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PII: S0963-9969(15)00087-3  
DOI: doi: [10.1016/j.foodres.2015.03.005](https://doi.org/10.1016/j.foodres.2015.03.005)  
Reference: FRIN 5710

To appear in: *Food Research International*

Received date: 22 September 2014  
Revised date: 30 January 2015  
Accepted date: 1 March 2015



Please cite this article as: Njoroge, D.M., Kinyanjui, P.K., Christiaens, S., Shpigelman, A., Makokha, A.O., Sila, D.N. & Hendrickx, M.E., Effect of storage conditions on pectic polysaccharides in common beans (*Phaseolus vulgaris*) in relation to the hard-to-cook defect, *Food Research International* (2015), doi: [10.1016/j.foodres.2015.03.005](https://doi.org/10.1016/j.foodres.2015.03.005)

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**Effect of storage conditions on pectic polysaccharides in common beans (*Phaseolus vulgaris*) in relation to the hard-to-cook defect**

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**Abstract**

The importance of common beans (*Phaseolus vulgaris*) in addressing food insecurity cannot be underestimated. However, their utilization is hampered by development of the hard-to-cook (HTC) defect i.e. the inability of cotyledons to soften sufficiently within a reasonable time during cooking, presence of flatulence causing oligosaccharides, antinutrients and low digestibility of macronutrients. The objective of this study was to determine the effect of storage conditions (time, temperature and relative humidity) on pectic polysaccharides of selected common bean varieties during the evolution of the hard cook problem. First, alcohol insoluble residue (AIR) was extracted from the bean flour. The AIR was fractionated into water, chelator and Na<sub>2</sub>CO<sub>3</sub> soluble pectin fractions and a hemicellulose fraction. The galacturonic acid content, neutral sugars, degree of methylesterification (DM), degree of acetylation (DAc) and molar mass distribution for pectin fractions were determined. In addition, filterable residual protein in various fractions was estimated. Results on the acidic and neutral sugars revealed that common beans contained highly branched, arabinose-rich pectic polysaccharides. Storage of common beans for more than 4 months at high relative humidity (83%) and high temperature (45°C) resulting in HTC development showed a decrease in pectin extractability in water paralleled by an increase in the alkaline soluble fraction. Other pectin characteristics such as DM and DAc showed minor variations upon storage of beans. The hydrolysis of both starch and proteins before AIR extraction decreased with increasing storage time, temperature and relative humidity. The increase in residual starch and protein might be linked to the protein-starch hypothesis where predominance of protein denaturation leads to restricted starch gelatinization. The results reveal that, the contribution of pectic polysaccharides to development of HTC defect during the storage of Canadian wonder and Red haricot common beans at elevated temperature and relative humidity is due to reduced pectin solubility. However, the influence of starch and proteins seems evident.

**Keywords**

Common beans; Galacturonic acid; Hard-to-cook; Neutral sugars; Molar mass; Pectin

## 1 Introduction

Legumes have a great potential in the fight against food insecurity and alleviation of protein energy malnutrition due to their relatively high levels of proteins and carbohydrates among other nutrients. The proteins from legumes are rich in lysine, the limiting amino acid in most staple cereal grains such as maize and rice, making it a good complement in providing a balanced amino acid ratio. Legumes are a relatively cheaper source of protein in the human diet, compared to the relatively more expensive animal protein sources. Worldwide, common beans (*Phaseolus vulgaris*) are the most important legumes in terms of production and consumption. Other primary dietary legumes include pea (*Pisum sativum*), chickpea (*Cicer arietinum*), broad bean (*Vicia faba*), pigeon pea (*Cajanus cajan*) and cowpea (*Vigna unguiculata*) (Bressani, 1993; Broughton *et al.*, 2003; Graham & Vance, 2003; Huma, *et al.*, 2008; Katungi *et al.*, 2009; Akibode & Maredia, 2011).

Legumes stored at elevated temperature (> 25 °C) and relative humidity (rh) (> 65%), conditions quite prevalent in tropical weather conditions, take a longer time to cook (Moscoso *et al.*, 1984; Garruti & Bourne, 1985; Hentges *et al.*, 1991; Liu *et al.*, 1992). The extended cooking time is due to either, the hard-shell (HS) effect or/and the hard-to-cook (HTC) defect. The HS effect is a phenomenon that restricts the moisture migration during the soaking and cooking process of legumes due to impermeability of the seed coat or outer shell to water. The impermeability of the seed coat to water is due to biochemical changes such as the oxidation of tannins, formation of protein-tannin complexes or biophysical changes such as size reduction (Stanley, 1992; Martin-Cabrejas *et al.*, 1997; Yousif *et al.*, 2007; Nasar-Abbas *et al.*, 2008; Katungi *et al.*, 2009; Pirhayati *et al.*, 2011). The HTC defect on the other hand is a phenomenon where seeds do not soften sufficiently during soaking and do not become tender after a reasonable cooking time. The HTC defect is characterized by extended cooking time of the seeds to allow softening to the desired texture. This results in high processing/cooking costs due to high energy consumption, inferior nutritional quality and negative changes in texture and palatability of the legumes. As a result, the acceptability and utilization of legume seeds by consumers is also reduced (Garcia & Lajolo, 1994; Liu, 1995; Garcia *et al.*, 1998; Mbofung, *et al.*, 1999; Yousif *et al.*, 2007; Pirhayati *et al.*, 2011). Several hypotheses have been postulated to explain the occurrence of the HTC defect in legumes. These hypotheses can be categorized into enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms include the pectin-cation-phytate theory (Mafuleka *et al.*, 1993; Reyes-Moreno & Paredes-Lopez, 1993; Liu, 1995; Garcia *et al.*, 1998; Reyes-Moreno *et al.*, 2000) and the lignification theory (Stanley, 1992; Reyes-Moreno & Paredes-Lopez, 1993; Nasar-Abbas *et al.*, 2008; Srisuma *et al.*, 1989). The non-enzymatic mechanisms include reduced pectin  $\beta$ -eliminative degradation (Liu *et al.*, 1993), protein denaturation in relation to

starch gelatinization (Garcia & Stanley, 1989; Liu, 1997) and lipid oxidation and/or polymerization (Reyes-Moreno & Paredes-Lopez, 1993). Although these hypotheses have been formulated, there is limited detailed work on each of the constituents involved in the suggested mechanisms.

