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1. INTRODUCTION

Cowpeas are an essential food grain legume in developing regions, especially in Africa. Cowpeas provide a good source of protein containing approximately 25% protein by dry mass and are quite easy to cultivate (Imbart, Régnault and Bernard, 2016). They offer an inexpensive, valuable potential source of protein and are an alternative to the much more expensive animal protein. Developing countries in Africa still face nutritional challenges especially women and children failing to meet their nutritional requirements regarding proteins, energy, vitamins, and minerals. In children, the causes of malnutrition are varied but in most cases are attributed to diets that are inadequate in quality and quantity associated with bulky, starch weaning foods (Achidi, Tiencheu, Tenyang, Womeni, Moyeh, Ebini and Tatsinkou, 2016).

However, cowpea remains underutilised due to its long cooking times, and the situation is worsened due to the development of a phenomenon known as the hard-to-cook (HTC) defect (Ndungu, Emmambux and Minnaar, 2012). HTC defect is a condition where seeds will not soften sufficiently during soaking and do not become tender after a reasonable cooking time (Liu, and McWatters, 1994). Therefore, HTC seeds need additional cooking energy during preparation (Hillocks, Bennett and Mponda, 2012), which is an inconvenience and they may also have inferior nutritional qualities such as reduced protein digestibility and quality (Martin-Cabrejas, Esteban, Perez, Maina and Waldron, 1997)

HTC defect develops when cowpeas are stored at high temperatures (>25^{o}C) and high relative humidity (>65%) (Hohlberg and Stanley, 1987). HTC defect remains a complex

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phenomenon, and several mechanisms have been proposed which include: (1) insolubilisation of pectin due to phytase activity (Galiotou-Panayotou, Kyriakidis and Margaris, 2008); (2) lignification of middle lamella (Nasar-Abbas, Plummer, Siddique, White, Harris and Dods, 2008); (3) membrane degradation (Stanley, 1991); (4) protein and starch interactions (Liu, 1997) and (5) the multiple mechanism theory (Garcia, Filisetti, Udaeta and Lajolo, 1998). The most widely accepted theory to explain the HTC defect in legume seeds is based on insolubilisation of pectin via binding with divalent cations (e.g. Ca^{2+} and Mg^{2+}) resulting from phytate breakdown by phytase at a relatively high temperature and humidity (Galiotou-Panayotou et al., 2008). The resultant pectates formed in the middle lamella region due to pectin binding to divalent ions do not dissolve sufficiently during a reasonable amount of cooking time, leading to failure of cell separation. Cell separation is essential in legume seeds since restricted cell separation results in longer cooking time (Shomer, Paster, Lindner and Vasiliver, 1990).

Gamma-irradiation has the potential to help to alleviate and understand the HTC phenomenon. Gamma rays are able to penetrate food and have enough energy to break chemical bonds in the process (Jeong and Jeong, 2017). Ionizing radiation leads to the degradation of polysaccharides such as starch, cellulose, and pectin by the cleavage of the glycosidic bonds (Cho, Kim and Rhim, 2003). This is important in legumes because the middle lamella contains pectin which is critical for cell separation during cooking (Pond, Church, Pond and Schoknecht, 2005). Gamma-irradiation of between 2-10 kGy has been shown to decrease the cooking time in normal cowpeas (Rao and Vakil, 1985) due to radical induced depolymerisation of the middle lamella pectin which enables easier cell separation during cooking. However, higher doses up to 50 kGy prolong cooking time (Abu and Minnaar, 2009) due to polymer crosslinking. Therefore, the aim of the study is to apply γ-

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irradiation to HTC cowpeas in order to better understand the mechanisms involved in the development of the HTC defect.

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2. LITERATURE REVIEW

2.1 INTRODUCTION

This review discusses the science and mechanisms involved in the development of the hard-to-cook (HTC) defect in legumes. It also reviews the current knowledge on the effects of high temperature and high humidity storage conditions (HTHH) on legume seed in respect of the HTC defect. It also focuses on the principles involved in γ-irradiation and reviews the effects of γ-irradiation on the major components of legume seeds, which have been cited as being involved in the HTC defect.

2.2 STRUCTURE AND CHEMICAL COMPOSITION OF COWPEA SEEDS

Cowpeas are small dicotyledonous legume seeds which vary in shape (kidney or globular) (Taiwo, 1998) and have a 100 seed weight of 2-28 g (Langyintuo, Ntoukam, Murdock, Lowenberg-Deboer and Miller, 2004). The two major structural components of cowpea seeds are the seed coat and the cotyledon (Figure 2.1). The cowpea seed coat like other typical legumes contains numerous specified tissues, which include the micropyle, hilium and raphe (Ma, Cholewa, Mohammed, Reterson and Gijzen, 2004). The seed coat (testa) serves as a protective barrier of the cotyledons from the external environment. The seed coat is rich in minerals such as magnesium, iron, calcium, zinc, potassium, and iron as well as phytate, tannins, and phenolics (Adebooye and Singh, 2007). The colour of the seed coat may vary depending on the phenolic compounds present. The range of colours is wide, and includes red, brown, purple, white and black, with the most common being white, brown and a combination of both (Taiwo, 1998; Affrifah, Chinnan and Phillips, 2005). The cowpea seed

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coat can be tightly bound to or loosely bound to cotyledons. The surface of the seed coat can be rough, smooth or wrinkled (Olapade, Okafor, Ozumba and Olatunji, 2002).

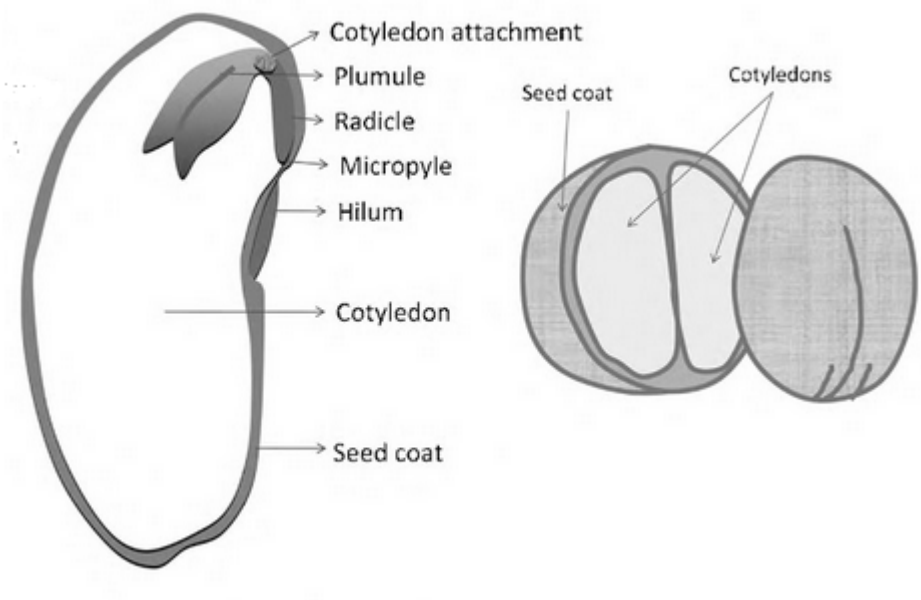

Figure 2.1 A general structure of a cowpea seed (from Tiwari and Singh, 2012)

The cowpea seed like all legumes contains two cotyledons protected by the seed coat. The cotyledons form the major part of the seed with respect to both weight and volume (Sefa-Dedeh, Stanley and Voisey, 1979). The cotyledons consist of large parenchyma cells, which range in size from 70 - 100 µm (Tiwari and Singh, 2012). The parenchyma cells are bound together by the cell walls and middle lamellae. The middle lamella contains pectic substances and protein which help bind adjacent cells together. The parenchyma cells contain starch granules and storage protein. The starch granules are embedded in a protein matrix and the protein bodies are surrounded by a membrane of lipoprotein (Figure 2.2) (Liu, McWatters, Phillips, 1992). The protein bodies contain crystalline inclusions known as globoids that are rich in phytin. The structure and composition of the cotyledons are of importance as they influence the cooking quality of the seeds.

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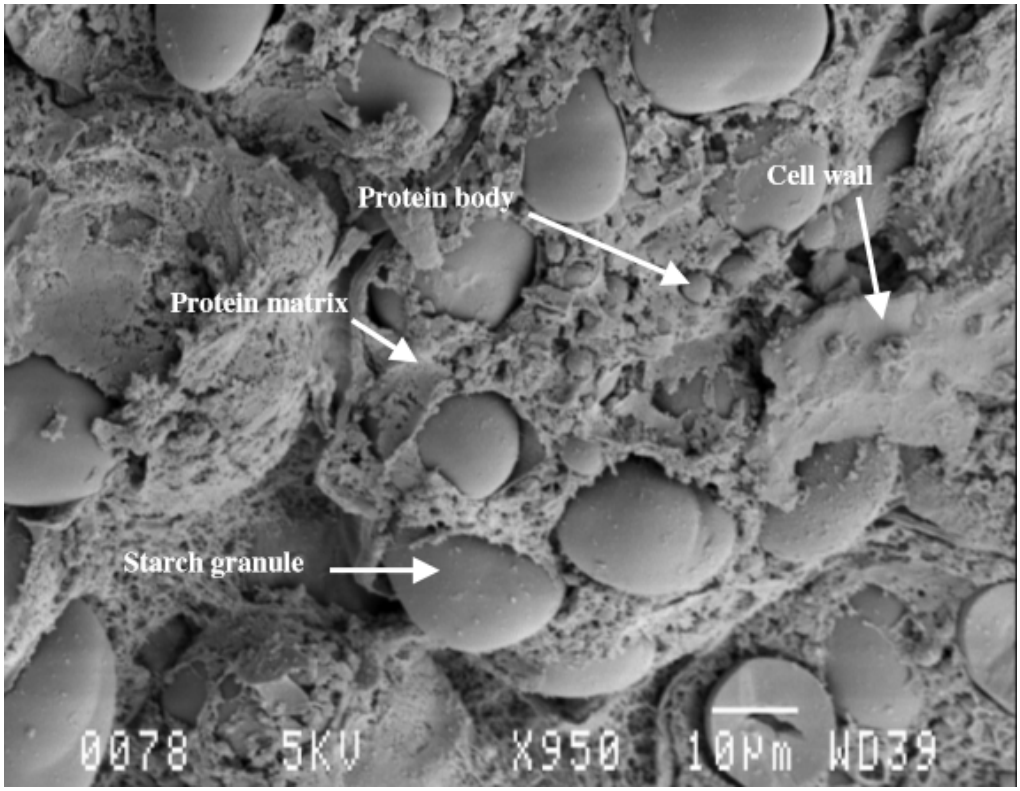

Figure 2.2 SEM of a cross section of tempered (41% moisture) Bechuana White cowpea seed cotyledon (Phadi, 2004)

The proximate composition of cowpea seeds varies according to variety, climate, growth location and agriculture practices (Hsieh, Pomeranz and Swanson, 1992). Cowpeas are not only an important source of proteins and carbohydrates but are also a good source of several B-complex vitamins, minerals and dietary fibre (Table 2.1). In addition, they are a rich source of minerals, such as calcium, magnesium, potassium, and phosphorus.

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Table 2.1 Proximate, mineral and vitamin content of fresh cowpeas (Blackeyes)

Nutrient	Unit	Value per 100 g
Proximates		
Water	G	11.95
Energy	kJ	336
Protein	G	23.52
Total lipid (fat)	G	1.26
Carbohydrates, by difference	G	60.03
Fibre, total dietary	G	10.6
Sugars, total	G	6.90
Minerals		
Calcium, Ca	Mg	110
Iron, Fe	Mg	8.27
Magnesium, Mg	Mg	184
Phosphorus, P	Mg	424
Potassium	Mg	1112
Sodium, Na	Mg	16
Zinc, Zn	Mg	3.37
Vitamins		
Vitamin C, total ascorbic acid	Mg	1.5
Thiamin	Mg	0.23
Riboflavin	Mg	0.226
Niacin	Mg	2.075
Vitamin B-6	Mg	0.357
Folate, DFE	µg	633
Vitamin B-12	µg	0.00
Vitamin A, RAE	µg	3
Vitamin A, IU	IU	50
Vitamin E (alpha-tocopherol)	Mg	0.39
Vitamin D (D2 + D3)	µg	0.0
Vitamin D	IU	0
Vitamin K (phylloquinone)	µg	5.0
Lipids		
Fatty acids, total saturated	G	0.331
Fatty acids, total monosaturated	G	0.106
Fatty acids, total polyunsaturated	G	0.542
Fatty acids, total trans	G	0

Adapted from the USDA Nutrient Database for Standard Reference (2016)

*RAE- Retinol activity equivalents, DFE-Dietary folate equivalents

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2.2.1 LEGUME COTYLEDON CELL WALL STRUCTURE AND COMPOSITION

Plant cell walls are dynamic structures, composed of complex polysaccharides (cellulose, hemicellulose, and pectin), small amounts of phenolic compounds (lignin and hydroxycinnamic acids esterified to the cell wall polysaccharides) and proteins (extensin and enzymes) with ionic and covalent linkages stabilising its components (Rodionova and Bezborodov, 1997). An understanding of the cell wall polysaccharide structure and composition is essential in order to understand the changes in the structural organisation of the polysaccharides that occurs during cooking. The mature cotyledon cell walls of pulses typically contain about 25-30% cellulose, 15-24% hemicelluloses, 0.4-0.6% lignin and 28-41% pectin (Tiwari and Singh, 2012). Structural proteins constitute about 1-5% on dry basis (Ochoa-Villarreal, Vargas-Arispuro, Aispuro-Hernández and Martínez-Téllez, 2012). The rigidity of the pulse cell wall is brought about by cellulose and hemicellulose polymers, with pectin providing fluidity through the gelatinous polysaccharides matrix, ensuring a strong yet dynamic and flexible properties of the cell wall (Ochoa-Villarreal et al., 2012). Cellulose and hemicelluloses are embedded in the amorphous pectin polymers with stability provided by the proteins and phenolic compounds (Sorieul, Dickson, Hill and Pearson, 2016). Hemicelluloses are bound to the surface of the cellulose network preventing direct contact among microfibrils, and pectin is linked to hemicelluloses forming a gel phase (Ochoa-Villarreal et al., 2012).

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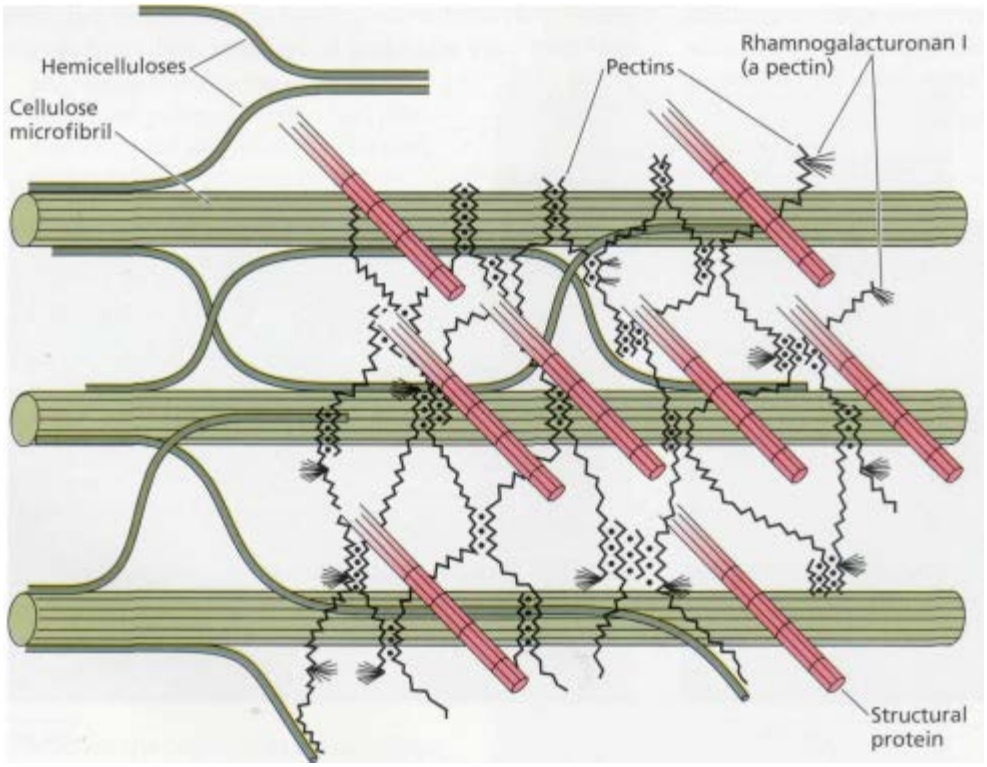

Figure 2.3 Schematic diagram of the major structural components of the plant primary cell wall and their likely arrangement (from Brett and Waldron, 1996)

2.2.1.1 CELLULOSE

Cellulose is the major biopolymer which provides structural support in the plant cell wall (Figure 2.3). Cellulose comprises a third of the total mass of the plant cell wall (Ochoa-Villarreal et al., 2012). It is a linear unbranched polymer composed of β-1,4-linked glucan chains organised in more or less crystalline microfibrils. The cellulose polymers are associated with each other via hydrogen bonding and van der Waals forces.

