the procedure of Christiaens et al. (2012). The fractionation was performed in triplicate.

2.4. Galacturonic acid content

All AIR samples and fractions obtained thereof (WEP, CEP, NEP and HP) were analyzed for their galacturonic acid (GalA) content according to the method described by Christiaens et al. (2012). The samples were first hydrolyzed with concentrated sulfuric acid, followed by a spectrophotometric determination of the GalA concentration using the method of Blumenkrantz and Asboe-Hansen (1973). All hydrolyses were performed in triplicate.

2.5. Degree of methoxylation (DM) and degree of acetylation (DAc)

The DM of pectin in AIR, WEP and CEP was determined as the ratio of the moles of methanol to the GalA content. To quantify the amount of methanol, saponification of the ester bonds in pectin with NaOH was performed according to the procedure of Ng and Waldron (1997). The amount of methanol released was subsequently determined spectrophotometrically as described by Klavons and Bennett (1986). The GalA content was determined as previously described. The DAc of AIR and WEP was calculated as the ratio of the molar amount of acetic acid to the molar amount of GalA and expressed as a percentage. The concentration of acetic acid was determined by means of an enzyme kit (K-ACETRM, Megazyme International Ireland, Wicklow, Ireland) (Njoroge et al., 2014). All hydrolyses were performed in triplicate.

2.6. Neutral sugars composition

Neutral sugar analysis was performed according the method described by Njoroge et al. (2014) and Sila, Smout, Elliot, Van Loey, and Hendrickx (2006). Lyophilized pectin fractions were hydrolyzed with 4 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h. Quantification of the neutral sugars were performed via high performance anion exchange chromatography (Dionex Bio-LC system) coupled with pulsed amperometric detection (HPAEC-PAD).

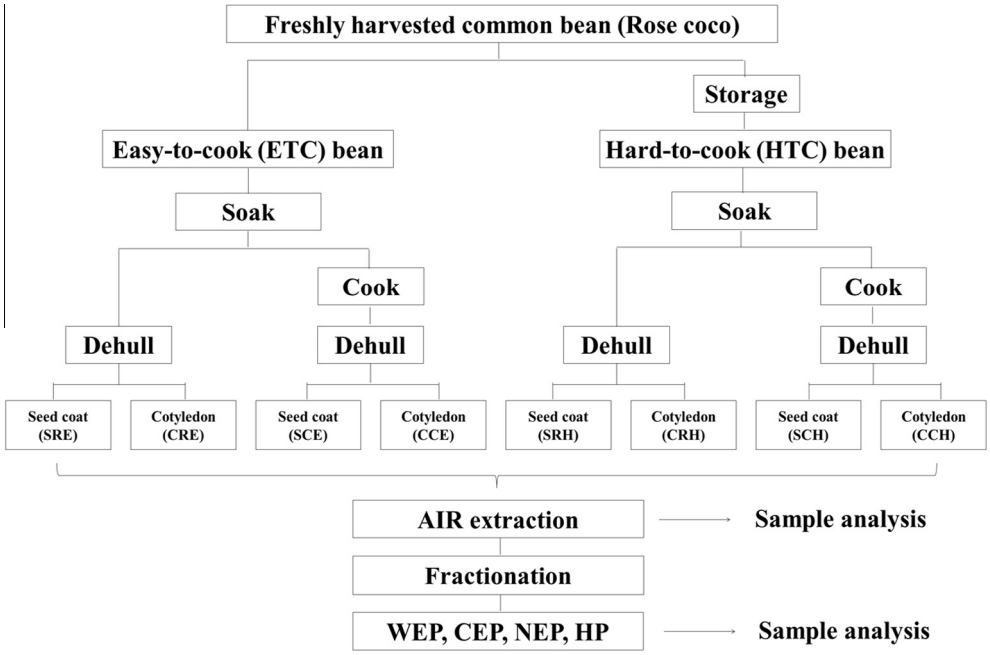

Fig. 1. Schematic overview of the experimental set-up. AIR: alcohol insoluble residue; SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean.

2.7. Molar mass distribution

Molar mass analysis was performed according the method described by Njoroge et al. (2014). Exact 3.0 mg of the lyophilized sample was dissolved in 1 mL of 0.1 M 4-morpholineethanesulfonic acid monohydrate (MES) buffer solution, pH 6.5, containing 0.1 M NaCl. The molar mass distribution was determined using a high performance size exclusion chromatography (HPSEC) coupled to multiangle laser light scattering (MALLS) (PN3621, Postnova analytics, Germany), refractive index (RI) detection (Shodex RI-101, Showa Denko K.K., Kawasaki, Japan) and diode array detector (DAD) (G1316A, Agilent technologies, Diegem, Belgium).

2.8. Protein, starch, hemicellulose pectin content of AIR

The composition of the AIR samples in terms of protein, starch, hemicellulose and pectin, as well as the residue content, were determined and expressed as percentage. The protein content of the AIR was determined using an automated Dumas protein analysis system (EAS, varioMax N/CN, Elt, Gouda, Netherlands), using 6.25 as the nitrogen to protein conversion factor. The starch content in the WEP, CEP and NEP fractions was estimated as the amount of glucose in the WEP, CEP and NEP fractions multiplied by 0.9 (during polysaccharide hydrolysis, performed before sugar analysis, one molecule of water is added per glucose residue) (Spielbauer et al., 2009). The amount of hemicellulose inclusive of starch was estimated in a similar way from the total sugar content in the HP fraction, while the pectin content was estimated from the GalA and pectic neutral sugar content (i.e. neutral sugar content without glucose and mannose) in the WEP, CEP and NEP fractions (Njoroge et al., 2014). Pectin polymers showing up in the HP fraction were considered as 'hemicellulose' as it was not possible to relate the different neutral sugars in HP to either hemicellulose or pectin. Residues were lyophilized and weighted after fractionation of the AIR.