In this study the focus was directed towards pectin changes, playing a role in an enzymatic as well as a non-enzymatic hypothesis for explaining the development of the HTC defect in common beans. Pectin is a complex polysaccharide found in cell walls and generally consists of three domains: homogalacturonan (HG) (smooth region), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II) (hairy regions) (Ridley *et al.*, 2001; Vincken *et al.*, 2003). Homogalacturonan, a linear homopolymer consisting of (1→4)- $\alpha$ -linked-D-galacturonic acid (GalA), is the major pectic polysaccharide generally accounting for approximately 60% of the total pectin amount in the cell wall (Willats *et al.*, 2001; Willats *et al.*, 2006; Mohnen, 2008; Voragen *et al.*, 2009). The carboxyl moieties of the polymer are esterified to a certain degree with methanol at C-6 (Mohnen, 2008; Voragen *et al.*, 2009). The ratio of the methanol to the GalA content is referred to as the degree of methylesterification (DM). The degree and pattern of methylesterification are important parameters for pectin functionality. GalA residues in HG can also be *O*-acetylated at C-2 and/or C-3. The ratio of acetyl groups to GalA content forms the degree of acetylation (DAc). In certain cases, up to 90% of *O*-acetylation may occur and this hinders enzymatic breakdown of HG and also alters solubility and gelation properties (Oosterveld *et al.*, 2000; Willats *et al.*, 2001; Vriesmann *et al.*, 2013). Rhamnogacturonan I is an acidic pectic domain consisting of as many as 100 repeats of the disaccharide (1→2)- $\alpha$ -L-rhamnose-(1→4)- $\alpha$ -D-galacturonic acid. The rhamnosyl residues of RG I can be substituted at O-4 with neutral sugar side chains composed of galactosyl and/or arabinosyl residues (Willats *et al.*, 2001; Voragen *et al.*, 2009). Rhamnogalacturonan II is a highly complex macromolecule with a (1→4)-linked  $\alpha$ -D-galacturonan backbone, partially methyl-esterified at C-6 of the GalA residues and substituted by four different side chains (Voragen *et al.*, 2009; Yapo, 2011). Several covalent and non-covalent linkages have been observed between pectin polymers. They include amongst others the cross-linking of pectin with calcium according to the 'egg box model' (Morris *et al.*, 1982; Voragen *et al.*, 2009) and the occurrence of ferulic acid dihydrodimers forming RG II dimers (Ishii, 1997; Ishii *et al.*, 1999; Voragen *et al.*, 2009). Pectic polysaccharides may also be cross-linked to other cell wall components such as hemicelluloses, phenolic compounds and wall proteins. The cross-linking provides an added structural and functional complexity to the wall (Caffall & Mohnen, 2009).

It is hypothesized that during storage of common beans at elevated temperatures (> 25 °C) and rh (> 65%), on the one hand, pectin may be hydrolyzed by pectin methylesterase (PME)

to pectinic acid and methanol. On the other hand, activation of phytase enzymes leads to hydrolysis of phytates resulting in the formation of phosphate esters of reduced chelating power while at the same time releasing bivalent ions such as calcium and magnesium. During soaking process prior to cooking of the beans, ion migration takes place hence leading to the formation of insoluble pectates. More specifically, pectinic acid reacts with the bivalent cations such as calcium and magnesium, released due to the hydrolysis of phytates, to form insoluble pectate compounds. The pectates formed do not dissolve easily on heating and therefore, they impose restriction on cell wall separation thus delaying softening of the beans to the desired texture during the cooking process (Jones & Bolter, 1983; Bhatti & Slinkard, 1989; Bhatti, 1990; Mafuleka *et al.*, 1993; Reyes-Moreno & Paredes-Lopez, 1993; Liu, 1995; Garcia *et al.*, 1998). A lower DM of pectin created during storage eventually also leads to reduced pectin  $\beta$ -eliminative degradation during cooking.

To improve legumes utilization and hence contribution to food security, a full understanding of the mechanisms involved in the development of the HTC defect is critical. This knowledge could subsequently be used to reduce and/or reverse the occurrence of the defect. The objective of this study was to investigate the effect of storage time, temperature and relative humidity (rh) on pectic polysaccharide structure and properties in common beans and relate the observed changes in pectin characteristics to the cooking quality of the differently stored beans. In contrast to previous studies, we attempt to evaluate the pectin nanostructural changes in detail.

## 2 Materials and methods

### 2.1 Storage of common beans

Four varieties of common beans (*Phaseolus vulgaris*) ((Pinto (GLP-x92), Rose coco (GLP-2), Canadian wonder (GLP 24) and Red haricot (GLP 585)), harvested on March 2013, were obtained from the Kenya Agricultural Research Institute (KARI). The beans were subdivided and placed in perforated trays and subsequently stored at four different storage conditions of temperature and relative humidity (rh) i.e. 25°C/75% rh, 35°C/75% rh, 45°C/75% rh and 35°C/83% rh for a maximum of 6 or 12 months (**Table 1**). Prior to storage, all beans were conditioned for two weeks at 25°C and 75% rh. The selected relative humidities were maintained during the storage period by the use of saturated solutions of sodium chloride and potassium chloride for 75% rh and 83% rh, respectively. The beans were sampled after 4, 8, and 12 months of storage for 25 and 35°C, and 75% rh conditions and after 2, 4 and 6 months for beans stored at 45°C and 75% rh and 35°C and 83% rh resulting in a total of 64

samples. After each sampling period, a portion of the sample was stored frozen (-20°C) while the other portion was used for determination of the bean cooking quality.