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2.2.1.2 HEMICELLULOSES

Hemicelluloses can be defined as cell wall non-starch polysaccharides that are not solubilised by hot water or chelating agents but are solubilised by aqueous alkali (Rose, 2003). In dicotyledonous plants, these include several polymers, mainly xylans, xyloglucans, and glucomannans which are characterised by having a backbone of β-1,4-linked sugars with an equatorial linkage configuration (Scheller and Ulvskov, 2010). The structural similarity of hemicelluloses and cellulose allows for the formation of strong non-covalent associations between the two types of polysaccharides.

Xyloglucans

Xyloglucans are heteropolysaccharides made up of a repeated structure of different monosaccharides. These are the predominant hemicellulosic polysaccharides in the primary cell walls of dicotyledons. Xyloglucans have a 'cellulosic backbone' consisting of 1,4-linked β-D-glucose, with several regularly spaced xylose side chains with D-xylose at C6 (α) for most glucose residues (Hedley, 2001). Disaccharides (α-L-fucose-1,2-β-D-galactose) and sometimes β (1→2)-linked L-arabinose may substitute some of the xylose residues (Gibeaut and Carpita, 1994). Cellulose and xyloglucans are found in equal proportions in Type I plant cell walls, which is predominant in dicotyledonous plants. Some xyloglucan chains play a role in supporting rigidity and cell maintenance, being crosslinked to cellulose microfibrils and pectin polymers (Ochoa-Villarreal et al., 2012).

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Xylans

Xylans are a heterogeneous group of polysaccharides with a backbone of β-(1→4)-linked xylose residues (Heinze, Koschella and Ebringerova, 2004). The substitutions which may occur vary widely in composition and distribution according to plant species. They may have substitutions with α-(1→2)-linked glucuronosyl and 4-O-methyl glucuronosyl residues (Ebringerova and Heinze, 2000). Xylans generally contain many arabinose residues attached to the backbone, and these are known as arabinoxylans and glucuronoarabinoxylans (Wertz and Bédué, 2013). Primary cell walls of dicotyledonous plants have small amounts of glucuronoarabinoxylans, while the endosperm of cereals has high amounts of arabinoxylans (Brett and Waldron, 1996). Xylan polymers in the cell wall can crosslink themselves through ferulic acid residues (De O Buanafina, 2009) and also be involved in crosslinking cellulose microfibrils and lignin (Imamura, Watanabe, Kuwahara and Koshijima, 1994; Balakshin, Capanema, Gracz, Chang and Jameel, 2011). Xylan polymers have a significant influence on the integrity of cell wall due to this immense cross-linking (Faik, 2013). Xylans have been documented as a constituent of legume seed cell walls (Stolle-Smit, Beekhuizen, van Dijk, Voragen and Recourt, 1995; Shiga and Lajolo, 2006).

Mannans and Glucomannans

The β-(1,4)-linked polysaccharides rich in mannose or with a backbone consisting of mannose are referred to as mannans and galactomannans (Boraston, Lammerts van Bueren, Ficko-Blean and Abbott, 2007). However, if the polysaccharide consists of mannose and glucose in a nonrepeating pattern, they are the glucomannans and galactoglucomannans. Mannans and glucomannans are often acetylated (Zhang, Rogowski, Zhao, Hahn, Avci, Knox

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and Gilbert, 2014). The mannans are present in low concentration in the plant primary cell wall of dicotyledons (Liepman, Nairn, Willats, Sørensen, Roberts and Keegstra, 2007).

2.2.1.3 PECTIN

Pectins are heterogenous cell wall polysaccharides characterised by a high content of α-(1,4)-D-galacturonic acid residues (Broxterman and Schols, 2018). In the primary plant cell wall, the pectic polysaccharides which can be detected in the cell wall include homogalacturonan (HG), xylogalacturonan (XYA), apiogalacturonan, rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) (Figure 2.4) (Harholt, Suttangkakul, and Scheller, 2010). The ratio between HG, XGA, RG-I, and RG-II varies in the cell wall, but typically the most abundant polysaccharide is HG, which constitutes about 65% of the pectin, with RGI representing 20% to 35% (Mohnen, 2008). The primary role of pectin in the plant cell wall in concert with other polymers is to provide physical strength and provide a barrier to the outside environment (Harholt et al., 2010). Pectin polysaccharides constitute a major part of the middle lamella, providing the principal adhesion between adjacent cells in legume seeds (Hoondal, Tiwari and Tewari, 2002). An understanding of pectin structure and composition is thus important to be able to understand and explain changes which occur during cooking and in textural defects that affect legume seeds.

The methods used to study pectin involve sequential extraction to acquire the different fractions. Pectins can thus be classified according to their method of extraction, with chelator soluble pectins (CSPs)/ cyclohexane-trans-l,2-diamine tetraacetate (CDTA) soluble pectin fractions representing the pectin which is ionically bound to Ca^{2+} (Brummell, 2006). The CSPs are enriched in homogalacturonan in the middle lamella (Brummell, 2006). Alkali can

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also be used in pectin extraction to isolate sodium carbonate-soluble pectins (SSPs) (Brummell, 2006). These pectins show characteristically high ratios of neutral sugars to uronic acid, suggesting enrichment in rhamnogalactorunan I (RG-I) from the primary wall (Brummell, 2006). Water soluble pectins (WSP) represent the freely soluble pectin in the apoplast which can be extracted using water (Redgwell, Melton and Brasch, 1992).

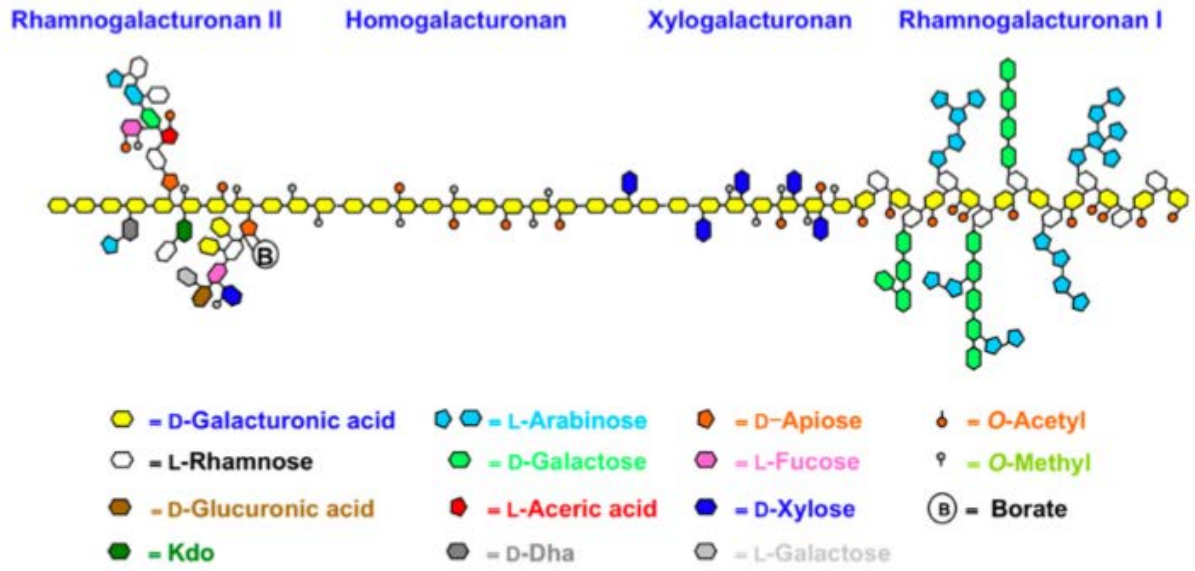

Figure 2.4 Schematic structure of pectin in the plant cell walls. Pectin consists of four different types of polysaccharides, and their structures are shown (Harholt et al., 2010)

Homogalacturonan (HG)

HG is the major pectin polymer in the primary cell walls of dicotyledonous plant seeds (Figure 2.4). It is a linear polymer made up of 1,4-linked α-D-galacturonic acid residues, with some of the carboxyl groups partially methyl-esterified at C-6 and acetyl-esterified at positions O-2 and/or O-3 (Ochoa-Villarreal et al., 2012). HG units with more than 50% of galacturonic acid residues esterified with methyl (or methoxy) are referred to as high methyl-

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esterified HGs and those with less as low methyl esterified HGs. Methyl esterification of HGs usually gets more attention as it influences the physical properties of pectin (Wolf, Mouille, and Pelloux, 2009). HG that is unmethylated has a negative charge and may ionically interact with Ca^{2+} to form a stable gel, which is associated with an increase in cell wall strength (Willats, McCartney, Mackie and Knox, 2001). This occurs when greater than 10 consecutive unmethylated esterified galacturonic acid residues are coordinated and is sometimes referred to as the egg-box model (Caffall and Mohnen, 2009). The egg-box model explains the gelation mechanism by which low methoxyl pectin forms a gel in the presence of calcium. Approximately 70% of pectin in plant cell wall is bound to Ca^{2+} (Jarvis and Apperley, 1995).

Rhamnogalacturonan-I (RGI)

RG-I is a group of pectin polysaccharides characterised by a backbone of alternating galacturonic acid and rhamnose residues $[\alpha\text{-}(1,2)\text{-D-GalA-}\alpha\text{-}(1,4)\text{-L-Rha}]_n$, consisting of large linear or branched arabinose and galactose as the main side chains (Harholt et al., 2010). The side chains are diverse depending on plant source and may include α-L-fucose; β-D-glucuronic acid and 4-O -methyl, as ferulic and coumaric acid. They represent the second most abundant pectin in the plant cell wall. RG-I has been suggested to function as linkage support to other pectic polysaccharides HG and RG-II, which are covalently attached as side chains (Ochoa-Villarreal et al., 2012). According to Zykwinska, Ralet, Garnier and Thibault (2005), RG-I can hydrogen bond to cellulose through some high molecular weight side chains, especially those rich in arabinose and/or galactose.

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Rhamnogalacturonan-II (RG-II)

RG-II is the most branched and complex group of pectic polysaccharides. In dicotyledonous plant cells, they are a minor pectic component and represent only 0.5 to 0.8% (Matsunaga, Ishii, Matsunamoto, Higuchi, Darvill, Albersheim and O'Neill, 2004). RG-II comprises a backbone consisting of at least eight α-GalA residues to which five different types of side chains are attached (Ulvskov, 2010). The cluster of side chains is linked to positions O-2 and O-3 on galacturonan backbone (Harholt et al., 2010). The side chains contain rare and unique glycosyl residues and glycosidic linkages (e.g 2-O-methyl-1-Fuc, L-aceric acid and α-1, 3-xylofuranose) (Harholt et al., 2010). RG-II exists in primary walls mainly as a dimer cross-linked by 1:2 borate-diol ester (Ishii, Matsunaga, Pellrin, O'Neill, Darvill and Albershiem, 1999). Almost 95% of RG-II polymers exist as dimmer complex form (dRG-II). The dimerization helps to ensure the integrity of the cell wall (O'Neill, Ishii, Albersheim and Darvill, 2004).

Xylogalacturonan (xyg) and apiogalacturonan (apa)

XYG is a group of pectic polysaccharides consisting of a HG backbone substituted by xylose residues at carbon 3 (O'Neill and York, 2003). In addition, xylose residues can connect to the first substituted xylose with β-1-4 linkage (Zandeleven, Beldman, Bosveld, Schols and Voragen, 2006). It is also a minor component of the cell wall, constituting less than 10% (Zandeleven et al., 2006; Mohnen, 2008). However, XYG was suggested to be in a higher proportion than HG in legumes such as common beans (*Phaseolus vulgaris)* (Shiga and Lajolo, 2006). XYG is present in peas (Le Goff et al., 2001) and soybeans seeds (Huisman, Brüll, Thomas-Oates, Haverkamp and Schols, 2001). APA is a group of pectin

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polysaccharides which are actually HG substituted by D-apiose. On the HG chain, apiose residues can be β-2-linked, 3-linked as well as 2- and 3-linked to single galacturonan residues (Wertz and Bédué, 2013).

2.3 THEORIES ON MECHANISMS RESPONSIBLE FOR THE HARD-TO-COOK DEFECT

Several mechanisms have been proposed to explain HTC development in legumes, with the earliest hypothesis dating back to 1946. The mechanisms involve both enzymatic and non-enzymatic processes, which affect the cotyledons, resulting in limited cell separation during cooking of legumes. The next section will explore the different mechanisms and the studies which are associated with each of the hypotheses.

2.3.1 PHYTASE-PHYTATE-PECTIN HYPOTHESIS

As stated, the most widely accepted theory for HTC development in legumes is phytase-phytate-pectin hypothesis (Galiotou-Panayotou et al., 2008), which relies on observations made by Sante Mattson in 1946. According to this theory, storage of legumes under HTHH conditions stimulates phytase enzyme activity, which hydrolyses phytate, the storage form of phosphate in seeds, releasing divalent ions which are bonded to the phosphate group. These cations (Ca^{2+} and Mg^{2+}) then migrate to the middle lamella where they crosslink pectic substances (Figure 2.5). The crosslinking of pectic substances prevents the dissolution of middle lamella during cooking. Pectin, a polymer of galactunoronic acid, cements the plant cells together (Yousif, Kato and Deeth, 2007) and exists mainly in a water-soluble form (Galiotou-Panayotou et al., 2008), permitting water uptake by the legume seeds during cooking. It is believed that pectin becomes more reactive after exposure to HTHH conditions

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as a result of the action of the pectin esterase enzyme, which demethylates the molecule, leaving a carboxyl group that can enter into crosslinking reactions (Mattson, 1946). Cross-linked pectins (calcium and magnesium pectates) in the middle lamella do not solubilize during cooking, leading to unseparated cells (Liu and Bourne, 1995). The “egg box model” is commonly used to explain how the cations bind pectin (Figure 2.6).

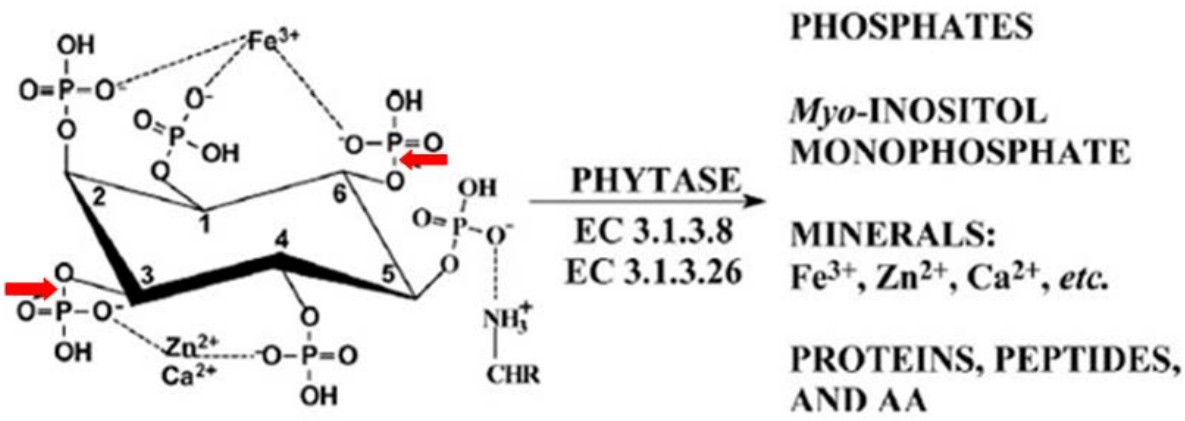

Figure 2.5 Summary of the reactions catalyzed by the enzymes 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The products released apart from inorganic P and myo-inositol monophosphate can include metal divalent and trivalent ions, proteins, peptides, and amino acids (AA). The red arrows indicate the ester bonds which are initially attacked by the 3- and 6-phytase, respectively (Yu et al., 2012).