2.9. Mineral element determination

Mineral elements (Ca, Mg, Fe, and Zn) of the bean material were analyzed by the methods as described by Kyomugasho, Willemsen, Christiaens, Van Loey, and Hendrickx (2015). Bean seed coat or cotyledon powder (20 mg) was incinerated at 550 °C for 20 h. The ash was dissolved in 10 mL demineralized water and 0.1 mL 69% HNO_3. The mixture was stirred overnight and diluted where necessary and stored at 4 °C until analysis. For analysis, inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7700x ICP-MS (Agilent Technologies, Santa Clara, USA) was used.

2.10. Phytate content

Phytate content of the bean seed coat or cotyledon was determined colorimetrically at 500 nm based on reduction of the pink color of the Wade reagent (0.3 g/L ferric chloride and 3 g/L sulfosalicylic acid) (Fruhbeck, Alonso, Marzo, & Santidrian, 1995).

Please see detailed methods of Sections 2.2–2.7 and 2.10 in supplementary methods.

2.11. Statistical analysis

Statistical analysis was conducted using the software SPSS Statistics (v.17.0, SPSS Inc., Chicago, USA), applying one-way analysis of variance (ANOVA) and Tukey's multiple range tests. Significant differences were defined at $p < 0.05$.

3. Results and discussion

3.1. Yield of alcohol insoluble residue

The yield of AIRs obtained from the eight samples is shown in Fig. 2a. The amount of AIR extracted from bean seed coats was about 4 times higher than that obtained from cotyledons. In general, there was no systematic difference between the amounts of AIR extracted from raw ETC and HTC bean seed coats, nor from

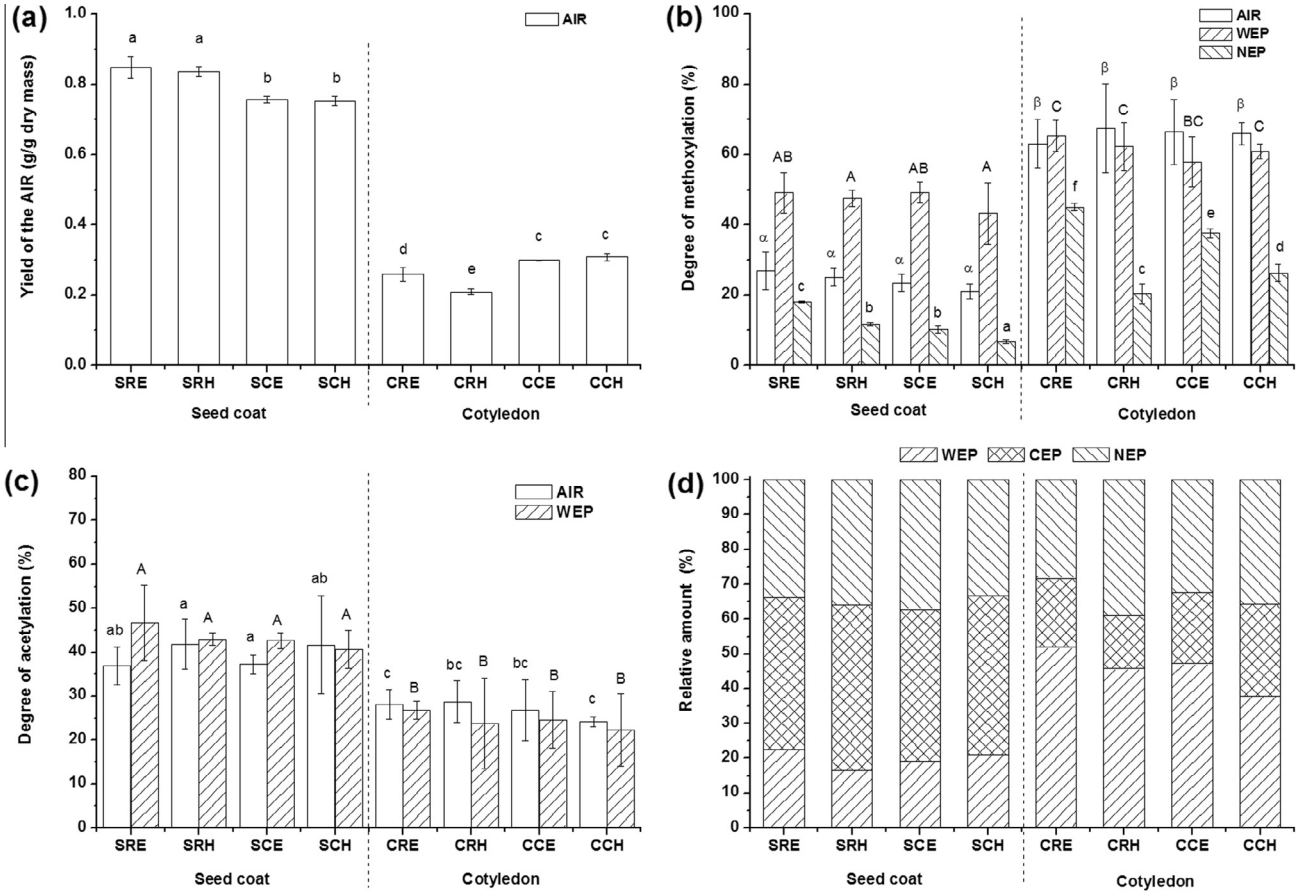

Fig. 2. Weight of alcohol insoluble residue (AIR) extracted from the bean seed coats and cotyledons (a); degree of methoxylation (b) and degree of acetylation (c) of the AIR and its non-starch pectic polysaccharide fractions; relative amounts of WEP, CEP and NEP fractions of the bean seed coats and cotyledons (d). WEP: water extractable polysaccharides; CEP: chelator extractable polysaccharides; NEP: Na_2CO_3 extractable polysaccharides; SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean. Same lower-case, upper-case, and Greek letters indicate no significant difference ($p < 0.05$).

cooked ETC and HTC bean seed coats. However, compared to the raw ETC cotyledon, a small decrease ($p < 0.05$) in the amount of AIR for the raw HTC cotyledons was observed. In addition, the amount of the AIR from the bean seed coats decreased while the amount of the AIR from the cotyledons increased after cooking. This is because it was difficult to separate the seed coats and cotyledons completely when dehulling the cooked beans, therefore, the cross contamination between the cooked seed coat and cotyledon samples is inevitable and thus influencing the yield of AIR. However, the extent of the cross contamination was minimal, which would not affect the results of the further analysis significantly.