## 2.2 Cooking quality of common beans

The cooking times of the beans were measured for each storage condition and sampling period. To determine the cooking time, bean seeds pre-soaked in deionized water for 16 h at 25 °C were subjected to a thermal treatment at 96 °C in a thermostated water bath (Memmert WBU-45, Germany) followed by texture estimation by finger pressing after every 30 minutes of cooking (Vindiola *et al.*, 1986; Kinyanjui *et al.*, 2015). Statistical data analysis was performed using a SAS statistical software package (SAS Enterprise Guide 4.3, USA). From the sigmoidal regression of all cooking curves the following parameters could be derived (i) 5% cooked beans (which is a measure for the lag phase before cooking), (ii), 95% cooked beans (which is a measure for total cooking time) and (iii) cooking rate. Significant differences in these parameters between the storage conditions were examined using the post-hoc Tukey test at a significance level of  $p \leq 0.05$  (Kinyanjui *et al.*, manuscript under preparation). Based on the cooking profiles 8 samples were selected for further detailed pectin analysis. Samples selected included bean samples that showed significant differences in their cooking times after different storage times and/or conditions as well as bean samples that did not, after being subjected to different storage times/conditions. Specifically, Canadian wonder and Red haricot bean samples were selected. For Canadian wonder, to determine the influence of storage time, temperature and rh, on bean pectic polysaccharides, samples stored for 0 month (CW/0), 4 months at 25 °C and 75% rh (CW/4/25/75), 4 months at 35 °C and 75% rh (CW/4/35/75), 6 months at 35 °C and 83% rh (CW/6/35/83), and 6 months at 35 °C and 83% rh (CW/6/35/83) were selected. For Red haricot, the samples selected were those stored for 0 months (RH/0), 6 months at 35 °C and 83% (RH/6/35/83) and 6 months at 45 °C and 75% (RH/6/45/75). The sample RH/6/35/83 was selected in order to compare with sample CW/6/35/83 since the former cooked faster after storage at the same condition.

## 2.3 Isolation of alcohol insoluble residue (AIR)

The AIR was isolated for each of the selected 8 samples representing the non-cooked (raw) beans for both Canadian wonder and Red haricot variety, before and after storage at different conditions of time, temperature and rh combinations. To obtain AIR, the selected bean samples were first soaked in deionized water at 25 °C for 16 h followed by extraction as described by Njoroge *et al.* (2014). The extraction process involved a stepwise incubation of the bean flour suspended in a phosphate buffer solution with  $\alpha$ -amylase, protease and amyloglucosidase enzymes for the respective temperature-time-pH combinations of 96 °C for

5 min at pH 6.0, 60 °C for 1 h at pH 7.5 and 60 °C for 1 h at pH 4.3, respectively. The thermal and enzymatic treatment was intended to remove starch and proteins.

#### **2.4 Cell wall polysaccharide fractionation**

The cell wall polysaccharides in the AIR extracted from the 8 samples were fractionated into water-solubilized pectin (WSP), chelator-solubilized pectin (CSP), Na<sub>2</sub>CO<sub>3</sub>-solubilized pectin (NSP) and a hemicellulose fraction (HF) following the method described by Njoroge *et al.* (2014).

#### **2.5 Galacturonic acid and neutral sugars analysis**

All the pectin fractions (WSP, CSP, NSP and HF) and AIR were analyzed for their galacturonic acid (GalA) content. The samples were first hydrolyzed with concentrated sulphuric acid according to the method described by Ahmed and Labavitch (1977). This was followed by a spectrophotometric determination of the GalA concentration using the method of Blumenkrantz & Asboe-Hansen (1973). All hydrolyses were performed in duplicate and for each hydrolysate obtained, three colorimetric analyses were performed.

Neutral sugars analysis of all pectin fractions (WSP, CSP, NSP and HF) was performed using the method described by Sila *et al.* (2006) and Christiaens *et al.* (2011).

#### **2.6 Degree of methylesterification (DM) and acetylation (DAc) determination**

The DM of pectin in AIR, WSP and CSP was determined as the ratio of the moles of methanol to the GalA content. The amount of GalA was determined as described in section 2.5, while methanol content was measured using the procedures described by Njoroge *et al.* (2014). The DAc of AIR and WSP was calculated as the ratio of the molar amount of acetic acid to the molar amount of GalA and expressed as a percentage. The amount of acetic acid was measured using procedures described by Njoroge *et al.* (2014).

#### **2.7 Molar mass distribution determination**

Samples were prepared for molar mass determination as described by Njoroge *et al.* (2014). The molar mass distribution of WSP, CSP and NSP was determined using size exclusion chromatography coupled to multiangle laser light scattering (MALLS) (PN3621, Postnova analytics, Germany), refractive index (RI) detection (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan) and diode array detection (DAD) (Agilent technologies 1200 Series, Diegem, Belgium) according to procedures described by Njoroge *et al.* (2014).



### 3 Results and discussion

#### 3.1 Cooking quality of the stored common beans

3.2 The cooking profiles of the selected common beans are presented in Figure 1. Two varieties of beans, Canadian wonder and Red haricot, showed approximately the same cooking profile prior to storage. Specifically, they cooked within the same time range of 120 min at 96°C. This is an indication that, for the varieties studied here, freshly harvested common beans have relatively similar cooking qualities. As can be seen from Figure 1, the sensitivity of common beans to the HTC defect is a function of both variety and storage conditions. The total cooking time of Canadian wonder bean seeds increased by 50% after 4 months of storage at 25 °C and 75% rh, while storage at 35 °C and 75% rh for the same period resulted in a 75% increase. Hence, the cooking time of Canadian wonder increased with increase in storage temperature although not significantly ( $p \leq 0.05$ ). Considering the effect of rh, after 4 months of storage at 35 °C, a surprising observation was made: the increase in total cooking time was 75% and 50% for 75% rh and 83% rh, respectively. Nevertheless, this difference in total cooking time was not significant ( $p \leq 0.05$ ) and, the lag phase of the cooking profiles, the time before the beans started to soften, was similar. The increase in total cooking time with increase in storage time was on the other hand significant ( $p \leq 0.05$ ). The total cooking time increased by 50% and 125% after storage at 35 °C and 83% rh for 4 and 6 months, respectively, for the Canadian wonder bean variety. Furthermore, after the 6 months storage at 35 °C and 83% rh, there was 90 min difference in total cooking time between Canadian wonder and Red haricot, with the former exhibiting a higher hardening upon storage. For Red haricot stored for 6 months at high temperature and/or rh (35°C/83% rh or 45 °C/75% rh) the cooking times increased significantly ( $p \leq 0.05$ ) (by 100% or 230% respectively). This is an indication that it is also possible to induce hardening in a bean variety that is in general more easy-to-cook. Sugar composition of stored common beans