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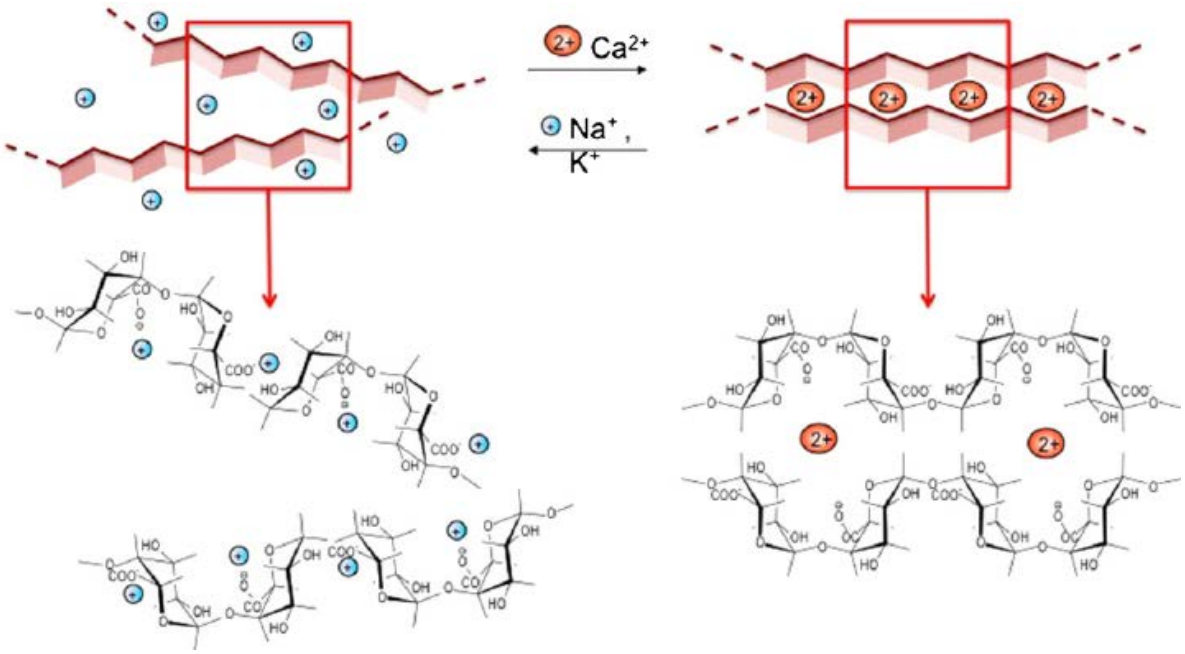

Figure 2.6 Egg box gelation mechanism of pectin: the carboxyl groups of pectin backbone form polyelectrolyte complexes with polyvalent cations (i.e. calcium ions) (Munarin, Tanzi and Petrini, 2012)

Most published research shows a relationship between phytate levels and the development of the HTC defect. Several authors have reported an increase in cooking time of legume seeds stored under HTHH conditions with a decrease in phytate levels (Coelho, de Mattos Bellato, Santos, Ortega and Tsai, 2007; Ndungu et al., 2012). Also in support of the phytase-phytate-pectin hypothesis, is that during the development of the HTC defect Ca^{2+} and Mg^{2+} have been shown to move to concentrate around cell wall-middle lamella (Garcia, Lajolo and Swanson, 1993; Kruger, Minnis-Ndimba, Mtshali and Minnaar, 2015) where they most probably bind pectin. Since the phytase-phytate-pectin theory also implicates insolubilisation of pectin during HTC development the decrease in water soluble pectin (WSP), which has been reported in legumes during the development of the HTC defect (Shomer et al., 1990; Shiga, Cordenunsi and Lajolo, 2009; Ndungu et al., 2012) could be related to pectin insolubilisation. Furthermore, to support this, an increase in molecular mass of WSP in HTHH stored legumes compared to control legumes has been reported (Shiga et al., 2009). However, drawbacks to

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phytase-phytate-pectin hypothesis have been related to the fact that other authors such as Njoroge, Kinyanjui, Makokha, Christiaens, Shpigelman, Sila and Hendrickx (2015) have reported minimal changes in chelator soluble pectin (CSP) in common beans, which is the pectin found associated with the divalent ions. Moreover, the same authors reported little to no changes in the degree of methylation (DM). DM should decrease during HTC development if the demethylation of carboxyl groups occurs. However, that was not the case in their study. Interestingly, with another pectin fraction, alkali-soluble pectin, the same authors found it actually to increase after HTHH storage in common beans. In another study, Shiga et al. (2009) also reported the increase in alkali-soluble pectin with HTHH storage. This may also possibly explain the reduction in soluble pectin that is observed during HTC development. Although some of the changes that the phytase-phytate-pectin hypothesis proposes may indeed occur, it appears that the mechanism may not solely be responsible for the HTC defect.

2.3.2 LIGNIFICATION HYPOTHESIS

Lignification is also one of the mechanisms postulated to be responsible for the development of HTC defect in legumes. According to the hypothesis, aromatic compounds migrate from the seed coat to cell wall surfaces where they act as precursors in reactions associated with lignification (Stanley, 1992). HTHH storage conditions have been shown to increase the number of free phenolics within legume seeds (Machado, FerruziI and Nielsen, 2008). Phenolic compounds in plants usually occur in their conjugated form, with one or more sugar residues linked to a hydroxyl group (Strack, 1997). Mechanisms by which phenolic compounds condense are thought to be both enzymatic and non-enzymatic (Stanley, 1992; Shoji, 2007). In the enzymatic pathway, peroxidase polymerizes the lignin precursors into

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lignin which is deposited into the cell wall causing it to thicken (Figure 2.7) (Mayer and Staples, 2002).

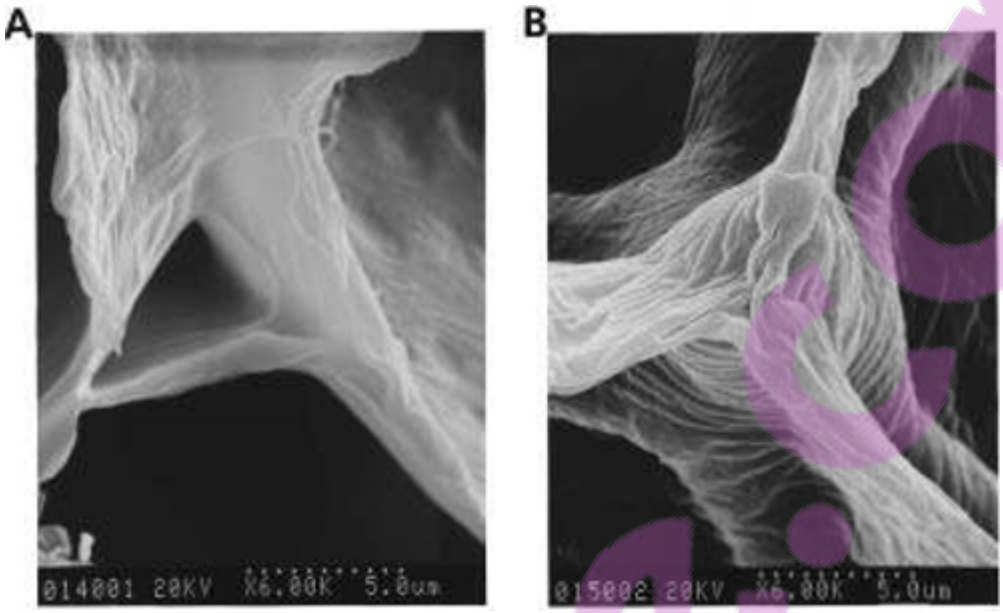

Figure 2.7 Scanning electron micrographs showing evidence of lignification with HTC defect development (A) control and (B) HTC bean cotyledons (Garcia et al., 1998).

The lignification (thickening) of legume cell walls is presumed not to allow the cotyledon cells to separate, and since it is impermeable to water, it does not allow the legume cells to absorb water, thus increasing the cooking time required to produce cooked seeds (Swanson, Hughes and Rasmussen, 1985). The cell wall is believed to become hydrophobic and impermeable to water due to the lignin matrix deposited around the polysaccharides (Medoua and Mbofung, 2006). Furthermore, the lignin synthesis which occurs in legume seeds is postulated to be a stress response to prevailing adverse conditions (Hincks and Stanley, 1987; Martin-Cabreja et al., 1997).

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Hincks and Stanley (1986) studied lignification in common beans using scanning electron microscopy (SEM) and found adhesion between cells was greater in HTC beans. They attributed the cellular changes to soluble phenolic compounds which can convert to lignin. Garcia et al. (1998) found an increase in phenolic compounds linked with the soluble pectin fraction. They related the increase in phenolics bound to the soluble pectin and their probable involvement in cross-linkages to changes in cell wall associations which affect cell wall separation during cooking. Similarly, Maurer, Ozen, Maurer and Nielsen (2004) found an increase in phenolic compounds in soluble pectin fraction of common beans. The consequence of the increased phenolics was reduced cell wall separation. However, with faba beans stored for 12 months, Nasar-Abbas, Plummer, Siddique, White, Harris and Dods (2008) observed a reduction in total phenolic compounds with increase in storage temperature especially $\geq$ 37 °C.

Common beans stored at 25 °C and 70% relative humidity (RH) were found to have increased lignified protein in the cotyledons (Molina, Baten, Gomez-Brenes, King and Bressani, 1976). These findings were corroborated by Martin-Cabrejas et al. (1997) who also found increases in lignin and lignified protein in HTC common beans. Furthermore, Nasar-Abbas et al. (2008) also found increases in acid detergent fibre and lignin contents in common beans. However, Srisuma, Hammerschmdt, Uebersax, Ruengsakulrach, Bennink and Hosfield (1989) found an increase in free hydroxycinnamic acids (especially ferulic acid) in common beans which they hypothetically linked to HTC development. The same authors did not detect any significant changes in lignin content in both seed coat and cotyledon during HTHH storage. Similarly, Ndungu et al. (2012) also found no substantial changes in acid detergent lignin content of cowpeas after HTHH storage.

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2.3.3 CHANGES IN STORAGE PROTEIN HYPOTHESIS

During the development of the HTC defect changes in protein occur, and a decrease in protein digestibility has been found (Tuan and Phillips, 1991; Nyakuni, Kikafunda, Muyonga, Kyamuhangire, Nakimbugwe and Ugen, 2008). According to the changes in storage protein hypothesis, under HTHH storage conditions, proteins are subjected to the action of proteases which hydrolyse the protein into smaller polypeptides and tyrosine. Such an increase in small polypeptides and free aromatic amino acids has been shown when legumes are stored under high temperature and high relative humidity (Hohlberg and Stanley, 1987). The small polypeptides and aromatic compounds migrate to the middle lamella of the legume seeds where they act as lignin precursors and are converted to insoluble lignin compounds by the activity of peroxidase. The aromatic amino acids which build up due to enzymatic hydrolysis of storage protein can enter the shikimate pathway, which is a major pathway of phenol biosynthesis (Tzin, and Galili, 2010). The lignin compounds remain deposited within the middle lamella causing it to become thicker, reducing the ability of the surrounding cells to separate and absorb water efficiently during cooking. The seeds exhibit the characteristic hardening associated with the HTC defect as a result of reinforced middle lamella (Hohlberg and Stanley, 1987). Such alterations to the legume protein were corroborated by the work of Hussain, Watts and Bushuk (1989), who found changes in the electrophoretic pattern of common bean cotyledon proteins under HTHH storage. In a recent study which focused on common beans, Parmar, Singh, Kaur, Virdi and Shekani (2017) reported an increase in low molecular weight proteins. The authors also observed an increase in protein β-sheet structures, which resulted in higher stability of pastes formed from their proteins.

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2.3.4 MEMBRANE DEGRADATION HYPOTHESIS

The other postulated mechanism to be important in HTC development is cell membrane deterioration. Biological cell membranes play a vital role as they help to maintain the structural integrity, organization, and flow of material through membranes (Waston, 2015). These functions are impaired if membranes lose their critical permeability (Stanley, 1991). Legumes stored under HTHH conditions have been shown to exhibit increased loss of solids and electrolyte leakage during soaking as compared to those treated more mildly, which suggests membrane disruption (Stanley and Aguilera, 1985; Richardson and Stanley, 1991; Liu, McWatters and Phillips, 1992). When legumes are stored under adverse conditions (HTHH), membrane lipids are hydrolysed by phospholipases to produce free fatty acids. The free fatty acids are susceptible to the action of lipoxygenase, which then converts them into free radicals. The free radicals formed destroy the membrane integrity by acting as destabilizing agents, altering the permeability (allowing solutes to leach out) and efficacy of the membrane tissues (Stanley, 1991). Kruger et al. (2015) reported on a novel *in situ* evaluation of the role of minerals in HTC development in cowpea, using proton induced X-ray emission (PIXE) spectrometry. They showed the movement of minerals towards cotyledon cell wall-middle lamella of HTC cowpeas. They suggested the movement of minerals was only possible after degradation of membranes, as also earlier suggested by Richardson and Stanley (1991).

2.3.5 PROTEIN-STARCH INTERACTIONS HYPOTHESIS

The starch granules in legumes are surrounded by a matrix of protein bodies (Phadi, 2004). Liu (1997) developed a hypothesis concerning the interactions of starch and protein in HTC

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seeds. In this hypothesis, the hardness of HTC seeds is attributed to partial gelatinisation of starch granules during cooking. In normal seeds, the proteins are highly water soluble and relatively thermal stable. This means that during cooking the proteins do not coagulate easily at a temperature lower than that of starch gelatinisation. This allows the embedded starch granule to absorb water freely through the native protein matrix, and starch swelling prevails over protein coagulation during cooking. However, during HTHH storage, it has been suggested that proteins become denatured, resulting in a decrease in their water solubility and a reduction in thermal stability occurs (Hohlberg and Stanley, 1987; Liu et al., 1992). The water soluble proteins which remain, readily coagulate at temperatures below that of starch gelatinisation because of their relative reduced heat stability. Protein coagulation prevails, resulting in restricted water absorption by starch and reduced starch swelling in HTC seed (Liu, 1997).

Garcia and Lajolo (1994) studied starch from HTC beans and reported an increased birefringence using light microscopy, increased resistance to amyloglucosidase attack and a higher degree of crystallinity. In addition, starch granules isolated from HTC beans (5 years storage) showed an increase in gelatinisation temperature compared to those from beans which had not been subjected to HTHH conditions. In another study, Parmar et al. (2017) isolated starch from HTC common beans and reported a lower proportion of small size starch granules. However, despite the findings from several studies on the starch in HTC legume seeds, Garcia and Lajolo (1994) suggested that the changes in starch during bean storage were an outcome of the HTC development rather than a mechanism of how the HTC defect develops. Similarly, Hohlberg and Stanley (1987) studying HTC black beans, despite observing an increase in starch gelatinisation temperature also suggested that the changes were not related to HTC development.

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2.3.6 MULTIPLE MECHANISMS HYPOTHESIS

Most studies have tried to explain the development of HTC defect by a single mechanism, but it is becoming evident that multiple mechanisms may be responsible. The multiple mechanism theory is based on both the reversible and irreversible components (Del Valle and Stanley, 1995). The authors propose that storage temperature is responsible for the irreversible component, and water activity is responsible for the reversible component. In the multiple mechanism theory, membrane degradation is thought to be part of the first mechanism which occurs before the other mechanisms. Phytate loss is considered as a minor contributor during the initial storage of the legumes and oxidation of phenolic compounds is regarded as a major contributor during extended storage (Hincks and Stanley, 1986). The mechanism proposes a reversible hardening caused mainly by phytate hydrolysis, resulting in its failure to chelate divalent ions which migrate to the middle lamella region where they cross link pectin. The irreversible hardening is as a result of deposition of lignin-like material, which strengthens the cell wall, and impairs water imbibition during cooking and soaking (Stanley, 1992).

2.4 IRRADIATION

Food irradiation can be defined as a process of exposing food to ionising irradiation to improve the safety and extend the shelf-life of food by reducing or eliminating microorganisms and insects (Li-Chan, Griffiths and Chalmers, 2010). Ionising irradiation can remove electrons from atoms and molecules converting them to ions (Ray and Bunia, 2013), which are inherently unstable and therefore reactive. The term ionising irradiation is used to describe many different types of rays; however, only specific types of irradiation are suitable to be used in food. Ionising irradiation like α-particles is not preferred due to its low

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penetrating power, while high energy electrons or X-rays generated above certain energy level may cause food to be radioactive. In considering the above challenges, a joint committee comprising the World Health Organization (WHO), Food and Agriculture Organization (FAO), International Atomic Energy Agency (IAEA) approved only the following types of ionising radiation for use in foods (WHO, 1981):

a) Gamma-rays from ^{60}Co and ^{137}Cs at energy levels of 5 MeV (1 eV = 1.6 x 10^{-19} J)

b) X–rays generated from machine sources at or below energy levels of 5 MeV, and

c) Electrons generated from machine sources at or below an energy level of 10 MeV

The interaction of irradiation energy and matter is complex and can depend on various factors such as the type of irradiation, the composition of absorbing material, temperature, the energy of irradiation and atmospheric environment (Al-Assaf, Coqueret, Dahlan, Sen, and Ulanski, 2016). The effect of irradiation on food can be either direct (Primary) or indirect (Secondary). The indirect effects involve the transfer of energy from the incident radiation to the food material, and the changes which occur after irradiation are as a result of the absorbed energy (Urbain, 1986). Concerning the indirect effects, the radiation energy first interacts with other food components such as water and the resultant free radicals interact with the food molecules.

2.4.1 PRIMARY CHEMICAL EFFECTS

The application of ionising radiation leads to all or part of its energy being absorbed by the food material. The energy absorbed into the food material results in the production of ions as

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well as excited atoms. Excited atoms have an electron that has been raised to energy comparable to or higher than the ionisation potential (Connerade, 1998). Excitation normally occurs when the amount of energy absorbed is low. The excitation of an atom in a molecule leads to recognition of the whole molecule as excited. They are two mechanisms for the production of an excited molecule, as reviewed by Urbain (1986):

1) Direct $A \rightarrow A^*$

2) Neutralisation of ions:

During the time of ionisation an excited ion $(A^+)^*$ may be formed $A \rightarrow (A^+)^* + e^-$

neutralisation occurs leading to the formation of an excited molecule A*

$$(A^+)^* + e^- \rightarrow A^*$$

After the excitement, the excited molecule retains its attained energy for a period of the order 10^{-8} sec, before the loss of excitation energy. However, if sufficient energy from irradiation is provided, it can cause ionisation of atoms. This is when an electron is ejected from the atom

$$RH \rightarrow RH^+ + e^-$$

The primary effects are the indirect consequence of radiation energy on the food material that has absorbed it. Primary effects are non-specific and can strike any molecule that is in the track of ionising radiation, interacting with any molecule or atom without preference. The changes that occur in a food material due to the interaction of primary products with themselves and or other components of the absorber are identified and termed as indirect effects of irradiation. The composition of the absorber affects the relative importance of the effects. Furthermore, the conditions under which irradiation occurs, influence the final change.