3.2. Degree of methoxylation (DM) and degree of acetylation (DAc)

The DM was determined for the AIR, WEP and CEP fractions of all samples, by quantification of both the GalA content and the amount of methanol groups. Significant differences were found in the DM between the bean seed coats and cotyledons of the same samples. The DM of AIRs extracted from seed coats was around 25%, while the DM of AIRs extracted from the cotyledons was generally higher (around 60%) (Fig. 2b). The fact that, compared with the bean cotyledons, the DM of the seed coat CEP of both ETC and HTC beans is rather low, makes the seed coat pectin more sensitive to Ca^{2+} cross linking than that of the bean cotyledon. The DM of the CEP fraction of the seed coat and even more of the cotyledon, decreased substantially ($p < 0.05$) after development of the HTC defect. Since the CEP fraction mainly contains low-esterified pectin that is ionically cross-linked, a decrease in DM indicates that increased ionic cross-linking might occur in the HTC beans. However, the trend of decreasing DM after storage was not reflected in the DM values of the AIR. This discrepancy may be ascribed to the influence of storage on the structure of pectic polysaccharides in the NEP and HP fraction. This hypothesis could not be verified experimentally, because the alkaline extraction procedure removes the methoxyl groups of these pectin fractions. In addition, no significant differences were found in the DM of the AIR and WEP fractions between the raw and cooked bean seed coats, nor between the raw and cooked bean cotyledons.

As shown in Fig. 2c, the DAc of AIR and WEP of bean seed coats was slightly higher than that of the cotyledon. However, for both AIR and WEP fractions, there were no systematic differences in the DAc between the ETC and HTC samples, as well as between the raw and cooked samples.

3.3. Galacturonic acid content and neutral sugar composition

Analysis of the composing sugars of different cell wall factions allowed to make an overall fingerprint of the non-starch polysaccharides. The concentration of the individual neutral sugars was determined using HPAEC-PAD, while the GalA content was measured by colorimetry. Remarkable differences in GalA content were

noticed between the seed coats and cotyledons, and storage and cooking significantly affected the sugar composition of the seed coat and cotyledon fractions as well (Fig. 3).

An overall amount of each sugar was calculated by the sum of the respective monosaccharide in the four fractions (WEP, CEP, NEP and HF). For the seed coat fractions, a high overall amount of Xyl (69.4–79.3 mg/g) and GalA (57.3–68.8 mg/g), as well as a considerable amount of Ara (21.3–28.2 mg/g) were present. Based on previous literature (Gooneratne, Majsak-Newman, Robertson, & Selvendran, 1994; Liu & Bourne, 1995; Shiga et al., 2011), the data of the sugar composition suggested that xylan, pectic xylogalacturonan (XGA), arabinans, galactans and xyloglucan (XG), together with small amount of rhamnogalacturonan (RG), comprised the bulk of the non-starch polysaccharides in bean seed coats. In this study, large amounts of Xyl and GalA that co-extracted from the CEP and NEP fractions suggested the predominant presence of XGA, which might be the main pectic polysaccharides in the bean seed coats. In addition, XGA was also documented in pea hulls by Le Goff, Renard, Bonnin, and Thibault (2001) and in mung seed coat by Gooneratne, Needs, et al. (1994).

As can be seen in Fig. 3, GalA was the most abundant monosaccharide in the WEP and CEP seed coat fractions, indicating the predominant presence of pectic polysaccharides that can be extracted by hot water and a chelating solution. The GalA residues decreased by 20.0% in the WEP fraction after development of the HTC defect, while the Xyl and Gal content also decreased (37.9 and 41.5%, respectively). This reduction is possibly due to a decreased extractability of the pectic polysaccharides and XG in HTC bean seed coats. The highest GalA content was observed in the CEP fraction indicating that large part of the pectic polysaccharides in the seed coat was ionically cross-linked. The increase of GalA in the CEP fraction after storage supports the hypothesis of decreased extractability of the pectic polysaccharides on HTC development. The increase in GalA (10.7% and 42.9% in CEP and NEP, respectively) and the decrease in neutral sugars in the CEP and NEP fractions (e.g. Ara by 12.2% and 19.8%, Gal by 37.4% and 26.4%, respectively) of HTC bean seed coats reflects an increase in pectin linearity in these fractions after storage. This is consistent with the report of Shiga et al. (2011), who also found that debranching of non-starch polysaccharides occurred in non-water extractable fractions during storage, which could be associated to the development of the HTC defect. Interestingly, Xyl in the NEP fraction decreased markedly (35.0%) after developing HTC defect. It is suggested that the decrease of Xyl might be attributed to a lignification-like mechanism (Shiga et al., 2011) that made xylans more tightly bound with cellulose, preventing full hydrolysis by TFA.

In case of cotyledons, high overall amounts of Ara (83.0–108.5 mg/g), GalA (31.5–50.85 mg/g), Gal (22.6–32.8 mg/g) and Xyl (22.1–36.1 mg/g) were found, indicating the possible presence of arabinan-rich pectic polysaccharides. Based on reported data for sugar composition (Shiga et al., 2009), it was suggested that branched arabinans, XG, short linear galactans and RG-I were the main non-starch polysaccharides in bean cotyledon. The GalA content in the cotyledon WEP fraction of HTC beans decreased by 19.4% compared to those in the ETC beans. On the other hand, considerable amounts of GalA were found in the non-water extractable cotyledon fractions, which showed an increase in GalA after development of the HTC defect. The fact that part of the galacturonans became less extractable by hot water in HTC beans and could only be extracted using a chelating agent and an alkali solution is also reflected in the DM changes as discussed above. In addition, a high amount of Ara residues were observed in the NEP and HP fractions, suggesting that linkages that predominated in Ara-rich polysaccharides are likely to be ester bonds, since they are known to be labile only in strong alkali solution. The increase of Ara in NEP and HP fractions during storage (HTC development) could be related to the further formation of strongly bound and/or entangled Ara-rich polysaccharides (Shiga, Lajolo, & Filisetti, 2004). Besides, a rather considerable amount of GalA was found in the cotyledon HP fraction, indicating that some pectic polysaccharides might be tightly bound with hemicellulose, thus requiring harsher extraction conditions to be released.