#### 3.2.1 Galacturonic acid and neutral sugars content

The amount of GalA extracted from the common beans AIR through solubilization in different solvents is shown in **Figure 2**. The amount of GalA was low in WSP for both Canadian wonder and Red haricot beans, with minor variations for all storage conditions investigated. A similar trend was observed for CSP. However, for NSP, the amount of GalA extracted increased for beans stored at high rh (83%) for Canadian wonder and at high temperature

(45°C) for Red haricot, compared to the samples before storage. The increase could be attributed to changes in extractability of alkaline solubilized pectin. Incidentally, the bean samples with higher amounts of GalA in NSP, also had longer cooking time except for the CW/4/35/83 sample. Therefore, it can be concluded that high GalA levels in NSP are related to bean hardening during storage, this means an increase of the cooking time. Finally, the HF contained high amounts of GalA for all samples, with Canadian wonder stored at 35 °C and 85% rh for 4 and 6 months being relatively higher. This may indicate that part of the pectin polymers in common beans are strongly bound to other cell wall polymers and hence were only extracted with strong alkaline solution.

The neutral sugars determined in WSP, CSP, NSP and HF included fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl) and mannose (Man) (**Figure 2**). In general, common bean pectic polysaccharides (in WSP, CSP, and NSP) are composed of mainly arabinose, medium amounts of galactose, xylose and minor amounts of rhamnose and fucose, not taking into account the non-pectic related neutral sugars glucose and mannose. These findings were consistent with those of Shiga *et al.*, (2004). Specifically for WSP, Ara was the most abundant sugar for both Canadian wonder and Red haricot. Furthermore, there was a large increase in the amounts of glucose in WSP with high storage rh (83%) or high storage temperature (45 °C). The high amounts of glucose in WSP of these samples could be attributed to residual starch that was not hydrolyzed (thermal and enzymatic hydrolysis) during the pre-processing steps of the AIR extraction. This would have led to higher amounts of starch left in the AIR, hence ending up in the WSP. The beans with high amounts of glucose in WSP (CW/4/35/83, CW/6/35/83, RH/6/35/83, RH/6/45/75) had in general longer cooking times. The absolute amounts of sugars in CSP were low and there were no considerable differences upon storage. For NSP, the amount of all sugars increased with storage except for Rha and Man. However, the increase was relatively large for arabinose, galactose and xylose with the increase in rh (83%) or high temperature (45 °C). Therefore, upon storage of beans, more NSP could be extracted from the AIR. In HF, arabinose, glucose and xylose increased with increase in rh (CW/4/35/83, CW/6/35/83, RH/6/35/83) and high temperature (RH/6/45/75). This may indicate the presence of higher amounts of alkali extractable xyloglucans and xylans upon these storage conditions. Alternatively, although less likely, high levels of glucose could also be an indication of the increase of non-hydrolyzed (during the pre-processing steps of the AIR extraction) starch with storage that could only be hydrolyzed by the strong alkaline solution used for extraction of hemicellulose fraction.

### 3.2.2 Sugar ratios

To better understand the polymeric structure of the common bean pectic polysaccharides, sugar ratios were calculated (Houben *et al.*, 2011; Koubala *et al.*, 2014). Ratio 1 of GalA to the total amount of pectin neutral sugars fucose, rhamnose, arabinose, galactose and xylose is a measure for the linearity of pectin, while, Ratio 2 of rhamnose to GalA indicates the contribution of rhamnogalacturonan (RG) to the entire pectin population. The extent of RG-I branching is indicated by Ratio 3, i.e. the sum of arabinose and galactose to rhamnose. The sugar ratios for the different pectin fractions calculated based on the sugar content are presented in **Figure 3**.

The linearity (sugar Ratio 1) of the pectin fractions decreased from CSP over NSP to WSP, for both Canadian wonder and Red haricot. The high linearity for CSP indicated the high contribution of the linear galacturonic acid rich part of pectin i.e. homogalacturonan (HG) while the low linearity for WSP and NSP indicated that they are more branched. The linearity of all pectin fractions showed no clear trend with the storage conditions for Canadian wonder beans. However, for Red haricot beans, CSP linearity decreased at high rh (83%) while the NSP linearity decreased with increased rh (83 °C) and high temperature (45 °C). The contribution of RG to the entire pectin population (Ratio 2) was high in WSP and low in both CSP and NSP. The high RG contribution to the pectin population for WSP corresponds with the low linearity as observed in Ratio 1 for this pectin fraction. Hence, pectic polymers of common beans extractable in hot water are rich in branched pectin domains of RG-I and RG-II. The Ratio 2 for WSP increased with storage time, high temperature and high rh for both Canadian wonder and Red haricot beans. However, there were minimal variations in the contribution of RG to the pectin population in CSP and NSP for both bean varieties upon storage. The degree of branching of RG-I (Ratio 3) was generally high for all pectin fractions i.e. WSP, CSP and NSP. Upon storage, there was no clear trend of changes in the degree of branching of WSP with different storage conditions. However, the degree of RG - I branching of CSP decreased for Canadian wonder and increased for Red haricot with an increase in storage time (6 months) and high rh (83%) or high temperature (45°C). In contrast, the degree of branching in NSP increased for both Canadian wonder and Red haricot. Notably, with increase in storage time (6 months) and high rh (83%) the degree of branching increased more than twice for Canadian wonder. This was an indication that the pectic polysaccharides extracted as NSP in stored beans are relatively enriched in arabinan (and to a lesser extent (arabino)galactan) side chains and hence highly branched. A high degree of branching for the NSP corresponded to a longer cooking time of the beans.