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2.4.2 SECONDARY EFFECTS

The net result of the primary chemical effects is the formation of new chemical compounds and also possibly, free radicals. The free radicals are short lived (Wasik and Bushuk, 1973) and have a short lifetime, usually less than 10^{-3} seconds (Hall, Hutter and Noble, 1963). Further chemical change, however, remains possible and is governed by the composition of the absorber and by other factors such as its physical state and temperature (Urbain, 1986). The products of primary effects and the unchanged molecules of the food can both interact with the formed free radicals from primary effects. The free radicals that form after irradiation have greater kinetic or excitation energy than the thermal energy of the absorber material/food. Although some free radicals have been observed to persist in some systems indefinitely, the radicals are usually highly reactive via reactions such as additional, combination, dissociation, rearrangement, disproportion and electron transfer (Urbain, 1986).

2.4.3 EFFECTS OF γ-IRRADIATION ON MAJOR COMPONENTS OF FOOD

2.4.3.1 CARBOHYDRATES

The effects of irradiation on carbohydrates are complex and can result in numerous possible radiolytic products. The major reactions involved are hydrolysis and oxidative degradation (Ibarz, 2008). In solid starch, bond breakage is centred on hydrolysis of the glucosidic bonds, leading to depolymerisation, and eventually to radicals centred on the C-1 and C-6 positions in the glucose units (WHO, 1999).

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During irradiation of aqueous systems there is the production of hydroxyl radicals, which is of primary importance in the radiolysis of carbohydrates (Urbain, 1986). The hydroxyl group can abstract hydrogen from C-H bonds forming α-hydroxy and α,β-hydroxyl radicals (Hasselmann and Marchioni, 1991). The α-hydroxy and α,β-hydroxyl radicals formed may disproportionate, dimerise or lose water (Figure 2.8). After disproportionation and/or dehydration a ketone, or aldehyde or an acid may form depending on the position of the C=O bond.

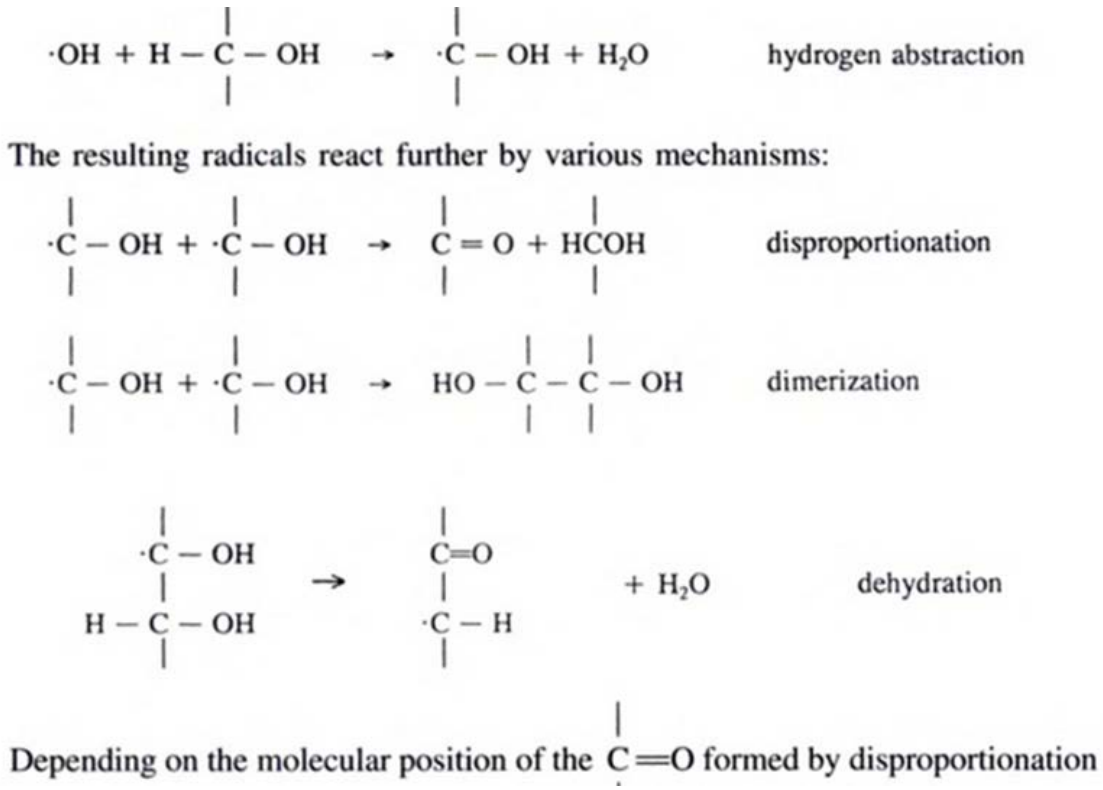

Figure 2.8 Hydroxyl radical reactions in carbohydrates (Diehl, 1995)

2.4.3.2 *Effect of irradiation on functional properties of carbohydrates*

There have been several studies on the effects of irradiation on the functional properties of legume seeds and flours. Gamma-irradiation can modify the physical and chemical properties

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of polysaccharides in foods via the free radical mechanism as previously highlighted. The changes that occur due to γ-irradiation are not visible to the naked eye, but the effects on physicochemical properties are always evident. Bashir and Aggarwal (2016) studied the effects of γ-irradiation (0.5 to 10 kGy) on the physicochemical, thermal and functional properties of chickpea flour, and found a reduction in pasting and swelling properties. Previously, Abu, Muller, Duodu and Minnaar (2005) studied the effects of γ-irradiation on the functional properties of the cowpea flour, they also reported a decrease in the swelling index and pasting properties, while oil absorption capacity increased significantly. The reductions were attributed to the γ-irradiation induced cleaving of the glycosidic bonds in polysaccharides via direct and indirect effects of irradiation. Notably, γ-irradiation fragments the amylopectin component of starch, leading to smaller molecular weight units (Rombo Taylor and Minnaar, 2004). Therefore, since amylopectin is primarily responsible for starch swelling (Tester and Morrison, 1990), the decrease in swelling index could be due to depolymerisation of amylopectin (Abu, Duodu and Minnaar 2006b; Wani, Jabeen, Geelani, Massodi, Saba and Muzaffar, 2014). When porridges (bean and maize) and their flours were irradiated, a dose dependent decrease in viscosity was observed (Rombo, Taylor and Minnaar, 2001). These authors further reported a maximal starch digestibility at 2.5 kGy. However, at higher doses there was a reduction in starch digestibility, and it was more significant in maize than bean flours. The same authors went on to study the effects of γ-irradiation on molecular properties of the starch in maize and bean flours and found an increase in β-bonded starch with increase in irradiation dose. This could explain the reduction in starch digestibility at higher doses.

Gamma-irradiation at 2 kGy has been shown to increase water absorption capacity (WAC) in cowpea starch, but above 10 kGy further increases were not observed (Abu et al. 2005). Similarly, Falade and Kolawole (2013), studying the effects of irradiation on cowpea, found

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an increase in WAC up to 6 kGy and after that, it decreased above 8 kGy. An increase in WAC has also been observed in dry bean starch (Rayas-Duarte and Rupnow, 1993; Gani Bashir, Wani, and Masoodi, 2012) after γ-irradiation. The increase in WAC could be due to starch degradation resulting in the formation of smaller dextrins and sugars with a higher affinity for water (Abu et al., 2006b).

Gani, Bashir, Wani and Masoodi (2012) studied the modification of bean starch by γ-irradiation at doses of 5, 10 and 20 kGy and reported no effect on the starch X-ray diffraction pattern. However, γ-irradiation caused a decrease in starch crystallinity in common beans with increased dose. Rayas-Duarte and Rupnow (1993) studying the effects of low, medium and high irradiation (2.5-20 kGy) on bean starch, did not observe a change in starch crystallinity with γ-irradiation. The authors also studied thermal properties of the bean starch using Differential Scanning Calorimetry (DSC) and found an increase in gelatinisation enthalpy. Gelatinisation generally refers to the disruption of molecular order within starch granules when they are heated above the gelatinisation temperature in the presence of sufficient water (Whistler and BeMiller, 1997). Rayas-Solis (1987) found an increase in gelatinisation temperature of great northern bean starch irradiated at 20 kGy, which was attributed to a more disordered granule structure. In contrast, Chung and Liu (2010) studied the molecular structure and physicochemical properties of irradiated potato and bean starches treated by γ-irradiation up to 50 kGy, and found a decrease in gelatinisation enthalpy that was greater in potato than in bean. However, Abu et al. (2006b) studied starch from irradiated cowpea flours and pastes at doses 2, 10, 50 kGy and observed no effect on enthalpy of gelatinisation in cowpeas after γ-irradiation.

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Gani et al. (2012) studied the modification of bean starch by γ-irradiation at doses of 5, 10 and 20 kGy. Above 10 kGy, as absorbed by SEM, γ-irradiation was found to cause surface fracturing of starch granules. The results were similar to those of Hussain, Wani, Suradkar and Dar (2014) who studied the modification of bean starch by γ-irradiation and found surface cracking and fractures on the surface of starch granules with increase in dose. However, as stated above in studies by Rayas-Duarte and Rupnow (1993) of beans and Abu et al. (2006b) of cowpea, no damage to the starch granules was observed after γ-irradiation.

2.4.3.3 *Protective effects on carbohydrates*

Legumes seeds exist as a matrix containing other components that may protect the carbohydrates from radiolysis during γ-irradiation (Urbain, 1986). Carbohydrates are more sensitive to radiation when in pure form and this was observed when comparing the radiolytic products of pure starch and wheat flour (Thakur and Singh, 1994). Radiolytic products produced when pure starch was irradiated at 5 kGy were equal to products at 50 kGy for wheat flour. When mixtures of sugars and amino acid are irradiated, polymerisation occurs followed by a browning effect (Urbain, 1986). The formation of carbonyl compounds in glucose solution is inhibited by addition of the sulphur-containing amino acids cysteine and methionine. Although proteins are also able to confer a protective effect during irradiation of sugars, they are less effective as compared to individual amino acids. The protective action of amino acids and proteins is due to interfering with the availability of hydroxyl radical to associate with sugar (Urbain, 1986). Different amino acids exert varying extents of protection, among other reasons because some are better scavengers such as sulphur-containing amino acids and because increasing chain length in aliphatic amino acids adds C-H bonds that interact with the hydroxyl radical (Molins, 2001).

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2.4.3.2 PECTIN

Gamma-irradiation can degrade the polymers in the primary plant cell wall, including pectin (D'Amour, Gosselin, Arul, Castaigne, Willemot, 1993). Gamma-irradiation can break the glycosidic linkages of pectin leading to smaller units (Cho, Kim and Rhim, 2003). The effects of γ-irradiation are more pronounced in aqueous pectin than dry pectin due to activity of radiolytic products such as the hydroxyl group (Džamiĉ and Jankoviĉ, 1966). Water soluble pectin in ripening papaya has been shown to increase upon irradiation (Zhao, Moy, and Paull, 1996). Furthermore, irradiation of aqueous pectin solutions causes a decrease in their molecular weight (Ayyad, Hassanien and Ragab, 1990) and viscosity (Zegota, 1999). However, Dogan, Kayacier and Erhan (2007) stated that irradiation does not greatly affect the rheological characteristics of pectin, and pectin can be irradiated for preservation purposes. Chen, Liu, Liu, Li, Liang and Luo (2015) explained that the difference could be due to the different pectin used, and they suggested the need for more research.

2.4.3.3 PROTEINS

The radiation chemistry of proteins is influenced by various factors such as the structure, the state of the protein and the irradiation conditions (Audette-Stuart Houée-Levin and Potier, 2005). Both direct and indirect effects of irradiation affect proteins. With proteins, like all macromolecules (in the presence of water), the indirect effects of irradiation predominate (Al-Assaf et al., 2016). In the absence of water, irradiation causes very few chemical changes in proteins (Diehl, 1990). During irradiation, the following may occur: folding of peptide chains, breaking of intramolecular disulphide bonds and secondary bonding forces such as hydrogen bonds, hydrophobic bonds, ionic bonds, or bonds that maintain subunits together as

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a functional protein (Stewart, 2001). Major permanent reactions that occur in proteins include deamination, decarboxylation, reduction of disulphide bonds, oxidation of sulphydryl groups, modification of amino acid moieties, valency change of coordinated metal ions, peptide-chain cleavage and aggregation (Kuan, Bhat, Patras and Karim, 2013). In the absence of oxygen, amino acids in aqueous solution undergo reductive deamination, hydrogen abstraction or decarboxylation during irradiation (Elias, 1987).

Given the complexity of proteins, changes that occur during irradiation have been studied using individual amino acids and peptides.

Below, are examples of reactions induced by irradiation that involve simple amino acids such as glycine and alanine (Figure 2.9)

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H abstraction: $\cdot OH + H_3\overset{+}{N}CHRCOO^- \longrightarrow H_3\overset{+}{N}\dot{C}RCOO^- + H_2O$

H abstraction: $\cdot H + H_3\overset{+}{N}CHRCOO^- \longrightarrow H_3\overset{+}{N}\dot{C}RCOO^- + H_2$

Reductive deamination: $e^-_{aq} + H_3\overset{+}{N}CHRCOO^- \longrightarrow NH_3 + \dot{C}HRCOO^-$

The radicals so produced can react further

$$2H_3\overset{+}{N}\dot{C}RCOO^- \longrightarrow \begin{array}{l} H_3\overset{+}{N}-CRCOO^- \\ \quad\quad | \\ H_3\overset{+}{N}-CRCOO^- \end{array}$$

diamino acid

$H_3\overset{+}{N}\dot{C}RCOO^- + H_3\overset{+}{N}\dot{C}RCOO^- \longrightarrow H_2\overset{+}{N}{=}CRCOO^- + H_3NCHRCOO^-$

$H_3\overset{+}{N}CHRCOO^- + \dot{C}HRCOO^- \longrightarrow H_3\overset{+}{N}\dot{C}RCOO^- + \underset{\text{fatty acid}}{CH_2RCOO^-}$

$H_3\overset{+}{N}\dot{C}RCOO^- + \dot{C}HRCOO^- \longrightarrow \underset{\text{imino acid}}{H_2\overset{+}{N}{=}CRCOO^-} + \underset{\text{fatty acid}}{CH_2RCOO^-}$

The imino acid can hydrolyse spontaneously to give

$H_2O + H_2\overset{+}{N}{=}CRCOO^- \longrightarrow \overset{+}{N}H_4 + \underset{\text{keto acid}}{RCOCOO^-}$

Decarboxylation may occur leading to the production of an amine with one carbon less than the original amino acids

$H_3\overset{+}{N}CHRCOO^- \longrightarrow H_3\overset{+}{N}\dot{C}HR + CO_2$

Figure 2.9 Reactions induced by irradiation involving simple amino acids glycine and alanine (Molins, 2001)

Since legume proteins are predominantly globular (Marcone, 1999), crosslinking induced by unfolding and aggregation may occur via intermolecular bonds (Delincèe, 2002). At low or mild irradiation doses globular proteins undergo unfolding and at higher doses aggregation

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occurs (Dogbevi, Vachon and Lacroix, 1999). Globular proteins have a tight structure, and radicals formed due to irradiation are held together in positions, which favour recombination reactions (Urbain, 1986). As a consequence, they are more resistant to change. As explained previously, irradiation at higher doses leads to protein crosslinking. These are usually observed as increased viscosity (Cieśla, Stéphane, Monique and Tien, 2004), reduced solubility (Abu, Müller, Duodu, and Minnaar, 2006a) and formation of higher molecular weight proteins (Gennadios, Rhim, Handa, Weller and Hanna, 1998).

2.4.5.1 *Effects of gamma-irradiation on protein functional properties*

Gamma-irradiation has been reported to modify the functional properties of legume proteins. Gamma-irradiation was shown to significantly increase protein digestibility in faba bean seeds (Osman, Hassan and Osman, 2014). A similar increase in protein digestibility was also observed in mucuna beans (Bhat, Sridhar, Chiu-Chung, Arun and Sanjeev, 2008) and cowpea (Tresina and Mohan, 2012). This has been attributed to the destruction of protein inhibitors or enhanced proteolysis resulting from exposure of peptide bonds (Koppelmann, Willem, Nieuwenhuizen, Gaspari, Knippels, Penninks, Knol, Hefle and de Jongh, 2005). Furthermore, increased protein digestibility could also be due to a reduction in phytate–protein complexes, which are less susceptible to proteolytic enzymes than the same protein alone (Ravindran, 1995). Abu et al. (2006a) reported a radical induced reduction in the nitrogen solubility index (NSI) of isolated proteins from cowpea flours and pastes. This was attributed to irradiation induced protein denaturation, which may expose previously buried non-polar sites (Zayas, 1997), leading to a decrease in solubility. Abu et al. (2006a) also observed an increase in protein gel strength with irradiation at 10 and 50 kGy. They proposed that this was due to protein crosslinking. The authors additionally observed that the oil absorption

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capacity (OAC) of isolated cowpea protein increased in a dose dependent manner with irradiation. Similarly, Falade and Adebiyi (2015) studying the effect of *γ*-irradiation on bambara groundnut, reported an increase in OAC at doses of up to 4 kGy. However, the OAC decreased with doses of above 4 kGy up to 10 kGy. The observed increase in OAC may be due, in part, to increases in protein hydrophobicity with increasing irradiation dose, as suggested by Abu et al. (2006a)

2.4.5.2 *Protective effects on proteins*

Proteins, like carbohydrates, are protected from radiolysis by other food components, which can make the effects of γ-irradiation on proteins practically negligible (Swallow, 1991). Hence, most of the effects of radiation energy lead to protein denaturation rather than the destruction of the individual amino acids themselves (Hasselmann and Marchioni, 1991). Since most studies are not done in environments that are similar to food matrixes but rather in isolated protein models, the results cannot be directly extrapolated to foods.