The sugar composition of fully cooked ETC and HTC samples is also shown in Fig. 3. No differences were found in the GalA contents of WEP between the fully cooked ETC and HTC seed coat/-cotyledon, but the GalA contents of the non-water extractable fractions in cooked HTC beans were generally higher than that in cooked ETC beans. Besides, no other systematic changes were found in the sugar composition of the bean seed coats and cotyledons after cooking, both for ETC and HTC beans. The Glu in NEP and HP fractions of cooked HTC cotyledon were significantly lower than that of ETC cotyledon, possibly because of the longer cooking time (3.5 h) compared to the ETC beans (1.5 h), thus resulting more leaching of Glu-rich polymers, possibly including some residual gelatinized starch.

3.4. Extractability of non-starch polysaccharide fractions

The relative percentages of non-starch/hemicellulose polysaccharides in WEP, CEP and NEP fractions in seed coats and cotyledons are presented in Fig. 2d. Since the HP fraction mainly contains hemicelluloses, the relative amounts of the non-starch polysaccharides of WEP, CEP and NEP fractions were calculated as the GalA plus sum of pectin neutral sugar content (Fuc, Rha, Ara, Gal and Xyl) in the specific fraction relative to the GalA plus sum of pectin neutral sugar content in the sum of the WEP, CEP and NEP fractions. WEP is generally made up of highly esterified polysaccharides (in this case, DM 47.5–49.1% and 62.2–65.3% for raw seed coats and cotyledons, respectively) loosely bound to the cell wall through non-covalent and non-ionic bonds, while CEP contains mainly low-esterified polysaccharides (in this case, DM 11.7–18.0% and 20.4–45.1% for raw seed coats and cotyledons, respectively) that are held in the cell wall by calcium bridges, and NEP is predominantly linked to the cell wall polysaccharides through covalent ester bonds.

Remarkable differences were found in the relative percentage of non-starch polysaccharide fractions between the seed coats and cotyledons. The seed coats contained generally a lower amount of WEP (16.54–22.52%) than the cotyledons (37.82–51.95%). In addition, a high amount of CEP (43.62–47.48%) was present in the seed coats. This indicates that the seed coat relatively contains a higher amount of ionically bound polysaccharides than the cotyledon (see also degree of methoxylation).

The changes in the percentage of non-starch polysaccharide fractions in seed coats and cotyledons after developing the HTC defect were similar. As shown in Fig. 2d, the WEP fractions slightly decreased both for seed coat and cotyledon after storage, while the non-water extractable fractions (CEP and NEP) increased. The increase in the sum of the CEP and NEP fractions indicates that a higher amount of ionically and/or ester-bound polymers were presented in the HTC beans both in the seed coat and the cotyledon. This might be responsible for the HTC defect of the cotyledon, possibly by hindering cotyledon cell separation during cooking. In addition, the decrease of the DM value of the CEP fraction after storage was in line with a decrease in water extractability. Differences in the different fractions between raw and fully cooked samples were minimal in the seed coats; however, the WEP fraction of the cotyledons deceased after cooking, which might partly be the consequence of WEP leaching during cooking.