### 3.3 Pectin extractability of stored common beans

The relative percentages of WSP, CSP and NSP fractions in Canadian wonder and Red haricot samples before and after storage at different conditions are presented in **Figure 4**. Relative amounts of the different fractions were determined as the GalA content plus the sum of pectin neutral sugars content (Fuc, Rha, Ara, Gal and Xyl) in a fraction relative to the GalA content plus the sum of pectin neutral sugars content in the sum of the WSP, CSP, and NSP fractions. For fresh Canadian wonder beans (before storage), the pectic polysaccharides solubilized in hot-water were approximately 50% with the rest being more or less equally solubilized in chelator (CDTA) and alkaline ( $\text{Na}_2\text{CO}_3$ ) solutions. However, for Red haricot beans before storage, hot water, chelator and alkaline soluble pectins were approximately 40%, 20% and 40% respectively. Looking at the percentage WSP, there were no changes in extractability of pectin in hot water for Canadian wonder stored for 4 months at 25 °C and 75% rh or 35 °C and 75% rh. However, there was a decrease in pectin extractability at higher rh (83%). Hot water pectin extractability decreased at high rh (83%) or high temperature (45 °C). From CSP, it can be deduced that variations in extractability of pectin in presence of a chelating agent under all the storage conditions investigated, for both Canadian wonder and Red haricot, were minor but decreasing. The amounts of NSP for Canadian wonder showed that, changes in pectin extractability in alkaline solutions did not differ much for beans stored for 4 months at 25 °C and 75% rh or 35 °C and 75% rh. However, the NSP content increased with an increase in storage rh (83%). Similarly, there was an increase in alkaline pectin extractability for beans stored at elevated rh (83%) or temperature (45 °C). The pectin extractability trends revealed that, on the one hand, the higher the pectin extractability in hot water the lower the cooking time of the beans and vice versa, while on the other hand, the higher the extractability in alkaline solutions the longer the cooking time. In this study, CW/6/35/83 and RH/6/45/75 showed the longest total cooking time (**Figure 1**). However, other underlying factors may be influencing the cooking quality or hardening of the beans during storage, since, the two varieties of beans stored under similar conditions (CW/6/35/83 and RH/6/35/83) had a similar pectin extractability pattern but differed in their cooking time. The decrease in pectin extractability in hot water during storage can probably not be explained by the pectin hypothesis. This hypothesis assumes the formation of insoluble calcium-pectate when the bivalent cation leaks from cytosol as a result of membrane damage during storage and binds with pectin (Jones and Bolter, 1983). However, since there were little to decreasing changes in CSP (the beans with the highest cooking times seems to contain the lowest levels of CSP), the pectin fraction that is usually bound to the cell wall through calcium bridges, the cause could be probably due to other mechanisms.

### 3.4 Degree of methylesterification and acetylation of stored common beans

The GalA and methanol content of AIR, WSP and CSP were determined and expressed as degree of methylesterification (DM), while the ratio of acetic acid to GalA expressed as a percentage for AIR and WSP represented the degree of acetylation (**Figure 5**). In general, the DM of AIR of both Canadian wonder and Red haricot beans was around 45 - 65%, while, the DAC for Canadian wonder and Red haricot was 50 – 75% and 40 – 50%, respectively. The influence of storage conditions on DM and DAC were minimal. Similar trends were observed for DM of WSP and CSP and DAC of WSP (results not shown). For the DM, this indicates that minimal pectin demethoxylation occurred during storage and the subsequent soaking process in deionized water for all investigated conditions for both Canadian wonder and Red haricot. According to the pectin hypothesis, it is assumed that, on the one hand, the activation of PME should lead to demethoxylation of pectin, while on the other hand, the activation of phytase should lead to release of calcium ions due to phytate hydrolysis. Consequently, cross-linking between pectin and calcium could occur leading to the formation of insoluble calcium-pectate. Therefore, it is expected that, the lower the DM, the longer the cooking time of the beans. However, based on our observations, the contribution of DM changes to the development of the HTC defect during storage (and the subsequent soaking process in deionized water) seems very limited, since, the variations with all storage conditions investigated was generally low. Therefore, it can be concluded that both DM and DAC, for the varieties studied here, would have a minor role in influencing the development of the HTC defect in common beans during storage.

### 3.5 Molar mass distribution of pectic polymers and presence of proteins in stored common beans

To investigate the effect of storage on the size of pectin polymers, the molar mass distribution of the Canadian wonder and Red haricot beans before and after storage at different storage time, temperature and rh was compared for WSP, CSP and NSP. The molar mass distribution of WSP for both Canadian wonder and Red haricot beans for all storage conditions investigated was similar (**Figure 6A**). The concentration elution profiles were similar for all the bean samples with Canadian wonder stored at high rh (83%) showing a higher concentration of low molar mass pectic polymers eluting between 52 – 56 min. The LS signal at 92° together with the absorbance at 280 nm is shown for WSP of both Canadian wonder and Red haricot beans in **Figure 6B**. The absorbance at 280 nm revealed the presence of proteins eluting between 55 – 70 min. The amounts of proteins appeared to be proportionally higher for the beans stored at high rh for both Canadian wonder and Red haricot (CW/4/35/83 CW/6/35/83 and RH/6/35/83) and at high temperature for Red haricot (RH/6/45/75). This could be attributed to the resistance of proteins to the protease enzyme

hydrolysis during the extraction process of the AIR. Consequently, more protein remains in the AIR, hence, showing up in WSP after fractionating. Therefore, these results suggest that high storage temperature and rh leads to a decrease in enzymatic protein hydrolysis of common beans. The resistance of the proteins to protease hydrolysis might suggest that protein changes contribute to the development of the HTC defect. In this study, the beans with high filterable residual proteins had in general longer cooking times for both Canadian wonder (CW/4/35/83 and CW/6/35/83) and Red haricot (RH/6/35/83 and RH/6/45/75). However, for the same storage conditions, Red haricot bean variety (RH/6/35/83) cooked faster than Canadian wonder (CW/6/35/83). For the CSP fraction, the light scattering (LS) 92° and absorbance at 280 nm signal intensities were very low (results not shown).

The NSP molar mass distribution for all storage conditions investigated for both Canadian wonder and Red haricot beans was similar (**Figure 7A**). The concentration elution profiles revealed relatively higher amounts of high molar mass polymers presence in beans stored at high rh for both Canadian wonder (CW/4/35/83 and CW/6/35/83) and Red haricot (RH/6/35/83) and those stored at high temperature for Red haricot (RH/6/45/75). Incidentally, the same samples exhibited poor cooking quality (longer cooking times), apart from RH/6/35/83. Absorbance at 280 nm revealed minor variations in the filterable residual proteins for all the storage conditions investigated for both Canadian wonder and Red haricot (**Figure 7B**). These observations might suggest pectin cross linking (e.g. by ferulic acid) during storage at high temperature/high relative humidity conditions.