2.4.3.4 PHYTATE

Several researchers have studied the effects of γ-irradiation on antinutrients in legumes, especially phytate. Phytate is the storage form of phosphate in legume seeds. The phosphate group binds divalent ions, which can be released due to activity of phytase under HTHH storage conditions. The divalent ions (Ca^{2+} and Mg^{2+}) then move to the middle lamella where they crosslink pectin, resulting in its insolubility. Gamma-irradiation can result in a dose dependent reduction in phytic acid in legume seeds. Tresina and Mohan (2012) studied the physicochemical and anti-nutritional effects of γ-irradiation of cowpeas at 2.5, 10, 15 and 25

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kGy and noted a dose dependent reduction in phytic acid. They attributed the reduction in phytic acid to cleavage of the phytic acid ring itself by γ-irradiation. In a similar study by Bhat et al. (2008) on the impact of irradiation on the phytic acid content of *Mucuna pruriens* (velvet bean) using doses 2.5, 5.0, 7.5, 10, 15, and 30 kGy, they found a dose depended reduction in phytic acid content, except at 2.5 kGy. Although γ-irradiation was found to result in a dose dependent reduction in phytic acid in the above studies, this was, however, not found to be the case in a study by Siddhuraju, Osoniyi, Makkar and Becker (2002). These authors studied effects of γ-irradiation on three different species of an unconventional legume, Sesbania (*S. aculeata*, *S. rostrata*, and *S. cannabina*) and one species of Vigna (*V. radiata*) (mung bean/green gram) at doses 2, 4 and 6 kGy after aqueous soaking. Gamma-irradiation did not significantly ($P>0.05$) reduce phytic acid in the respective raw and soaked seeds. They suggested that the ineffectiveness of irradiation to cleave phytic acid could be due to low radiation dose (2–6 kGy) used in their study. Furthermore, they stated that the effectiveness of irradiation depends more on the combined hydrothermal and germination processing methods than on the irradiation treatment alone.

2.5 CONCLUSIONS

The storage of legume seeds under adverse conditions of high temperature and high relative humidity leads to the HTC phenomenon. Despite several hypotheses that have been proposed to explain the mechanisms responsible for the development of HTC, its cause is still incompletely explained. The most common and widely accepted mechanism for HTC development is the phytase-phytate-pectin hypothesis. More recently, the multiple mechanism theory has been proposed to explain HTC development in legumes. The theory suggests that more than one mechanism is responsible for HTC development.

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Gamma-irradiation has not been used to explore the mechanisms responsible for HTC development in legumes. Changes in the polysaccharides pectin and starch have been shown to occur during development of HTC phenomenon. Gamma-irradiation effectively depolymerises polysaccharides, which may help in understanding the mechanisms involved in the development of HTC defect. The literature specifically reviewed the principles involved in γ-irradiation and its effects on legume components, which include depolymerisation of carbohydrates and pectin, unfolding, and aggregation of protein and decrease in phytate. Furthermore, γ-irradiation has been shown to reduce cooking time in easy-to-cook cowpeas. Therefore, there is logic in studying the effect of γ-irradiation on HTC cowpeas with the aim of trying to elucidate the mechanisms responsible for the development of the HTC defect.

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3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESIS 1

Moderate γ-irradiation (at 11 kGy) will depolymerise insoluble pectates in hard-to-cook (HTC) cowpeas resulting in low molecular weight pectin molecules, which are more water soluble, reducing the cooking time of HTC cowpeas.

Irradiation of pectin in aqueous solutions (1.0 to 10 kGy) has been shown to cause depolymerisation of macromolecules (pectin and starch) due to the radical induced cleaving of glycosidic bonds rather than radical-induced de-esterification of polysaccharide chains, as shown by the decrease in the viscosity of pectin solutions (Zegota, 1999). Effects of irradiation in aqueous solutions are more pronounced than on dry pectin due to the activity of radiolytic products of water such as the hydroxyl group which can cleave the glycosidic bonds (Džamiĉ and Jankoviĉ, 1966).

3.1.2 Objective 1

To determine the effects of moderate γ-irradiation (at 11 kGy) on insoluble pectates (Ca^{2+} and Mg^{2+} pectates) with the aim of reducing HTC defects in cowpeas.

3.2 HYPOTHESIS 2

Gamma-irradiation will hydrolyse the glycosidic bonds in starch and pectin, resulting in low molecular weight molecules carbohydrates and affecting the functional properties (water absorption capacity, water solubility index and swelling power) of HTC cowpeas.

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Gamma-irradiation leads to degradation of polysaccharides such as starch, cellulose and pectin via hydrolysis of glycosidic bonds (Cho, Kim and Rhim, 2003). Gamma-irradiation has been shown to decrease the molecular size of amylopectin in bean and maize starches (Rombo et al., 2004). Gamma-irradiation was also shown to decrease starch related functional properties in cowpeas flours and pastes, such as swelling index, gel strength and viscosity (Abu et al., 2005).

3.2.1 Objective 2

To determine the effects of γ-irradiation on thefunctional properties of HTC cowpea flours with the aim of understanding the HTC defect.

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4 RESEARCH

4.1 EFFECTS OF γ-IRRADIATION ON COTYLEDON CELL SEPARATION AND PECTIN SOLUBILISATION IN HARD-TO-COOK COWPEAS

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[1] Research Chapter published: Jombo, T.Z., Minnaar, A. and Taylor, J.RN., 2018. Effects of gamma-irradiation on cotyledon cell separation and pectin solubilisation in hard-to-cook cowpeas. *Journal of the Science of Food and Agriculture*, 98, 1725-1733.

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4.1.1 Abstract

Cowpeas stored at high temperature and humidity develop the hard-to-cook defect (HTC). This defect greatly increases cooking times and energy costs. To better understand the mechanisms involved in the HTC defect development, the effects of γ-irradiation on cotyledon cellular structure and pectin solubility in two cowpea cultivars with different susceptibility to HTC defect were investigated. Gamma-irradiation increased cell size intercellular spaces in both cowpea cultivars and reduced cooking time of the less HTC susceptible cultivar. However, it did not reverse the HTC defect in the more HTC susceptible cultivar. Gamma-irradiation also increased the levels of cold water- and hot water-soluble pectin. The irradiation effects were thus mainly due to hydrolysis of pectin fractions in the cell walls. However, chelator-soluble pectin (CSP) solubility was not affected. As the cell wall changes brought about by γ-irradiation were associated with pectin solubilisation, this supports the phytase-phytate-pectin theory as a major cause of the HTC defect. However, the non-reversal of the defect in HTC susceptible cowpeas and the absence of an effect on CSP indicate that other mechanisms are involved in HTC defect development in cowpeas, possibly the formation of alkali–soluble, ester bonded pectins.

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4.1.2 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important grain legumes (pulses) in Africa, Latin America and Asia in the fight against food insecurity and malnutrition, and in improving human health (Nedumaran, Abinaya, Jyosthnaa, Shraavya, Rao and Bantilan, 2015). It offers a relatively cheap source of protein compared to animal protein, which can improve the diets of poor households (Akibode and Maredia, 2016). However, its utilisation is limited by its long cooking time, which translates into high energy and time demands. In rural Africa, firewood is the most used source of energy, but its increasing use has a severe impact on the environment (Girard, 2002). Importantly, cowpea utilisation is also specifically limited by the development of the hard-to-cook (HTC) defect, which further increases the cooking time of the seeds up to greater than 4 hours (Ndungu, Emmambux and Minnaar, 2012). The HTC defect occurs in legumes which are stored under tropical conditions of high temperature (>25^{o}C) and high relative humidity (>65%) (Liu, McWatters and Phillips, 1992), referred to as high temperature, high humidity (HTHH) conditions.

Several theories have been proposed to explain the HTC defect: (1) the phytase-phytate-pectin theory (Galiotou-Panayotou, Kyriakidis and Margaris, 2008); (2) the lignification theory (Nasar-Abbas, Plummer, Siddique, White, Harris and Dods, 2008); (3) protein and starch interactions (Liu, 1997) and (4) a multiple mechanism theory (Garcia, Filisetti, Udaeta and Lajolo, 1998). Despite much research, the mechanisms responsible have not been completely elucidated. The most widely accepted is the ‘phytase-phytate-pectin’ theory (Galiotou-Panayotou et al., 2008). According to this theory, increased activity of phytase due to the HTHH storage conditions degrades phytate leading to release of divalent ions which crosslink pectin, rendering it insoluble which causes failure of cell separation during wet cooking (Coelho, Bellato, Santos, Ortega and Tsai, 2007). With cowpea, this theory is

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supported by the fact that both cold water soluble pectin and hot water soluble pectin have been shown to decrease with increase in storage time under HTHH conditions (Ndungu et al., 2012).

Pectin is one of the main components of the plant cell wall middle lamella. It is responsible for “cementing” adjacent cells together (Pond, Church, Pond and Schoknecht, 2005). During the cooking of legumes, tissue softening occurs due to the disintegration of the middle lamella, which allows cell separation (Ilker and Szczesniak, 1990). Ultrastructural and histochemical studies have revealed that the cotyledon cells in HTC seeds are poorly separated, showing failure of cell separation (Garcia, Lajolo and Swanson, 1993). This restricted cell separation leads to extended cooking time of the seeds (Shomer, Paster, Lindner and Vasiliver, 1990).

Gamma-irradiation could be a valuable tool to help better understand the mechanisms responsible for the HTC defect. Gamma-irradiation hydrolyses polysaccharides such as starch, cellulose, and pectin via cleavage of glycosidic bonds (Cho, Kim and Rhim, 2003; Rombo, Taylor and Minnaar, 2004). This is important in legumes since, as indicated, the middle lamella which is critical for cell separation during cooking is pectin rich (Shiga, Lajolo and Filisetti, 2003). Gamma-irradiation at 2-10 kGy has been shown to decrease the cooking time in cowpeas (Rao and Vakil, 1985). More recently, γ-irradiation has been investigated to modify physical, functional and pasting properties of cowpea (Falade and Kolawole, 2013). The effects of γ-irradiation on the legumes seeds were related mainly to changes in the major macromolecules: starch and protein.

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The application of γ-irradiation to study the mechanisms involved in the development of HTC defect in legumes has not been previously reported. This study investigated the effects of γ-irradiation on cotyledon cellular structure and pectin solubility in two HTC cowpea cultivars with different susceptibility to the HTC defect.

4.1.3 Materials and Methods

4.1.3.1 Cowpeas

Two cowpeas cultivars were selected for this investigation which had been found to have different susceptibility to the HTC defect (unpublished data): Agrigold (more susceptible) and Bechuana White (less susceptible). The two cowpea cultivars were cultivated in South Africa. After harvesting, they were stored in polypropylene plastic containers at 8°C and 61% Relative Humidity (RH) until analysis.

4.1.3.2 Accelerated HTHH storage conditions

HTC defect was induced by incubating the cowpea seeds (3.6 kg) at 40°C and 80% RH for 20 and 40 days in airtight polypropylene plastic containers. To obtain the desired RH, saturated potassium chloride solution was used according to ASTM E104-02 (ASTM international, 2012). Temperature and RH were monitored using a humidity/temperature logger, which was placed inside the plastic container.

4.1.3.3 Irradiation

The HTHH stored and control stored (at 8 °C) cowpea seeds were then vacuum sealed in low density polyethylene bags and placed into cardboard boxes and subjected to γ-irradiation.

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They were irradiated by Synergy Sterilisation SA (Pty) Ltd (Isando, South Africa) using a ^{60}Co source. The target dose was 11 kGy. Actual dose delivered was an average of 11.2 kGy at a dose rate of 1.7 kGy h^{-1}. Harwell Perspex dosimeter (Didcot, UK) was used. The un-irradiated cowpea seeds (0 kGy) were used as controls.

4.1.3.4 Preparation of cowpea flours

After HTC defect induction and control storage (as applicable) and irradiation treatment (as applicable), the cowpeas were ground to pass through a 0.25 mm opening screen using an air cooled laboratory hammer mill (Falling Number mill 3100, Perten Instruments, Huddinge, Sweden). The cowpea flours were packaged in zip-lock type polyethylene bags. The flours from the cowpeas where HTC had been induced were stored at ambient temperature (22ºC), whereas flours from the control cowpeas were stored at 4^{o}C. Different storage temperatures were used because reversibility of the HTC defect (decreased cooking time) has been observed by storage of HTC cowpea seeds under refrigerated conditions (6.5°C, 71% RH) (Hentges, Weaver and Nielsen, 1990). The reversible components of the HTC defect have been associated with the phytate-pectin mechanism of HTC development (Del Valle and Stanley 1995).

4.1.4 Analyses

4.1.4.1 Moisture

Moisture content was determined using AACC method 44-15A air-oven method (AACC International, 2000).

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4.1.4.2 Cooking time

Cooking time was determined using a Mattson bean cooker, as described by Mwangwela, Waniska, McDonough and Minnaar (2006). For each treatment, 25 cowpeas were positioned on the perforations in the cooker, placed in an aluminium pan with deionised water and boiled at 95ºC (the boiling temperature at 1,200 m altitude where the research was conducted). The cooking time of the cowpeas was recorded as the time when 80% of the pins (pin weight 50 g) had penetrated the cowpeas and plunged through the holes in the base of the cooker.

4.1.4.3 Isolation of pectin fractions

Three pectin fractions were isolated:

Cold water soluble pectin (CWSP) - comprising pectic substances that are not strongly associated with the cell walls (Jansen, Jang, Albersheim and Bonner, 1960) (bonding interactions involve weak bonds/van der Waals forces (Chang, Tsai and Chang, 1993); **Hot water soluble pectin (HWSP) -** comprising pectic substances associated with the cell walls by intensive hydrogen bonding interactions (Chang et al., 1993) plus the CWSP; **Chelator soluble pectin** (**CSP) -** comprising pectin cross-linked by ionic bonds, particularly involving Ca^{2+} ions (Brummell, 2006).

Cowpea flour (5 g) was mixed with 30 mL 746 g kg^{-1} ethanol for 10 minutes at ambient temperature (22^{o}C) to remove soluble sugars. The mixture was centrifuged at 17300 × g for 10 minutes. Aqueous ethanol extraction was repeated twice. The final extraction was performed with absolute ethanol. The residual pellet (alcohol-insoluble solids) (AIS) was vacuum dried and stored in a desiccator at 22ºC. AIS (1 g) was extracted three times with 10

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mL cold distilled water for 10 minutes at 22^{o}C and the combined extracts were considered as CWSP (Ndungu et al., 2012). HWSP was determined according to Bernal-Lugo, Parra, Portilla, Pena-Valdivia and Moreno (1997) by extraction of 1 g AIS with hot distilled water at 80ºC. CSP was determined essentially as described by Hentges, Weaver, and Nielsen (1991). After CWSP extraction, the resulting pellet was extracted three times with 10 mL of 5 g kg ethylenediaminetetraacetic acid (EDTA) for 10 minutes at 22^{o}C. The three pectin fractions were each analysed for their galacturonic acid content using the metahydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (1973), with galacturonic acid (Sigma-Aldrich, catalogue no. G2125, Johannesburg, South Africa) as the standard.

4.1.4.4 Phytate

Phytate was determined using an indirect quantitative analysis method by measuring organic phosphate, as described by Frubeck, Alonso, Marzo and Santidrian (1995). Anion exchange chromatography using Dowex 1; anion-exchange resin-AG 1 x 4, 4% cross-linkage, chloride form, 100-200 mesh (Sigma-Aldrich) was applied to remove inorganic phosphate. Phosphate was determined colorimetrically at 500 nm based on the pink colour of Wade reagent.

4.1.4.5 Confocal laser scanning microscopy (CLSM)

Tissue blocks (approx. 1 mm^{3}) were cut from the inside edge of the cowpea seed cotyledons. These were fixed in 2.5 g kg^{-1} (w/v) formaldehyde overnight. The fixed cotyledon tissues were rinsed three times in 0.075 M sodium phosphate buffer for 10 minutes. The tissues were dehydrated at 22^{o}C for 15 minute periods in a graded series of aqueous ethanol (236 g kg^{-1}, 393 g kg^{-1}, 550 g kg^{-1}, 707 g kg^{-1}, and 785 g kg^{-1}). The dehydrated tissues were infiltrated with 500 g kg^{-1} (v/v) London Resin White (LR) in ethanol for 1 hour and then with 1000 g

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kg^{-1}(LR overnight at 4^{o}C). The tissues were then placed in gelatin capsules, filled with LR and polymerised for 48 hours. Sections of 2.0 µm thickness were cut and placed on a glass slide. They were then stained with the fluorescent stain Calcoflour white MR2 (Sigma-Aldrich) for 5 minutes, to identify the cell walls. The stained sections were washed in running water and dried. Sections were then viewed using a Zeiss LSM 510 META Confocal Laser Scanning Microscope (Zeiss, Jena, Germany). The microscope system was equipped with a Plan-Apochromat 20 x/0.75 objective lens. Excitation was at 405 nm and emission at 514 nm using a 420 nm long pass filter, with a pinhole set at 48 µm. The digital images obtained were processed using Zeiss LSM image browser software.