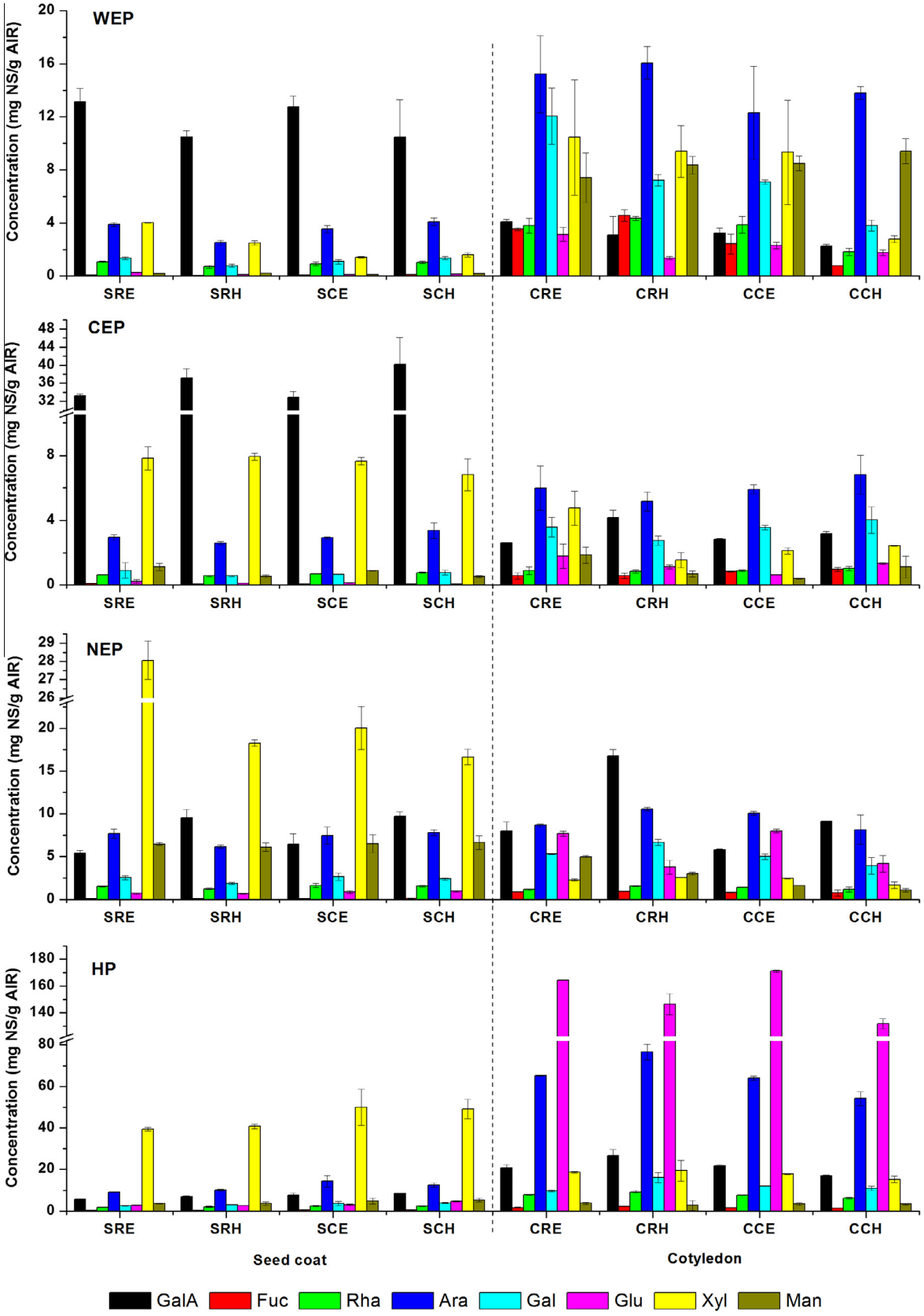

Fig. 3. Galacturonic acid and neutral sugar (NS) contents of water extractable polysaccharides (WEP) and chelator extractable polysaccharides (CEP), Na_2CO_3 extractable polysaccharides (NEP) and hemicellulose polysaccharides (HP) fractions of the bean seed coats and cotyledons. GalA, galacturonic acid; Fuc, fucose; Rha, rhamnose, Ara, arabinose; Gal, Galactose, Glu, glucose; Xyl, xylose; Man, mannose. SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean.

3.5. Molar mass distribution

Fig. 4 illustrates the elution profiles of the WEP and NEP fractions obtained by HPSEC-MALLS/RI/DAD. The refractive index (RI) intensity is proportional to the concentration, whereas the multi-angle laser light scattering (MALLS) intensity was used to calculate the molecular weight (MW) (Vriesmann, Teófilo, & de Oliveira Petkowicz, 2011). For the same fractions of the same sample, distinct differences were observed between the MW/concentration chromatograms obtained for seed coats and cotyledons. For the WEP fractions, the molecular weight of the polymers from bean cotyledons was slightly higher than that of the bean seed coat in the elution time range from 44 min to 57 min, possibly due to the differences in polymer constitution and molecular conformation, which could influence the elution behavior (Andersson, Wittgren, & Wahlund, 2003). The molar mass distribution of the NEP fractions for seed coat and cotyledon were similar.

The elution times of the main concentration peak for cotyledon WEP and NEP fractions were generally earlier than that of the corresponding fractions of seed coats, suggesting that the average molar mass of non-starch polysaccharides in the cotyledons was higher than that in the seed coats. The light scattering (LS) 92° and RI intensity for the CEP fractions were very low, nevertheless, the CEP fractions showed a similar molar mass distribution as the NEP fractions (data not shown).

For the seed coats, peaks with low concentration eluting at around 42 min were observed in the WEP fractions, implying that the molecular weight of the non-starch polysaccharides from seed