#### 4 Conclusions

Storage of Canadian wonder and Red haricot at high temperature and high rh resulted in decreased pectin extractability in hot water (WSP) and an increased extractability in alkaline solutions (NSP). In addition, the NSP fraction contains a higher concentration of high molecular weight pectin polymers. However, other pectin characteristics such as DM and DAc did not show much variation. The high amount of glucose in the WSP fraction of beans stored at high rh (83%) and temperature (45°C) indicated changes in thermal and enzymatic hydrolysis of starch, during AIR isolation. In addition, higher residual protein concentrations in the WSP fractions suggests protein changes resulting in a higher resistance to protease catalyzed hydrolysis during AIR preparation from beans stored at high rh and/or temperature. These results suggest that the contribution of pectin changes to the development of the HTC defect during storage of Red haricot and Canadian wonder common beans at elevated temperature and rh is due to reduced pectin solubility not as a result of PME catalyzed pectin demethoxylation but rather due to covalent pectin cross linking. In addition there seems to be role for protein changes supporting the protein-starch hypothesis where protein denaturation leads to restricted starch gelatinization and maybe even pectin extractability.

## Acknowledgments

This research has been carried out with the financial support from the VLIR-UOS-Belgium through the Food and Nutrition Security Project (ZEIN2011PR385). We sincerely thank the Kenya Agricultural Research Institute (KARI-Thika) for providing the plant raw materials required for the research, and Heidi Roba and Margot De Haes for their technical support. S. Christiaens is a Postdoctoral Researcher funded by Research Foundation Flanders (FWO).

## Figure captions

**Figure 1:** Percentage cooked seeds as a function of cooking time at 96 °C for Canadian wonder and Red haricot common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 2.** Neutral sugars fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glu), xylose (Xyl) and mannose (Man) and galacturonic acid (GalA) content for WSP, CSP, NSP and HF of Canadian wonder and Red haricot common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 3.** Sugar ratios for WSP, CSP and NSP, representing linearity of pectin (1), the contribution of RG to the pectin population (2) and branching of RG-I (3) of Canadian wonder and Red haricot common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 4.** Relative amounts of water-, chelator- and  $\text{Na}_2\text{CO}_3$ - soluble pectin fractions for Canadian wonder and Red haricot common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 5.** Degree of methylesterification (DM) and acetylation (DAc) of alcohol insoluble residue for Canadian wonder and Red haricot common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 6. (A)** Log molar mass distribution (dashed lines) versus concentration (solid lines) as a function of elution time (min), and **(B)** light scattering signal at 92° (solid lines) and absorbance at 280 nm (dotted lines), for WSP of Canadian wonder (CW) and Red haricot (RH) common beans before and after storage at varying time, temperature and relative humidity combinations

**Figure 7. (A)** Log molar mass distribution (dashed lines) versus concentration (solid lines) as a function of elution time (min), and **(B)** light scattering signal at 92° (solid lines) and absorbance at 280 nm (dotted lines), for NSP of Canadian wonder (CW) and Red haricot

(RH) common beans before and after storage at varying time, temperature and relative humidity combinations

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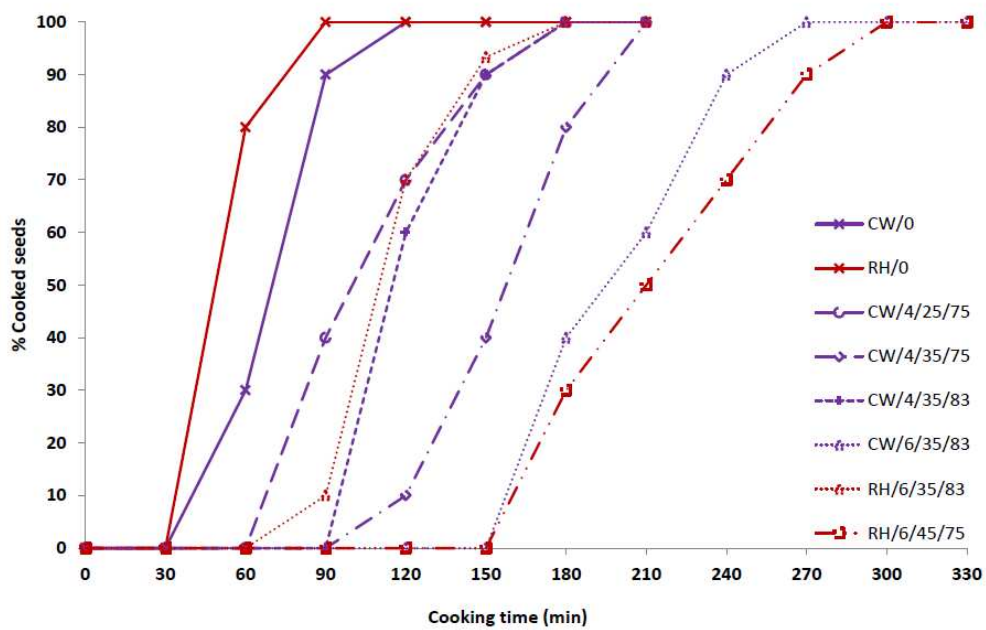
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Figure 1