4.1.4.6 Scanning electron microscopy (SEM)

The tissue blocks embedded in gelatin used for CLSM (above) were also used for SEM. The gelatin capsules were cut into 10 mm long pieces and mounted on an aluminium stub using double sided carbon tape. After which, they were sputter coated with carbon. The coated samples were viewed at 1.0 kV using a Zeiss Gemini Ultra Plus Field Emission SEM (Oberkochen, Germany).

4.1.4.7 Statistical analysis

All experiments were repeated at least twice. The data were subjected to one-way ANOVA (Statistica® 8.0) and multifactor analysis of variance (MANOVA) with the means separated using Fisher's least significant difference (LSD) test at a 95% level of probability ($P \leq 0.05$).

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4.1.5 Results and Discussion

4.1.5.1 Effects of HTHH storage and irradiation on cooking time

Variety, HTHH storage, and irradiation all had a highly significant ($P \leq 0.001$) on the cooking time of both cowpea cultivars (Table 4.1.1). The storage of both cowpeas cultivars under HTHH conditions substantially ($P \leq 0.05$) increased cooking time, indicating the successful development of the HTC defect (Table 4.1.2). HTHH storage increased cooking time of Bechuana White cowpeas by 45% and 303% after 20 and 40 days of storage, respectively. With Agrigold cowpeas, which are more susceptible to the development of HTC defect, the cooking time increased by greater than 600% after 20 days of HTHH storage. The increase in cooking time due to storage of legumes under adverse conditions has been reported previously (Ndungu et al., 2012; Coelho et al., 2007). This could be due to the formation of water insoluble pectates as according to phytate-phytase-pectin theory (Galiotou-Panayotou et al., 2008). The softening of beans during cooking is usually linked to the separation of parenchyma cells along middle lamella in the cotyledon of legumes (Ilker and Szczesniak, 1990) which allows for water absorption by legume seeds. The formation of water insoluble pectates in the middle lamella could have limited cell separation and minimised water uptake resulting in an increase in cooking time.

Table 4.1.1 Multifactor ANOVA of the effect of cowpea variety and in combination with HTC storage days and Irradiation dose on the cooking time Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTC days	1	0.000000
Irradiation dose	1	0.000000
Variety x HTC days	2	0.000000
Variety x Irradiation dose	1	0.000003
HTC days x Irradiation dose	2	0.000164
Variety x HTC days x irradiation dose	2	0.000001

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Table 4.1.2 Effects of HTHH storage at 40ºC and 80% RH and γ-irradiation on the cooking time of Bechuana White and Agrigold cowpea varieties

Cowpea variety[1]	Storage time (days)	Cooking time (minutes)				
		Irradiation dose (kGy)				
		0		11		
			% Change due to HTHH		% Change due to HTHH	% Change due to Irradiation
Bechuana White	0	$58^{a} \pm 7^{2}$		$45^{a} \pm 2$		(-22)
	20	$84^{b} \pm 9$	(+45)	$63^{a} \pm 5$	(+40)	(-25)
	40	$234^{d} \pm 22$	(+303)	$149^{c} \pm 12$	(+231)	(-36)
Agrigold	0	$92^{b} \pm 4$		$71^{a} \pm 8$		(-23)
	20	$>540^{c}$	(> 487)	$>540^{c}$	(> 661)	(0)
	40	$>540^{c}$	(>487)	$>540^{c}$	(> 661)	(0)

[1] For each cowpea variety, means followed by different letters are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of three independent experiments

Gamma-irradiation significantly ($P \leq 0.05$) reduced the cooking time of both cowpea cultivars. Cooking time of Bechuana White cowpeas was reduced by 25% and 36% after 20 and 40 days of HTHH storage, respectively. Gamma-irradiation reduced the initial cooking time of Agrigold cowpeas by 23% before HTHH storage. However, it did not evidently result in a reduction in cooking time of HTC Agrigold after 20 and 40 days of storage and cooking time remained extremely long, greater than 540 minutes. The reduction in cooking time after γ-irradiation has been previously shown in easy to cook cowpeas (Abu and Minnaar, 2009) and Bambara groundnuts (Falade and Adebiyi, 2015). This could be due to loss of membrane

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integrity resulting in increased water absorption (Falade and Adebiyi 2015; Bhat, Sridhar, Young, Bhagwath and Ganesh, 2008). The increased water absorption properties and starch gelatinisation may have influenced the observed reduction in cooking time (Falade and Adebiyi, 2015).

4.1.5.2 Effects of HTHH storage and irradiation on cotyledon cellular structure

The development of HTC defect in both cultivars of cowpeas by HTHH storage resulted in closely packed cotyledon cells, which showed only limited cell separation after cooking for 60 and 120 minutes, as viewed by CLSM (Fig 4.1.1e,g Bechuana White, Fig 4.1.2e,g Agrigold). This is in contrast to the situation where after 120 minutes of cooking of both cowpea varieties which had not been subjected to HTHH storage, there was more general cell separation (Fig 4.1.1c,d Bechuana White, Fig 4.1.2c,d Agrigold).

Gamma-irradiation had no visible effect on cell separation in cooked cowpeas that had not been subjected to HTHH storage (Fig 4.1.1d Bechuana White, Fig 4.1.2d Agrigold). Furthermore, there was no cell separation with both cowpea cultivars that had been subjected to HTHH storage (Fig 4.1.1f,h Bechuana White, Fig 4.1.2f,h Agrigold). This is despite the fact that irradiation substantially reduced the cooking time of HTHH stored Bechuana White (Table 4.1.1). Although not significant ($P>0.05$), it was observed that γ-irradiation reduced cotyledon cell wall thickness in both cowpea varieties. The reductions were from approximately 4.0 µm to 3.8 µm in Bechuana White (Fig 4.1.1a,b) before storage and from 4.0 µm to 3.5 µm in Bechuana White (Fig 4.1.1e,f) after HTHH storage. In Agrigold, reductions were from approximately 2.5 µm to 2.0 µm (Fig 4.1.2a,b) before storage and from 3.1 µm to 2.8 µm in Agrigold (Fig 4.1.2e,f) after HTHH storage. However, γ-irradiation

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substantially increased the size of the intercellular spaces between the cotyledon cells for both cowpea cultivars. For Bechuana White, the increase in intercellular space was from an average of 7.1µm to 18.5µm (Fig 4.1.1e,f) after storage and 13.5µm to 22µm (Fig 4.1.2a,b) with Agrigold. There was also a significant (P<0.05) increase in cell size after γ-irradiation and cooking for 120 minutes for both cowpea varieties. With Bechuana White, the increase in cell size was from an average of 60.3 µm to 82.6 µm (Fig 4.1.1c,d) and 57.2 to 80.6 µm for Agrigold cowpeas (Fig 4.1.2c,d) which had not been subjected to HTHH storage. Legume cotyledon cell expansion during cooking as result of water uptake normally occurs in advance of cell separation (Narasimha, Srinivas and Desikachar, 1989) and the separation of cells is enhanced by the disintegration of the middle lamella (Falade and Adebiyi, 2015). The reduction in cell wall thickness, increase in intercellular space size and cell expansion with γ-irradiation suggests that it was causing partial disintegration of the middle lamella.

To better understand the changes in cellular structure brought about by γ-irradiation observed by CLSM, the cowpea seed tissues were also studied using SEM, which provides a 3-dimensional surface image. Generally, after HTHH storage and γ-irradiation treatment, starch granules increased in size and were more clearly defined in both cowpea cultivars. With the seeds cooked for 120 minutes that had not been subjected to HTHH storage but had been subjected to γ-irradiation treatment, the starch granules in Bechuana White were more clearly defined and had increased in size to approximately 15 µm (Fig 4.1.3d) from 11 µm (Fig 4.1.3c) with the corresponding treatment without irradiation. For Bechuana White that had been HTHH stored for 40 days and subjected to γ-irradiation and then cooked for 120 minutes, the starch granules were also clearly defined and had increased in size to approximately 16 µm (Fig 4.1.3h) from 12 µm (Fig 4.1.3g) with the corresponding treatment without γ-irradiation. This shows that the starch granules had enhanced water uptake as a

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result of the γ-irradiation treatment and supports the data in Table 4.1.1 that γ-irradiation reduced cooking time. However, with Agrigold which was HTHH stored for 40 days and cooked for 120 minutes, there was no clear difference in starch granule appearance between those that had been subjected to γ-irradiation treatment (Fig 4.1.4h) and the corresponding treatment without (Fig 4.1.4g). With both treatments, the starch granules were of a similar size, approximately 14 μm. This is a clear reflection of the greater susceptibility of the Agrigold variety to the HTC defect and suggests that changes had taken place in the middle lamella and the cell wall that prevented water uptake.

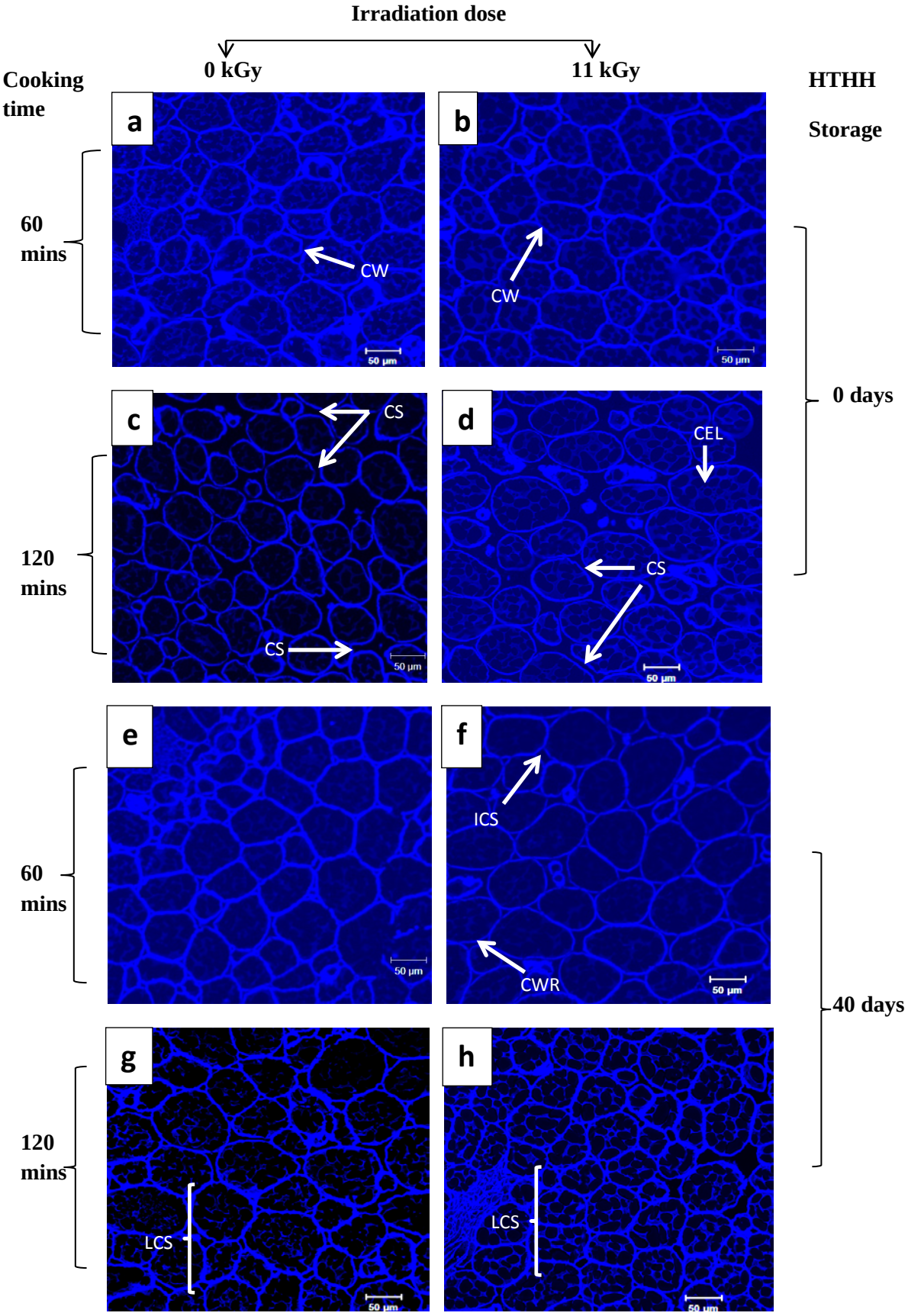

Figure 4.1.1 Confocal laser scanning microscopy illustrating effects of cooking for 60 and 120 minutes on cell separation in high temperature high humidity stored and irradiated Bechuana White cowpeas. CS- Cell separation, CW- Cell wall, LCS - Limited cell separation, CWR - Cell wall reduction,CEL – Cell Elongation, ICS - Intercellular space

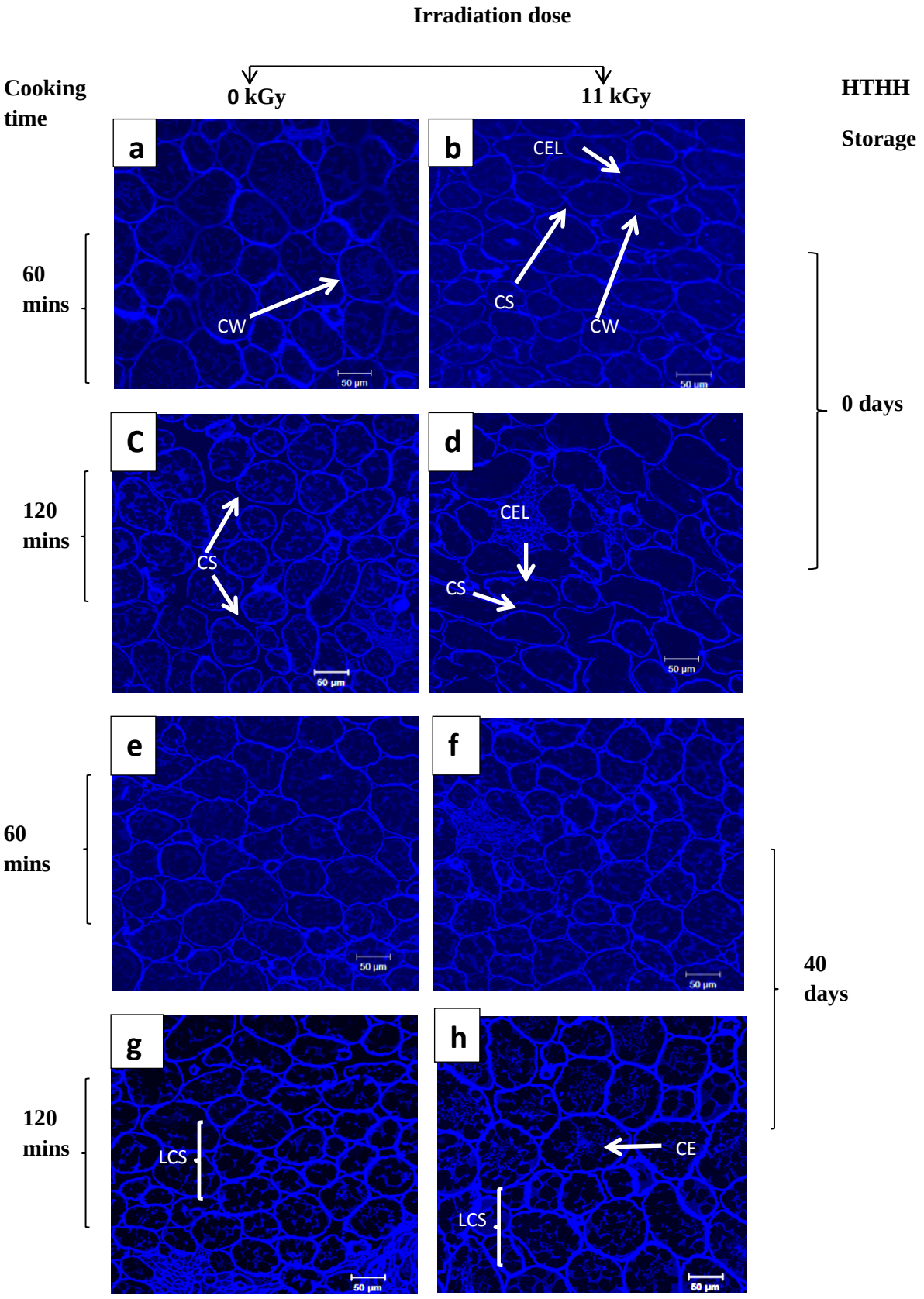

Figure 4.1.2 Confocal laser scanning microscopy illustrating effects of cooking for 60 and 120 minutes on cell separation in high temperature high humidity stored and irradiated Agrigold cowpeas. CEL - Cell Elongation, CS- Cell separation, LCS - Limited cell separation, CW – Cell wall