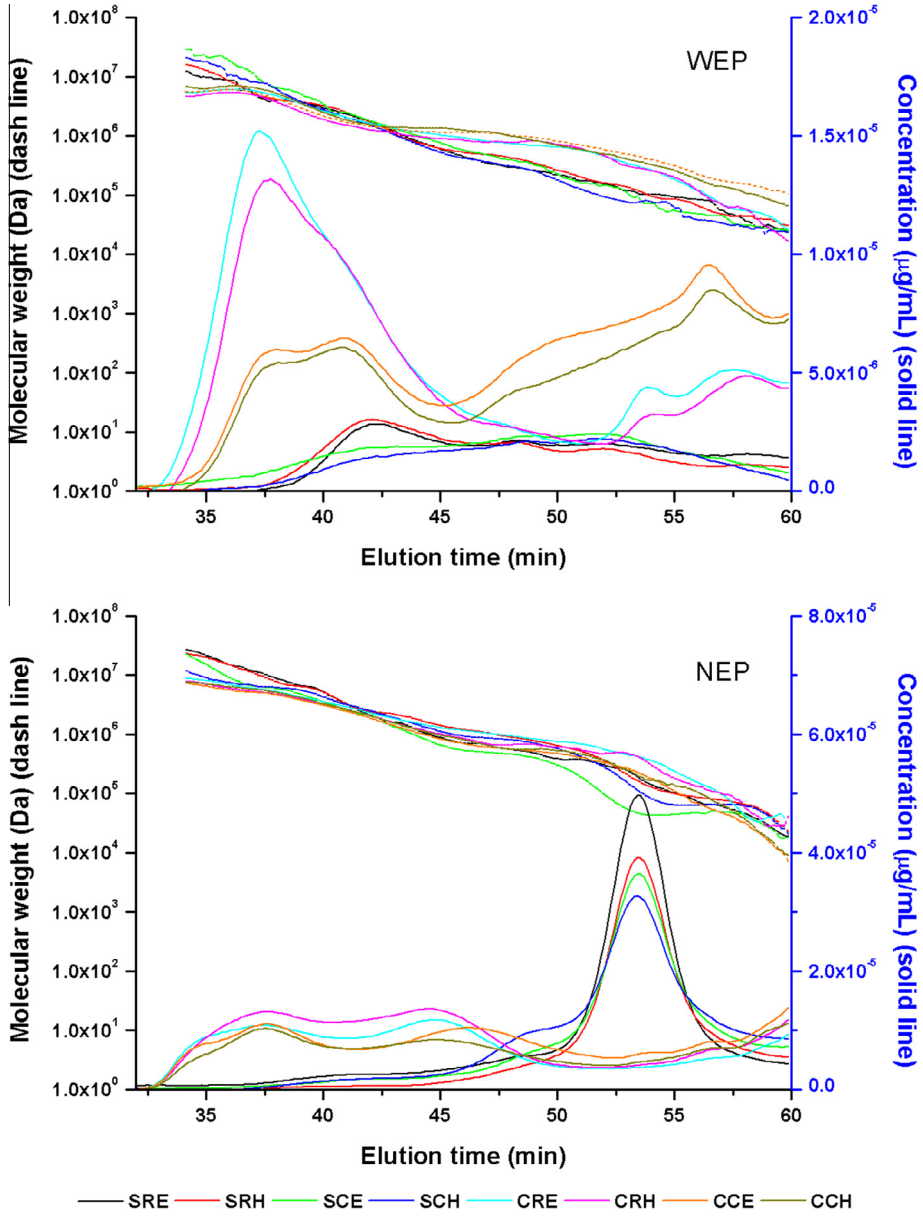

Fig. 4. Molar mass distribution (dash line) and concentration (solid line) of the non-starch polysaccharides in water extractable polysaccharides (WEP) and Na_2CO_3 extractable polysaccharides (NEP) fractions of the bean seed coats and cotyledons. SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean.

coat was relatively low, compared with the seed coat main concentration peaks eluted at 37 min. Large and sharp main peaks were found at elution times around 53 min in the NEP fractions of seed coats, indicating the predominant presence of families of polymers with lower molar mass, such as RG (Vriesmann, Amboni, & de Oliveira Petkowicz, 2011). Njoroge et al. (2014) also found extra peaks at elution time of approximate 56 min in the NEP fractions of whole bean flour, which might be the same polymers that represented the main peaks of seed coats in Fig. 4b.

For the cotyledons, large concentration peaks eluting at approximate 37 min were found in the WEP fractions, indicating the predominant presence of high molar mass components, which probably, based on the elution time, are mainly composed of pectic polysaccharides (Njoroge et al., 2015). In the WEP fraction, extra concentration peaks were found at an elution time around 57 min, together with the large UV (280 nm) peaks showed at the same elution time (data not shown), suggesting the presence of residual protein that co-extracted with the polysaccharides. Two concentration peaks were found in the NEP fractions at the elution time of 37 min and 45 min, respectively, indicating the presence of two different types of polysaccharides, which molar masses were larger than that from the seed coat NEP fractions.

No significant differences were found in the elution times and shapes of the concentration peaks between the ETC and HTC beans in the WEP and NEP fractions, both for the raw seed coats and cotyledons, indicating there was no substantial change of molar mass distribution in these fractions after developing the HTC defect.

The distribution of the molar mass was significantly affected by the cooking process. The decrease of concentration at elution time of 37 min (cotyledon) and 42 min (seed coat), together with the increase of the concentration at elution time after 45 min, in the WSP fractions from cooked bean material indicated that the molar mass of some polymers decreased compared with the raw material, implying that polysaccharides degradation occurred to a certain extent during cooking. Similar concentration decreases were also observed in the NEP fractions after cooking, both for the seed coats and cotyledons. The decreases in the WEP and CEP fractions after cooking could partially due to the leaching out during cooking.

3.6. Mineral and phytate contents

The mineral and phytate contents of the bean seed coats and cotyledons are presented in Table 1. No significant differences were found in the Ca, Mg, Fe and Zn contents between the ETC and HTC beans, both for the seed coats and cotyledons. As expected, the HTC defect did not result in changes in mineral contents of the seed coats and cotyledons. In addition, the Ca, Mg, Fe and Zn contents of the seed coats were significantly higher than that of the cotyledons, calcium being around 8-fold higher in the seed coats compared to the cotyledons. Besides, due to leaching, the mineral content for all the samples decreased after cooking, but no significant differences were found between the ETC and HTC beans, both for the seed coats and cotyledons. The phytate content of the seed coats are substantially lower than that of the cotyledons. The cotyledons of the HTC (aged) beans contain a significantly lower amount of phytates compared to the ETC beans, indicating hydrolysis due to phytase activity during storage (Galiotou Panayotou et al., 2008). In addition, for all samples, the cooking process resulted in a loss of phytates due to leaching.

The molar ratio between the cation concentrations (Ca^{2+} or all minerals including Ca^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+}) and the pectic free carboxyl group (COO^-) concentrations were calculated and presented in Table 1. The calculation is based on the stoichiometric R-value (representing two times the molar concentration of the minerals considered divided by the free carboxyl group molar concentration, in line with the egg box or shifted egg box model (Braccini & Pérez, 2001) that has been presented for the interaction of pectin with divalent minerals, in particular Ca^{2+}). This ratio that has been used in the past in relation to rheological properties of pectin based gels (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010). Two R-values have been calculated: R_{ca} representing the R-value for Ca^{2+} ($2[Ca^{2+}]/[COO^-]$) and the R_{mn} representing the R-value for all minerals taking into account the complexing capacity of phytate ($2[Ca^{2+} + Mg^{2+} + Fe^{2+} + Zn^{2+} - 6\text{-phytate}]/[COO^-]$).

The R_{ca}-value of the raw seed coats (0.81–0.88) was lower than that of the corresponding raw cotyledons (1.61–2.35). Theoretically, at a ratio up to 1.0 all Ca^{2+} (or all minerals with similar pectin interaction behavior) can be bound in the egg box structure but even above 1.0 further increase of pectin gel strength has been observed (Ngouémazong et al., 2012). Ca^{2+} has been reported to be a more crucial cation than Mg^{2+} for the development of the HTC defect, because Ca^{2+} is the most suitable divalent cation to be complexed with pectin by ionic cross linking (Braccini & Pérez, 2001).