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Figure 2

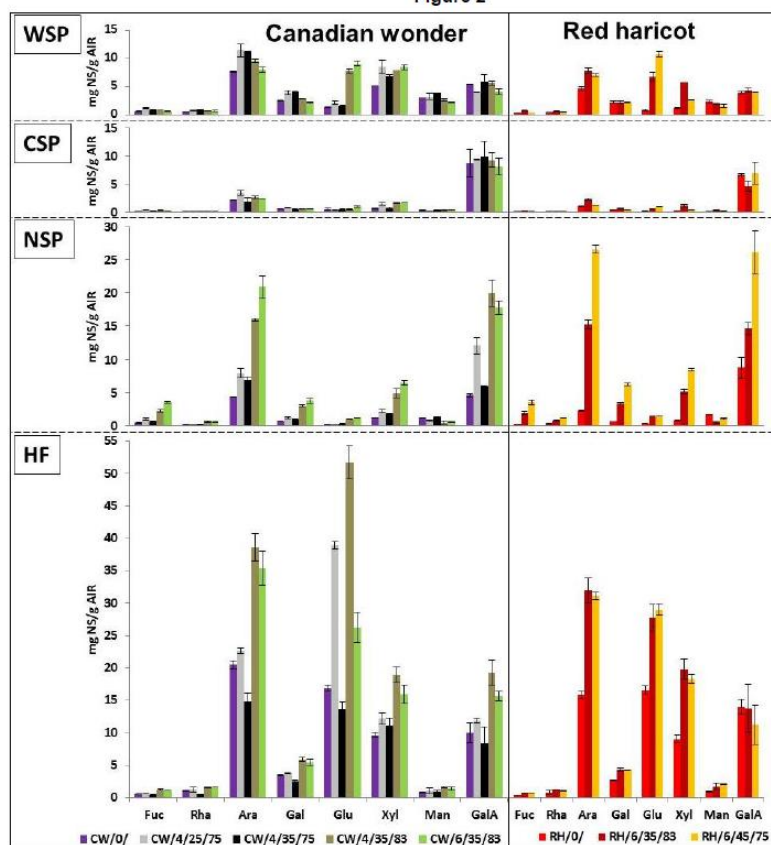


Figure 3

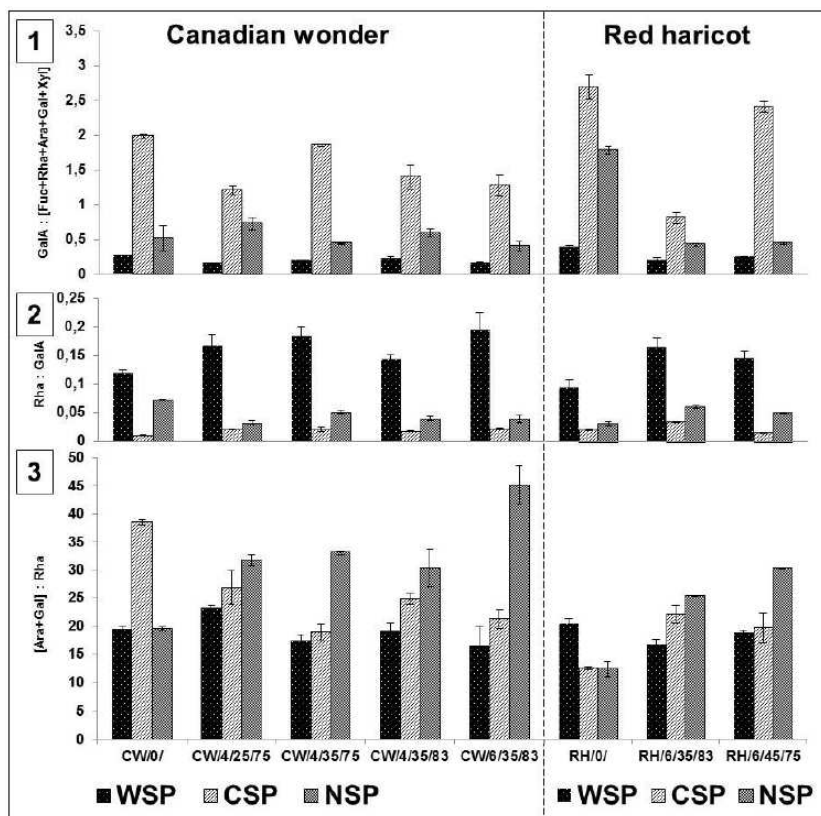
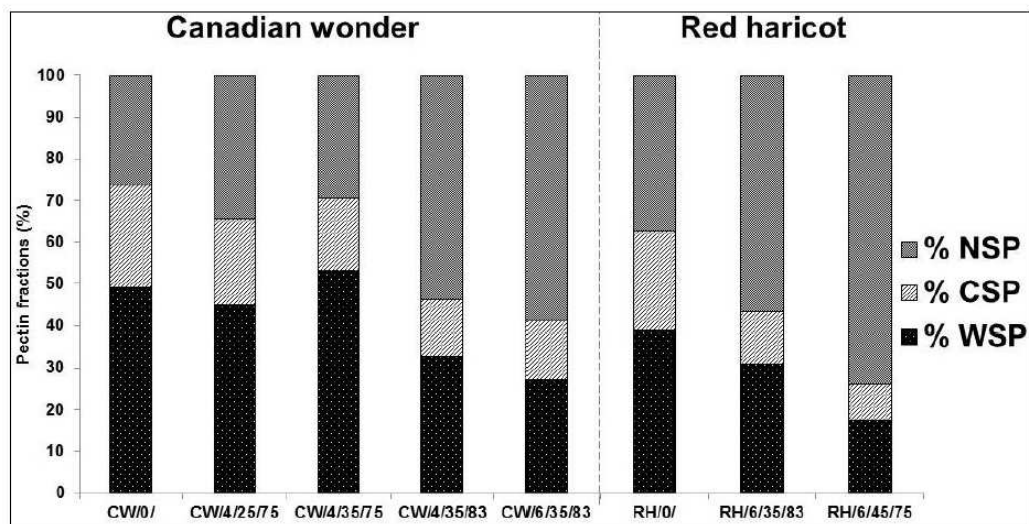