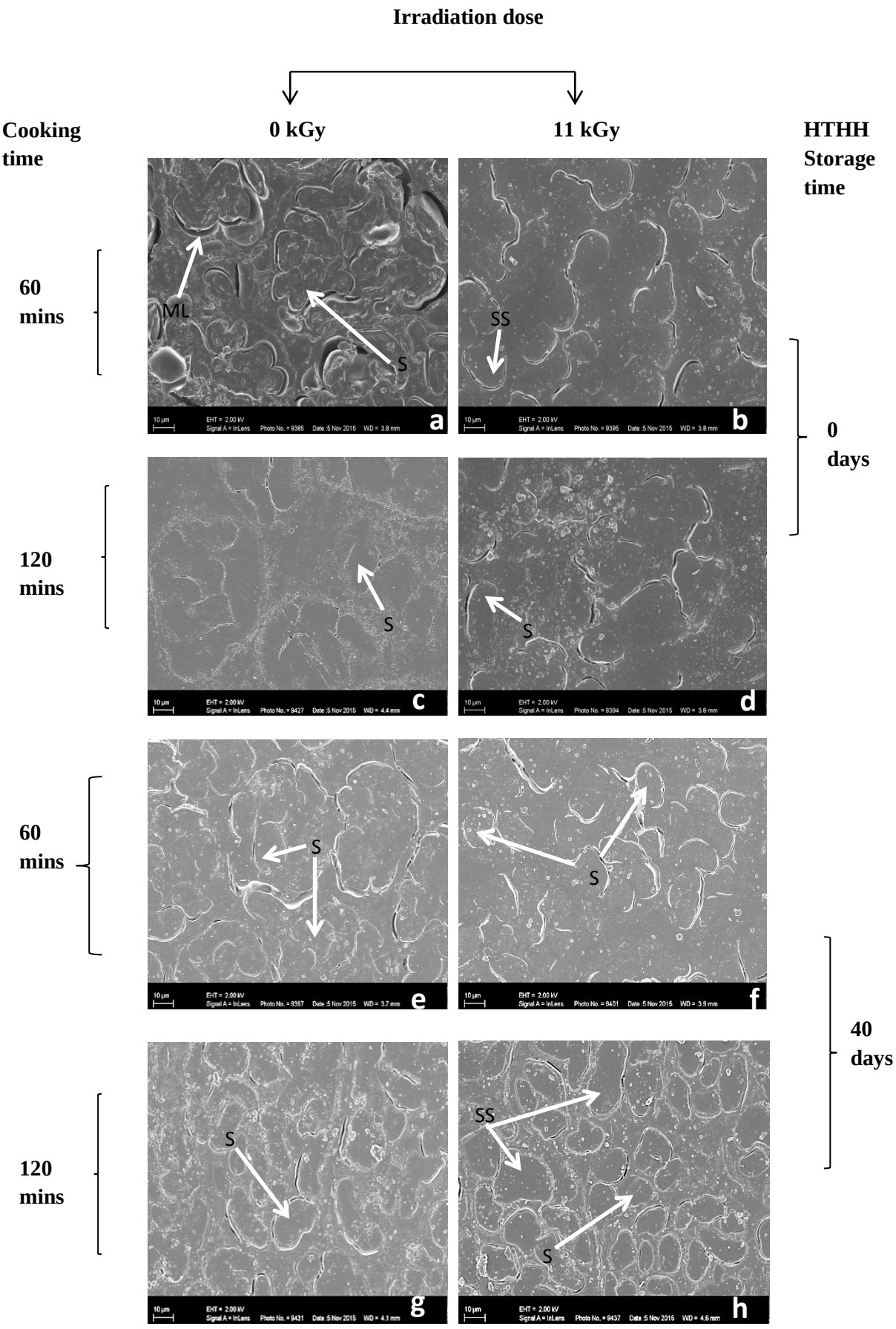

Figure 4.1.3 Scanning electron microscopy illustrating effects of cooking for 60 and 120 minutes on microstructure of high temperature high humidity stored and irradiated Bechuana White cowpeas. M-Middle Lamella; S-Starch granule; SS-Starch swelling

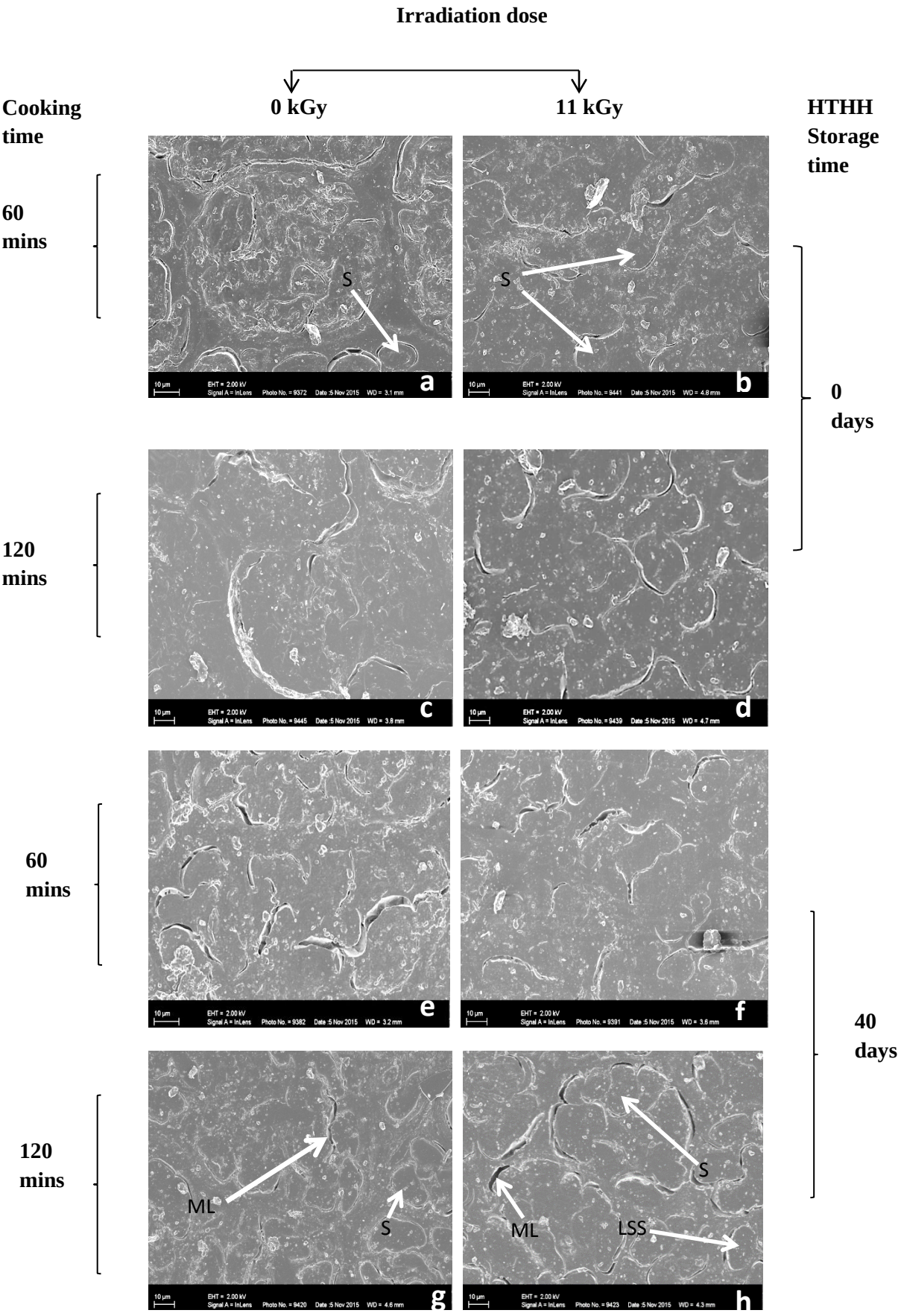

Figure 4.1.4 Scanning electron microscopy illustrating effects of cooking for 60 and 120 minutes on microstructure of high temperature high humidity stored and irradiated Agrigold cowpeas. M-Middle Lamella, S – Starch granule, LSS-Limited starch swelling

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4.1.5.3 Effects of HTHH storage and irradiation on phytate and pectin

To explain the visual changes in the cotyledon cells caused by HTHH storage and γ-irradiation, the levels of phytate, CWSP, HWSP and CSP (middle lamella components) were determined in the variously treated samples. Variety, HTHH storage, and irradiation all had a highly significant effect ($P \leq 0.001$) on phytate in cowpea (Table 4.1.3). Irradiation and HTHH storage also had a highly significant effect ($P \leq 0.001$) on CWSP of cowpeas (Table 4.1.4). Variety had no significant effect ($P > 0.05$) on CWSP of cowpeas. Variety, HTHH storage, and irradiation all had a highly significant effect ($P \leq 0.001$) on the HWSP of cowpeas (Table 4.1.5). Variety had a highly significant effect ($P \leq 0.001$) on CSP of cowpeas (Table 4.1.6). HTHH storage had a significant effect ($P \leq 0.01$) on the CSP of cowpea cultivars. Irradiation had a significant effect ($P \leq 0.05$) on the CSP of cowpea cultivars.

Concerning phytate, overall there was a decrease in phytate level in both cowpea varieties with HTHH storage (Table 4.1.7). The decreases were 8% and 10% for Bechuana White and 10% and 18% for Agrigold of storage, respectively. The decrease was probably due to increased activity of phytase during HTC development which hydrolyses phytate releasing divalent minerals, inorganic phosphates and produces lower level inositol phosphates (Yu, Cowieson, Gilbert, Plumstead and Dalsgaard, 2012).

With γ-irradiation treatment, there were some small reductions in the level of phytate as a result of irradiation treatment (Table 4.1.7). The reduction of phytate by irradiation has been previously reported in soya beans (Siddhuraju, Makkar and Becker, 2002). Its action is probably by the cleavage of phytate inositol ring (Stanley and Aguilera, 1985). However, generally, the γ-irradiation treatment had only a limited effect on the phytate levels after HTHH storage for both cowpea varieties. This is presumably because after the development

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of the HTC defect the amount of available phytate had been reduced by the action of the phytase. This is supported by the fact that the highest reduction in phytate (11%) was with the Agrigold cowpeas that had not been subjected to HTHH storage.

Table 4.1.3 Multifactor ANOVA of the effect of cowpea variety and in combination with HTC storage days and Irradiation dose on phytate of Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTC days	1	0.000000
Irradiation dose	1	0.000039
Variety x HTC days	2	0.050675
Variety x Irradiation dose	1	0.295272
HTC days x Irradiation dose	2	0.095379
Variety x HTC days x irradiation dose	2	0.000927

Table 4.1.4 Multifactor ANOVA of the effect of cowpea variety and in combination with HTC storage days and Irradiation dose on the cold water soluble pectin of Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.182717
HTC days	1	0.000000
Irradiation dose	1	0.000010
Variety x HTC days	2	0.002132
Variety x Irradiation dose	1	0.693276
HTC days x Irradiation dose	2	0.000000
Variety x HTC days x irradiation dose	2	0.000001

Table 4.1.5 Multifactor ANOVA of the effect of cowpea variety and in combination with HTC storage days and Irradiation dose on the HWSP of Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000001
HTC days	1	0.000010
Irradiation dose	1	0.000004
Variety x HTC days	2	0.258386
Variety x Irradiation dose	1	0.000327
HTC days x Irradiation dose	2	0.003219
Variety x HTC days x irradiation dose	2	0.088500

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Table 4.1.6 Multifactor ANOVA of the effect of cowpea variety and in combination with HTC storage days and Irradiation dose on chelator soluble pectin of Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTC days	1	0.002312
Irradiation dose	1	0.015220
Variety x HTC days	2	0.000849
Variety x Irradiation dose	1	0.182717
HTC days x Irradiation dose	2	0.618625
Variety x HTC days x irradiation dose	2	0.063470

Table 4.1.7 Effects of HTHH storage at 40ºC and 80% RH and γ-irradiation on the phytate content of Bechuana White and Agrigold cowpeas

Cowpea variety[1]	Storage time (days)	Phytate (g/kg^{-1} dry basis)				
		Irradiation dose (kGy)				
		0		11		
			% Change due to HTHH		% Change due to HTHH	% Change due to Irradiation
Bechuana	0	$9.67^{c} \pm 0.29$[2]		$9.63^{c} \pm 0.19$		0
White	20	$8.87^{b} \pm 0.08$	(-8)	$8.72^{b} \pm 0.04$	(-9)	(-2)
	40	$8.72^{b} \pm 0.04$	(-10)	$8.15^{a} \pm 0.05$	(-15)	(-7)
Agrigold	0	$12.07^{d} \pm 0.15$		$10.78^{bc} \pm 0.09$		(-11)
	20	$10.83^{c} \pm 0.21$	(-10)	$10.28^{bc} \pm 0.09$	(-5)	(-5)
	40	$9.84^{a} \pm 0.28$	(-18)	$9.95^{a} \pm 0.30$	(-8)	(+1)

[1] For each cowpea variety, means followed by different letters are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of two independent experiments

Regarding pectin, overall HTHH storage generally caused reductions ($P \leq 0.05$) in the levels of CWSP (pectic substances bound by interactions involve weak bonds/van der Waals forces (Chang et al., 1993) and not strongly associated with the cell walls (Jansen et al., 1960) and HWSP (pectic substances associated with the cell walls by intensive hydrogen bonding interactions (Chang et al., 1993) plus the CWSP) in both cowpea varieties, but not CSP (pectin cross-linked by ionic bonds, particularly involving Ca^{2+} ions) which increased slightly but significantly ($P \leq 0.05$) with the cowpea seeds that had not been subjected to γ-irradiation.

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The reduction in the levels of pectin that were solubilised with hot and cold water with HTHH storage can be attributed to the formation of water insoluble Ca and Mg pectates in accordance with the phytase-phytate-pectin theory (Galiotou-Panayotou et al., 2008). During the development of the HTC defect in cowpeas Ca and Mg have been shown to migrate to concentrate around the cell wall-middle lamella (Kruger et al., 2015) where they most probably bind pectin. This in part explains the slight increase in the CSP fraction. However, the reduction of CWSP and HWSP cannot be directly explained by the phytase-phytate-pectin theory. It is probable that the decrease in the amount of pectin solubilised with hot and cold water was due to formation of alkali–soluble, ester bonded pectins (Shiga et al., 2004; Njoroge et al., 2015). The authors found that these more difficult to extract alkali-soluble, ester bonded pectins, increased with HTC development in common beans (*Phaseolus vulgaris*).

Gamma-irradiation increased the CWSP fraction in both cowpea varieties (Table 4.1.8). Likewise, γ-irradiation of Bechuana White resulted in increased HWSP fraction. In contrast, γ-irradiation had no effect on the HWSP fraction in Agrigold. The increase in pectin that was solubilised by cold and hot water was probably as a result of hydrolysis of glycosidic bonds by γ-irradiation (Cho et al., 2003), which results in readily soluble lower molecular weight fragments. This would be the cause of the indicative partial disintegration of the middle lamella, as evidenced by a reduction in cotyledon cell wall thickness, increase in intercellular space size and cell expansion as observed by CLSM (Figs 4.1.1 and 4.1.2). Interestingly, as shown in Table 4.1.8 γ-irradiation affected CWSP and HWSP solubilisation differently in the two cowpea cultivars. More of these pectin fractions was solubilised with Bechuana White than with Agrigold. This is probably related to the fact that Bechuana White (which is less susceptible to the HTC defect than Agrigold) showed a decrease in cooking time after γ-

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irradiation treatment (Table 4.1.1). The fact that γ-irradiation can modify pectin resulting in a decrease in cowpea cooking time after HTHH storage strongly supports the concept that pectin modification plays a key role in the development of the HTC defect in cowpeas. Furthermore, the absence of an effect in the case of HWSP in Agrigold (the variety which was more susceptible to the HTC defect) after γ-irradiation treatment was likely due to a difference in pectin composition.

Also, γ-irradiation of the cowpeas had no significant (P>0.05) effect on the CSP fraction. This could be due to the conformation of CSP, which is described by the "egg box model" (Munarin, Tanzi and Petrini, 2012). This might hold the pectin molecules in position leading to reformation of glycoside bonding between the galacturonic acid units, limiting irradiation effects. CSP is the pectin fraction that is usually bound to cell wall via calcium bridges (Brummell, 2006). Since γ-irradiation did not modify this pectin fraction in either cowpea variety, this further supports the earlier proposed concept that formation of alkali–soluble, ester bonded pectins could also be involved in the development of the HTC defect. This is because Bechuana White had a reduction in cooking time even though γ-irradiation did not affect the proportion of this fraction after HTHH storage.