Phytate is highly charged with 6 phosphate groups and capable of chelating divalent cations. The phytate content was 9.35–12.54 μmol/g in the raw cotyledons of HTC and ETC beans respectively, which are capable of chelating up to 56.12–75.22 μmol/g of divalent cations (a maximum binding Ca^{2+}/phytate ratio of 6 has been suggested by Martin and Evans (1986). Therefore, considering the overall divalent cation concentration and the chelating power

Table 1
Mineral, galacturonic acid, free carboxyl group, phytate contents, and R-values of the bean seed coats and cotyledons.

	Ca* (μmol/g)	Mg* (μmol/g)	Fe* (μmol/g)	Zn* (μmol/g)	GalA (μmol/g)	COO^- (μmol/g)	Phytate (μmol/g)	R_{Ca}-value	R_{mn}-value
SRE	215.6 ± 5.4^{a}	105.2 ± 0.3^{a}	1.39 ± 0.06^{a}	0.79 ± 0.10^{ab}	728.7 ± 32.2^{a}	532.2	4.92 ± 0.05^{e}	0.81	1.10
SRH	213.7 ± 2.5^{a}	103.7 ± 1.3^{a}	1.38 ± 0.17^{a}	0.84 ± 0.08^{a}	646.6 ± 26.0^{b}	484.1	4.27 ± 0.24^{f}	0.88	1.22
SCE	184.0 ± 8.0^{b}	84.7 ± 0.6^{b}	0.75 ± 0.03^{b}	0.81 ± 0.04^{a}	591.0 ± 18.6^{c}	451.9	3.45 ± 0.11^{g}	0.81	1.11
SCH	166.2 ± 8.4^{c}	86.7 ± 1.9^{b}	0.73 ± 0.08^{b}	0.77 ± 0.19^{ab}	549.2 ± 30.3^{c}	433.3	1.94 ± 0.35^{h}	0.77	1.12
CRE	25.2 ± 5.2^{d}	67.6 ± 1.1^{c}	1.08 ± 0.07^{c}	0.63 ± 0.08^{bc}	85.0 ± 10.5^{d}	31.3	12.54 ± 0.16^{a}	1.61	1.24
CRH	28.9 ± 4.2^{d}	64.7 ± 0.8^{d}	1.11 ± 0.04^{c}	0.74 ± 0.10^{ab}	75.4 ± 13.5^{de}	24.6	9.35 ± 0.56^{c}	2.35	3.21
CCE	19.8 ± 1.3^{e}	45.0 ± 1.7^{e}	0.90 ± 0.10^{d}	0.55 ± 0.03^{c}	63.1 ± 12.0^{de}	14.9	10.11 ± 0.05^{b}	2.67	0.78
CCH	21.6 ± 3.3^{e}	39.1 ± 0.8^{f}	0.96 ± 0.09^{d}	0.53 ± 0.02^{c}	65.1 ± 4.0^{e}	22.2	6.51 ± 0.53^{d}	1.95	2.10

SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean. Minerals, galacturonic acid (GalA), free carboxyl group (COO^-), and phytate contents are expressed as μmol/g bean seed coat or cotyledon (dry basis). Mineral, GalA, COO^-, and phytate contents are expressed as mean value ± SD (n = 3). Different superscript letters in the same column indicate significant differences ($p < 0.05$).
COO^-: Content of free carboxyl group based on amount of GalA taking into account the degree of methylesterification (DM) of cell wall pectic polysaccharides: (μmol GalA/g bean material) × (1 − DM%).
R_{ca}: R_{ca} − value based on Ca^{2+}: $2[Ca^{2+}]/[COO^-]$.
R_{mn}: R-value based on all minerals taking into account the phytate content: $2[Ca^{2+} + Mg^{2+} + Fe^{2+} + Zn^{2+} - 6 \times \text{phytate}]/[COO^-]$.

of phytate in the cotyledon, R_{mn}-values of 1.24 and 3.21 are obtained respectively for ETC and HTC beans, indicating that the decrease of phytate released a substantial amount of cations largely changing the cross pectin linking intensity for HTC compared to ETC, possibly explaining the HTC behavior. This hypothesis requires migration of minerals, this was recently studied by Kruger et al. (2015), who found that the Ca^{2+} and Mg^{2+} inside the parenchyma cells relocated and concentrated to the cell wall-middle lamella where they probably bound to the carboxyl group of pectin, during development of the HTC in cowpeas. The effect of HTC development on the R_{mn}-values for the bean seed coat is less pronounced, R_{mn}-values being 1.10 and 1.22 for ETC and HTC beans. The different ratios and their changes for seed coat and endosperm can explain the phenomena of hard shell and/or HTC that have been described in literature. Upon cooking mainly the endosperm R_{mn}-values are changing, in particular for the ETC beans; changes of R_{mn}-values of the seed coat seem minimal. These observations are completely in line with the pectin structure observations we made (see above), e.g. the seed coat pectin and in particular the CEP fraction showed a much lower degree of methoxylation compared to the cotyledons therefore being far less sensitive to the beta-elimination reaction explaining the rather limited change in R_{mn}-value for the seed coat upon cooking.

It is has been reported that soaking in $CaCl_2$ containing solutions significantly extended the cooking time of beans (Kinyanjui et al., 2014), implying that the bean cell wall pectic polysaccharides were still susceptible to exogenous Ca^{2+}. Our observations on phytate and mineral contents are in line with these ideas. In particular the pectin of the seed coat (R_{mn}-values around 1.0) has the capacity of binding additional Ca^{2+} leading to enhanced pectin cross linking. Also the enhancement of cooking when soaking and cooking beans in $NaHCO_3$ or Na_2CO_3 solutions is in line with the observations in this study, the slightly alkaline pH of these soaking/cooking media prevents the release of divalent cations from phytate and at the same time enhances the β-eliminative reaction leading to enhanced pectin depolymerization and associated solubilization. Also the retardation of bean softening under slightly acidic conditions is in line with the observations in this study, slightly acidic conditions (e.g. pH < 5) will lead to divalent cation release from phytate cross-linking pectin and at the same time slow down the β-eliminative reaction. At pH conditions below the pectin pKa, the pectin cross linking will be limited while the β-eliminative reaction reduces to zero resulting in long cooking times.