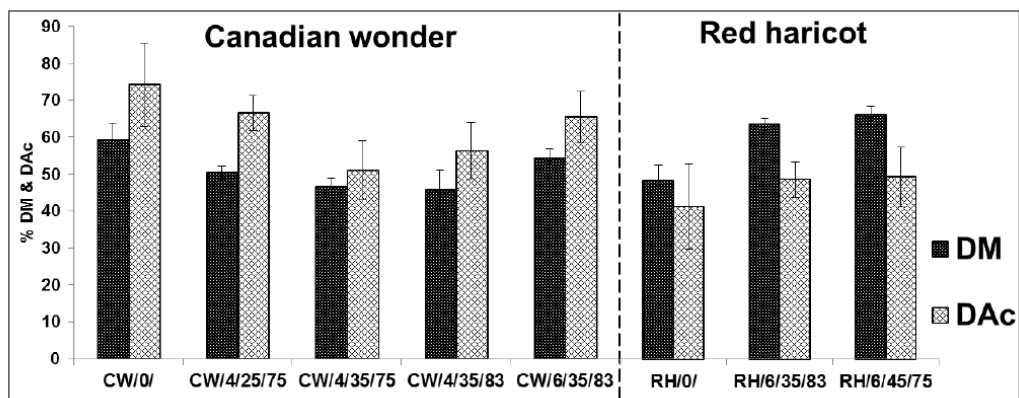


Figure 4



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Figure 5



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Figure 6

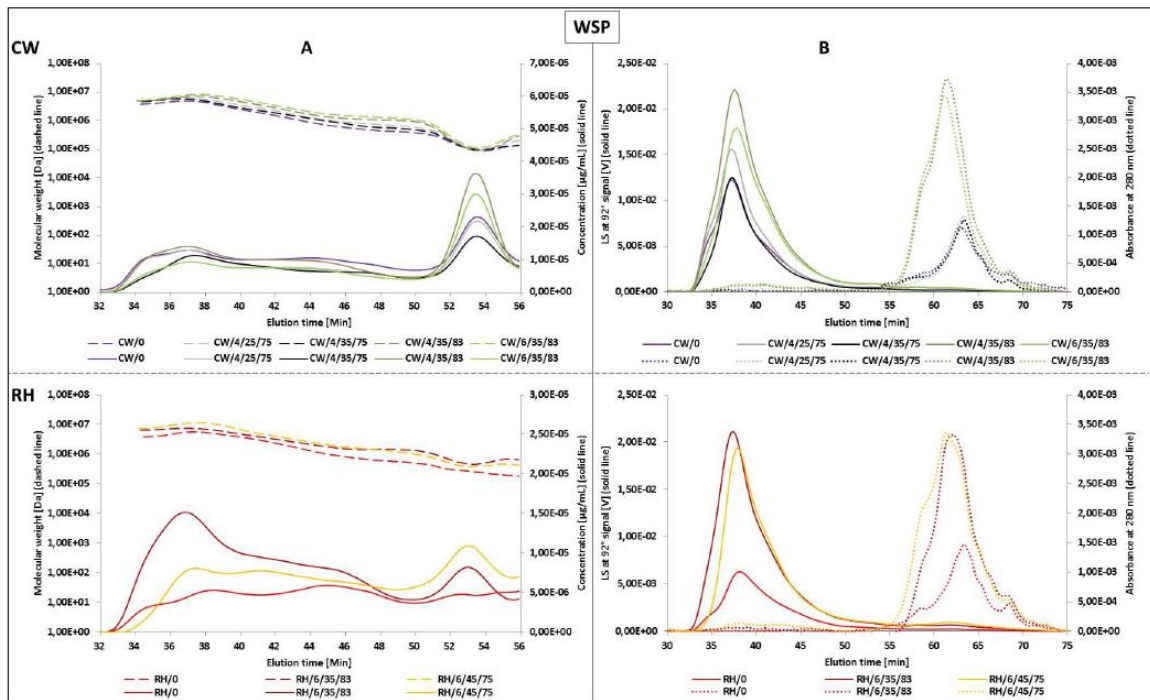
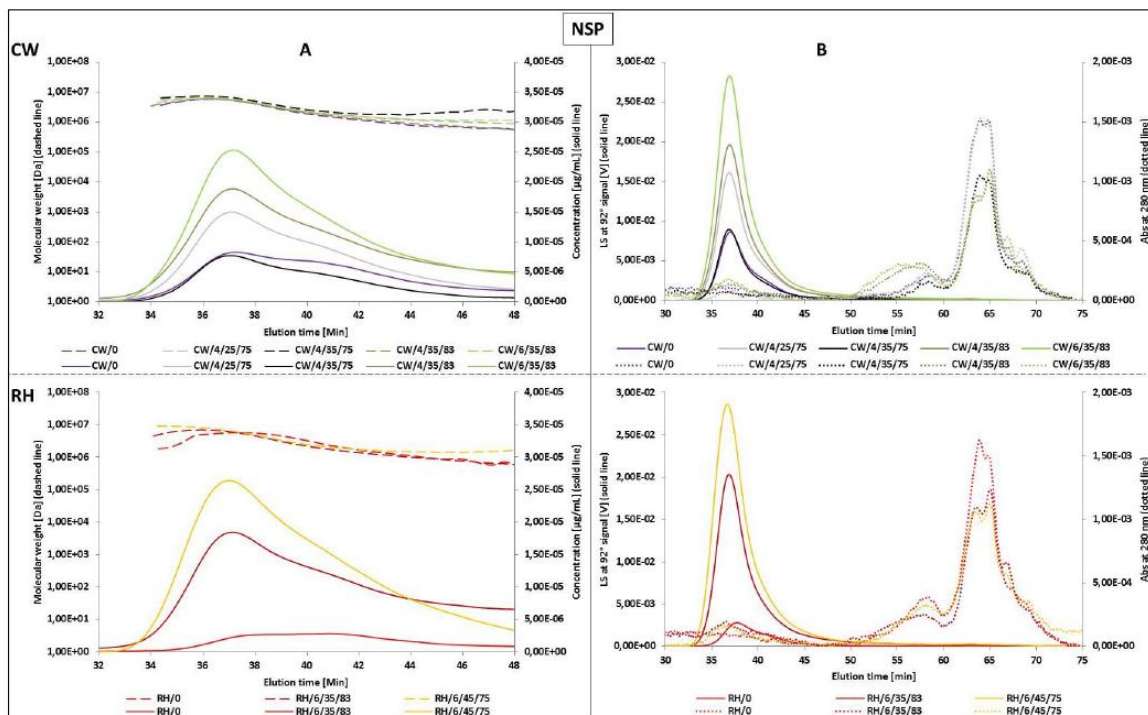


Figure 7



**Table 1.** Sampling plan for common beans stored at varying time, temperature (T) and relative humidity (rh) combinations for Pinto, Canadian wonder, Rose coco and Red haricot varieties (x = sampling period)

T (°C)/rh (%)	Storage time (months)					
	0	2	4	6	8	12
25/75	X		X		X	X
35/75	X		X		X	X
45/75	X	X	X	X		
35/83	X	X	X	X		

**Highlights**

- Beans storage at high temperature and relative humidity affects pectin properties.
- DM and DAc changes during storage have minor contribution in HTC defect development
- Reduced extractability of hot water soluble pectin is associated with beans hardness.
- There is possible role of protein changes in influencing development of HTC defect.

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