All together, the high concentration of Ca^{2+} in the seed coats of both ETC and HTC beans, together with its high relative amount of CEP, which contained a marked amount of GalA and exhibited a rather low DM (see Table 1, Figs. 2 and 3), indicate a predominant presence of Ca^{2+} cross-linked pectic polysaccharides in the seed coats. Furthermore, the seed coats exhibited a R_{mn}-value of around one, together with the high amount of AIR (Fig. 2), which showed similar pectin concentration (10.56-10.71%) to the cotyledon AIR (Table 2), makes pectin Ca^{2+} cross linking a dominant factor in the seed coat. Although the R_{mn}-value was relatively high for the cotyledon, the bulk of divalent minerals in the cotyledon might be chelated by the predominant amount of phytate rather than cross-linked with pectin. In this sense, the release of divalent minerals induced by phytate hydrolysis during storage could substantially contribute to the development of the HTC defect.

Table 2
Percentage composition (%) of alcohol insoluble residue (AIR) of the bean seed coats and cotyledons.

	Protein	Starch in WEP, CEP, NEP	Hemicellulose inclusive of starch	Pectin	Residual
SRE	5.63	0.11	6.24	10.71	52.17
SRH	7.08	0.08	6.69	10.56	59.41
SCE	8.59	0.10	8.34	10.29	61.20
SCH	10.81	0.11	8.25	11.27	69.16
CRE	29.60	1.14	12.77	9.30	7.23
CRH	30.05	0.56	15.29	11.27	8.16
CCE	38.13	0.98	12.83	9.28	17.48
CCH	38.65	0.65	10.85	6.94	11.70

SRE: seed coat of raw easy-to-cook bean; SRH: seed coat of raw hard-to-cook bean; SCE: seed coat of cooked easy-to-cook bean; SCH: seed coat of cooked hard-to-cook bean; CRE: cotyledon of raw easy-to-cook bean; CRH: cotyledon of raw hard-to-cook bean; CCE: cotyledon of cooked easy-to-cook bean; CCH: cotyledon of cooked hard-to-cook bean.

3.7. Protein, starch, hemicellulose, pectin and residue contents of the AIR

The percentage amounts of protein, starch, hemicellulose, pectin and residue contents in the AIRs from bean seed coats and cotyledons are presented in Table 2. The AIRs of the cotyledons contained markedly higher amounts of protein than the AIRs of the seed coats. This was in line with the UV (280 nm) peaks at elution time of 57 min of the cotyledon WEP fractions detected by the HPSEC-MALLS/RI/DAD (data not shown), indicating the possible cross-linking of protein with cell wall polysaccharides in the bean cotyledon, limiting its solubility and increasing its resistance to hydrolysis by proteases. The increase in protein content in the cooked cotyledon compared to the raw cotyledon might be attributed to thermal denaturation and breakage of the linkage between protein and polysaccharides during the cooking, resulting in increasing extractability. The total amounts of starch in WEP, CEP and NEP fractions were lower than 1.14% in all samples, verifying that most of the starch was hydrolyzed by the amylase and amyloglucosidase. Therefore, the abundant Glu in the cotyledon HP fractions (Fig. 3) might be attributed to the presence of large amount of β-glucans and/or XGs. The amounts of hemicellulose in the cotyledons were generally higher than those in the seed coats. The percentage amounts of pectin were between 9.28% and 10.27% in both cotyledon and seed coat, except for the low amount of pectin in the cooked cotyledon of the HTC bean, which might be attributed to leaching and/or degradation of soluble pectic polymers caused by the longer cooking time. Large amounts of residues were found in the AIRs of seed coat, which primarily will consist of cellulose (Njoroge et al., 2014).

4. Conclusions

Differences in composition, structure and extractability of non-starch polysaccharides between bean seed coat and cotyledon were observed. The amount of AIR extracted from seed coats was about 4 times higher than that from cotyledons. >50% of the AIR from seed coats was non-extractable residues, whereas higher contents of protein and hemicellulose were found in the AIRs of cotyledons. Sugar composition analysis showed that the seed coats comprised large amounts of xylose-rich polymers, while cotyledons contained abundant amounts of arabinose-rich polymers. The seed coats contained higher relative percentages of CEP and lower percentages of WEP fractions than that of the cotyledons. In addition to that, a high ratio of divalent cation pectic/free carboxyl group after storage, taking into account the alteration of the phytate content, suggested increased Ca^{2+}-pectin polysaccharide cross-linkage in the cell wall of HTC beans. The molar mass of non-starch polysaccharides in the cotyledons was generally higher than that in the seed coats, indicating the presence of polysaccharides with a longer backbone and/or more branches.

Some compositional and structural of non-starch polysaccharides from the HTC beans differed significantly from the ETC beans. The DM of the CEP fractions from the HTC beans was lower than from the ETC beans. No systematic changes were found in the DM of the AIRs and WEP fractions. The relative percentages of WEP fractions decreased and the sum of non-water extractable fractions (CEP and NEP) increased after development the HTC defect. Besides, no substantial changes were found in the polymer molar mass distribution of WEP and NEP fraction after development the HTC defect.

The increase in the amount of the seed coat CEP fraction, which is the largest fraction in the seed coat, together with the decrease in the DM of this fraction after the storage, suggests strengthening of the cell wall network by forming new ionic linkages during storage. This might enhance seed coat impermeability to water during soaking and cooking, and it could contribute to the hard shell phenomenon. On the other hand, the decrease in the amount of WEP fraction and increase in the amount of non-water extractable fractions in the cotyledon indicates that the decrease in hot water extractability for cell wall non-starch polysaccharides might contribute to the hard-to-cook defect of the cotyledon, possibly by hindering cotyledon cell separation during cooking. In conclusion, differences in the structure, composition and extractability of non-starch polysaccharides between the ETC and HTC common beans confirmed the significant role of the pectic polysaccharides in interaction with divalent ions in the HTC development, which consequently affect their cooking behaviors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.05.018.

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