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Table 4.1.8 Effects of HTHH storage days at 40°C and 80% RH and for γ-irradiation on the content of different pectin fractions of Bechuana White and Agrigold cowpeas

Cowpea Variety[1]	Storage Time (days)	Cold Water Soluble Pectin (CWSP) (g/kg^{-1} dry basis)			Hot Water Soluble Pectin (HWSP) (g/kg^{-1} dry basis)				Chelator Soluble Pectin (CSP) (g/kg^{-1} dry basis)				
		Irradiation dose (kGy)			Irradiation dose (kGy)				Irradiation dose (kGy)				
		0		11	0		11		0		11		
			% Change due to HTHH storage			% Change due to HTHH Storage		% Change due to HTHH storage		% Change due to HTHH Storage		% Change due to HTHH Storage	
Bechuana White	0	1.16[d] ±0.02[2]		1.39[e] ± 0.04	(+20)	1.28[d] ±0.01		1.39[e] ±0.01	(+9)	0.35[a] ±0.00		0.36[b] ±0.01	(+3)
	20	1.03[b] ±0.01	(-11)	1.19[d] ±0.01	(-14)	1.14[a] ±0.01	(-11)	1.37[e] ±0.00	(-1)	0.36[b] ±0.00	(+3)	0.36[b] ±0.00	(0)
	40	1.10[c] ±0.01	(-5)	0.90[a] ±0.00	(-35)	1.18[b] ±0.01	(-8)	1.25[c] ±0.01	(-10)	0.36[b] ±0.00	(+3)	0.36[b] ±0.00	(0)
Agrigold	0	1.29[d] ±0.01		1.31[d] ±0.01	(+2)	1.39[abc] ±0.01		1.44[c] ±0.02	(+4)	0.39[a] ±0.00		0.39[a] ±0.00	(0)
	20	1.06[b] ±0.04	(-18)	1.18[c] ±0.03	(-10)	1.40[b] ±0.01	(+1)	1.40[bc] ±0.08	(-3)	0.40[c] ±0.00	(+3)	0.40[c] ±0.00	(+3)
	40	0.92[a] ±0.01	(-29)	0.95[a] ±0.01	(-27)	1.32[ab] ± 0.00	(-5)	1.31[a] ±0.00	(-9)	0.39[a] ±0.00	(0)	0.39[a] ±0.00	(0)

[1] For each cowpea variety, means followed by different letters are significantly different at $P \leq 0.05$ for each pectin type.
[2] Means (±) standard deviations of two independent experiments

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4.1.6 Conclusions

The application of γ-irradiation has helped to further understand the mechanisms by which the HTC defect prevents cotyledon cell wall separation in cowpeas during cooking. It increases cell size and intercellular spaces in both highly and less susceptible HTC defect type cowpeas. It does not, however, reverse the HTC defect in cowpeas that are very susceptible to the HTC defect. The effects of γ-irradiation are as a result of hydrolysis of pectin fractions in the cell walls. The CWSP, HWSP, and CSP are affected differently, with large increases in CWSP and HWSP particularly in the cowpeas that are less susceptible to HTC development but essentially no change in CSP. The fact that the cell wall changes brought about by γ-irradiation were associated with pectin solubilisation supports the phytate-phytase pectin theory to some extent. However, the non-reversal of the HTC defect in HTC susceptible cowpeas and the absence of an effect on CSP indicate that other mechanisms are also involved in the HTC development in cowpeas, possibly the formation of alkali–soluble, ester bonded pectins.

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4.1.7 References

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4.2 EFFECTS OF γ-IRRADIATION ON THE FUNCTIONAL AND THERMAL PROPERTIES OF INDUCED HARD-TO-COOK COWPEA (*VIGNA UNGUICULATA* L. WALP.) SEEDS AND COOKED PREPARED PASTES

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4.2.1 Abstract

Cowpea is an important food grain legume for food and nutrition security in sub-Saharan Africa. However, its utilisation is limited by the development of the hard-to-cook (HTC) defect caused by storage under adverse conditions, resulting in extended cooking time. To further understand mechanisms involved in HTC development, the effects of γ-irradiation on the functional and thermal properties of the more HTC susceptible and less susceptible dehulled cowpea seeds and cooked prepared pastes were investigated. Gamma-irradiation decreased water solubility index, swelling power, peak-, breakdown- and setback viscosities in both cowpea varieties, presumably due to starch polymerisation. Water absorption capacity (WAC) increased in less HTC variety after γ-irradiation, but with no effect in more HTC variety. Furthermore, γ-irradiation did not affect thermal properties in either variety. Gamma-irradiation had no effect on most of the functional properties of cooked prepared pastes in both varieties, with the exception of WAC, which increased in the more HTC variety. Since γ-irradiation affected all starch related functional properties with the exception of thermal properties, the non-reversal of the HTC defect suggests that changes in starch during HTC development are a consequence and not a cause of the defect. Furthermore, there were no differences in the functional properties of pastes from both varieties after γ-irradiation; hence dehulling, cooking, and preparation of pastes eradicates differences in functional properties of cowpea pastes. Thus, preparation of dehulled cooked pastes eliminates HTC phenomenon in cowpeas.

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4.2.2 Introduction

Cowpeas (*Vigna unguiculata* (L.) Walp.) are a very important food grain legume in sub-Saharan Africa since they offer a relatively inexpensive source of high quality protein (Kyei-Boahen, Savala, Chikoye and Abaidoo, 2017) compared to animal protein. This makes them a vital component in the efforts to achieve food and nutritional security in households. However, the utilisation of cowpeas is limited by their long cooking times and further worsened by the development of the hard-to-cook defect (HTC).

The HTC defect occurs in legumes which have been stored at high temperature (>25 ºC) and high relative humidity (>65%) (HTHH) (Liu et al., 1992). The two main hypotheses postulated to explain the development of HTC defect in legumes are phytase-phytate-pectin hypothesis (Galiotou-Panayotou et al., 2008) and the lignification theory (Hincks and Stanley, 1987). However, of interest also, is the protein and starch interaction hypothesis which has been proposed to explain HTC development (Liu, 1997). The hypothesis proposes that under HTHH storage, changes occur to legume protein that results in protein coagulation (56°C) occurring before starch gelatinisation during cooking of HTC legumes, hence restricting starch from absorbing water and swelling (Liu et al., 1992). When legume seeds which have not developed the HTC defect are cooked, starch gelatinisation is expected to occur first, at a lower temperature than protein coagulation, which allows it to absorb water and swell adequately. Starch is one of the polymers which is responsible for soft texture associated with cooked legumes (Yousif, Kato and Deeth, 2007). During development of the HTC defect, changes have been reported to occur to the starch and protein. These include a decrease in water solubility and thermal stability (Hohlberg and Stanley, 1987) and an increase in low molecular proteins and protein β-sheet conformation in common bean

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(Parmar, Singh, Kaur, Virdi and Shevkani, 2017). Regarding starch and HTC, common bean starch was found to have a higher degree of crystallinity (Garcia and Lajolo, 1994). Furthermore, the gelatinisation temperature of starch in HTC increased by 6.9°C in beans which had been stored under HTHH conditions for 5 years. Moreover, starch from HTC common beans was found to have a higher proportion of small size granules (Parmar et al., 2017).

The application of γ-irradiation on legumes to study its effect on the functional properties has been previously studied in cowpea (Abu and Minnaar, 2009), bambara (Falade and Adebiyi, 2015) and chickpea (Bashir and Aggarwal, 2016). In cowpeas that had not been subjected to HTC defect, after γ-irradiation, swelling and pasting properties decreased (Abu and Minnaar, 2009). However, the effect of γ-irradiation on functional and thermal properties of HTC cowpea seeds to understand HTC defect has not been studied.

To better understand the mechanisms involved in HTC development, the effects of γ-irradiation on the functional and thermal properties of the Agrigold (more HTC susceptible) and Bechuana White (less susceptible) cowpea seeds and cooked prepared pastes were investigated. In research chapter 4.1, it was shown that changes associated with γ-irradiation were mainly related to pectin solubility and supported the phytase-phytate-pectin theory to an extent. However, this did not reverse HTC defect in the more HTC susceptible variety. Therefore, the findings from this chapter will enhance the previous study on effects of HTHH stored and γ-irradiation on cellular and pectin solubility in efforts to understand mechanisms involved in HTC development.

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4.2.3 Materials and Methods

4.2.3.1 Cowpeas

Two cowpea varieties were selected for this investigation which had been found to have different susceptibility to the HTC defect (unpublished data): Agrigold (more susceptible) and Bechuana White (less susceptible). The two cowpea varieties were cultivated in South Africa. After harvesting, they were stored at 8°C and 61% Relative Humidity (RH) until analysis.

4.2.3.2 Accelerated HTHH storage conditions

HTC defect was induced by incubating the cowpea seeds at 40°C and 80% RH for 20 and 40 days in airtight plastic containers. To obtain the desired RH, saturated potassium chloride solution was used according to ASTM E104-02 (ASTM international, 2012). Temperature and RH were monitored using a humidity/temperature logger, which was placed inside the plastic container. The same sample used for analyses in chapter 4.1 was used.

4.2.3.3 Irradiation

The HTHH stored and control stored cowpea seeds were then vacuum sealed in low density polyethylene bags and placed into cardboard boxes and subjected to γ-irradiation. They were irradiated by Isotron SA (Isando, South Africa) using a ^{60}Co source. The target dose was 11 kGy. The actual dose delivered was an average of 11.2 kGy as a dose rate of 1.7 KGy h^{-1}

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4.2.3.4 Preparation of cowpea flours and pastes

After HTHH storage followed by γ-irradiation, the untreated, HTHH stored (Day 20 and 40), irradiated (11 kGy) cowpea seeds (Agrigold and Bechuana White varieties) were dehulled and milled to a minimum particle size of 500 µm using an air cooled laboratory hammer mill (Falling Number mill 3100, Perten Instruments, Huddinge, Sweden).

Cowpea cooked pastes were prepared by first soaking the dehulled cowpea for 3 hours in distilled water (22ºC), followed by cooking (95ºC) for 120 minutes in an aluminium pan. The cooked dehulled cowpeas were then formed into a paste using a pestle and mortar. The paste was freeze-dried and milled to a maximum particle size of 500 µm prior to analyses.

4.2.3.5.1 Analyses

4.2.3.5.1.1 Moisture

Determination of moisture content of dehulled cowpea flours and cooked prepared pastes were conducted using AACC method 44-15A (AACC International, 2000) air-oven method.

4.2.3.5.1.2 Pasting properties

Pasting was performed using the method described by Abu et al. (2005) with slight modifications. Pasting properties of the HTC dehulled cowpea seeds and cooked prepared pastes was determined using an Anton Paar Rheometer (Physica MCR 301, Ostildern, Germany). Cowpea flour and freeze dried paste (about 1.7 g db) weighed accurately were suspended in distilled water and adjusted to a total weight of 17 g. The pasting condition was initiated by stirring the slurry at 960 rpm, at 50 °C for 30s. Stirring was at 160 rpm for the

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rest of the cycle. The suspension was heated from 50 °C to 91 °C (at a heating rate of 5.5 °C/min) and held at this temperature for 15 min before cooling down to 50 °C (at a cooling rate of 5.5 °C/min). The parameters recorded were the peak viscosity (PV), breakdown viscosity (BV) and setback viscosity (SV).

4.2.3.5.1.3 Nitrogen Solubility Index (NSI)

NSI of the dehulled cowpea seeds and cooked prepared pastes were determined according to the AACC Method 46-23 (AACC International, 2000) with modification. Cowpea flour and freeze dried paste (1 g) were dispersed in 20 mL of 0.1M NaCl solution and stirred continuously for 1 hour at 30°C at pH 7. The suspension was centrifuged (7000 x g, for 15 min, at 4°C) and the supernatant filtered through a Whatman No. 1 filter paper. The residue from the suspension was re-washed twice in 10 mL of 0.1 M NaCl solution at pH 7. The filtrate was frozen (-18°C) overnight, freeze dried and weighed. The nitrogen content of the freeze dried sample was determined using DUMAS combustion analysis, AACC Method 46-23 (AACC International, 2000). NSI was expressed as a percentage of the total nitrogen content of freeze dried sample divided by total nitrogen content in flour sample on a dry basis.

4.2.3.5.1.4 Water absorption capacities (WAC)

WAC of the dehulled cowpea seeds and cooked prepared pastes were determined according to the AACC method 56-20 (AACC international, 2000) with slight modifications. Cowpea flour and freeze dried paste (1 g) (M0) were accurately weighed and dispersed in 20 ml deionised water (at 22 °C) and vortexed for 10 min. The samples were centrifuged (1000 x g,

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for 15 min at 20 °C) and the supernatant decanted. The centrifuge tubes were then inverted for 5 min on a paper towel, followed by weighing of the residue (M2). WAC was calculated as follows:

where M0 is the sample weight (db) and M2 is the weight of residue.

$$WAC = \frac{M2 - M0 \times 100}{M0}$$

4.2.3.5.1.5 Water Solubility Index (WSI) and Swelling power

The WSI of HTC cowpea seeds and cooked prepared pastes was determined using the method described by Ocloo, Minnaar and Emmambux (2014). About 125 mg (dry basis) accurately weighed cowpea flour and freeze dried paste samples were heated in 20 mL distilled water at 95 °C for 30 min in shaking water bath (100 rpm). The samples were then allowed to cool and centrifuged at 3000 × g for 15 min at 25 °C. The supernatant was decanted and evaporated in a forced draught air-oven at 105 °C for 16 h. The WSI was determined as the ratio in weight of dried supernatant to the weight of the flour and expressed as a percentage (%). The residue obtained after centrifugation was then weighed to obtain the swelling power. Swelling power was calculated as ratio of the final residue to the initial dry sample weight.

4.2.3.5.1.6 Thermal properties

Thermal properties of the cowpea seeds were determined using a Metler Toledo HPDSC-827 Differential scanning calorimeter (Schwerzenback, Switzerland). Cowpea flour (10 mg) was accurately weighed into a 100 µL aluminium sample pan, and 30 mg of distilled water was

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added. The pans were hermetically sealed and the samples were equilibrated at ambient temperature (22 °C). The instrument was calibrated using indium, and an empty aluminium pan was used as reference. Thermal properties were measured using a heating rate of 10 ºC min between 30 and 120°C. The following parameters were determined: The melting enthalpy ($\Delta H\ Jg^{-1}$) peak onset (T_o), peak (T_p) and peak end (T_c) temperatures.

4.2.3.5.1.7 Statistical analyses

All experiments were repeated at least twice. The effects of irradiation on functional properties and thermal properties before and after storage under HTHH conditions were determined using one-way analysis of variance (ANOVA) and multifactor analysis of variance (MANOVA) with the means separated using Tukeys honest significant difference (HSD) test at a 95% level of probability ($P \leq 0.05$).

4.2.4 Results and Discussion

4.2.4.1 Effects of HTHH storage and γ-irradiation pasting properties

Variety, HTHH storage, and irradiation all had a highly significant effect ($P \leq 0.001$) on the peak viscosity of dehulled cowpea seeds (Table 4.2.2). Variety and HTHH storage also had a significant effect ($P \leq 0.05$) on the peak viscosity of dehulled cowpea cooked prepared pastes (Table 4.2.3). Irradiation had no significant effect ($P > 0.05$) on the peak viscosity of dehulled cowpea cooked prepared pastes. HTHH storage increased the peak viscosity of Bechuana White dehulled cowpea by 14.8% and 40.7% after 20 and 40 days, respectively (Table 4.2.1). With the more prone HTC Agrigold dehulled cowpea, peak viscosity increased by 101.1% and 108.1% after 20 and 40 days, respectively. The more HTC Agrigold had a substantially

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higher peak viscosity compared to Bechuana White (Figs 4.2.1 and 4.2.3). Peak viscosity is indicative of the water binding capacity of the starch granule (Shimelis, Meaza and Rakishit, 2006). The increase in peak viscosity could probably be due to interactions involving pectin and starch which facilitated gel formation as suggested by Fu and Rao (2001). Calcium liberated due to HTC development can interact with pectin and starch resulting in the greater capacity to hold water. Norzia, Kong, Karim and Seow (2001) found that dispersions of pectin alone or in combination with sucrose exhibited a more liquid like behaviour. However, the viscosities increased in the presence of Ca^{2+}. Gamma-irradiation reduced the peak viscosity of Agrigold cowpea seeds by 70.3% and 53.9% after 20 and 40 days of storage, respectively.

Variety, HTHH storage, and irradiation all had a highly significant effect ($P \leq 0.001$) on the breakdown viscosity of cowpea seeds (Table 4.2.4). Variety, HTHH storage, and irradiation had no significant effect ($P \leq 0.05$) on breakdown viscosity of cowpea cooked prepared pastes. HTHH storage increased the breakdown viscosity of Bechuana White cowpea seeds by 190.4% and 438.1% after 20 and 40 days, respectively (Table 4.2.1). With Agrigold cowpea seeds, breakdown viscosity increased by 871.1% and 891.9% after 20 and 40 days, respectively. Peak viscosity value minus trough viscosity determined the breakdown viscosity. Breakdown viscosity indicates the stability of peak viscosity during processing (Moorthy, 1985). Agrigold showed a higher breakdown viscosity than Bechuana White indicating that Agrigold is more susceptible to disintegration upon heating and shearing than Bechuana White (Figs 4.2.1 and 4.2.3). Gamma-irradiation reduced the breakdown viscosity of Agrigold dehulled cowpea by 76.3% and 41.5% after 20 and 40 days of storage, respectively. As for Bechuana White dehulled cowpea, decrease in breakdown was 10.4% and 70.8% after 20 and 40 days of storage, respectively. The decrease in breakdown viscosity

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can be attributed to the instability of calcium, pectin and starch interactions, which were highlighted earlier, upon heating and shearing.

Variety and irradiation had a highly significant effect ($P \leq 0.001$) on the setback viscosity of cowpea seeds (Table 4.2.6). HTHH storage had no significant effect ($P > 0.05$) on setback viscosity of cowpea seeds. After HTHH storage, they were insignificant ($P > 0.05$) increases in the setback viscosity of both Bechuana White and Agrigold cowpea seeds (Table 4.2.1). The setback viscosity from less HTC variety Bechuana White was higher compared to those of the more HTC variety Agrigold (Figs 4.2.1 and 4.2.3). Since setback is generally regarded as a measure of the retrogradation tendency of starch (Shelton and Lee, 2000), the higher setback value of Bechuana White variety indicates that it has a fast retrogradation tendency compared to Agrigold. Gamma-irradiation significantly decreased ($P \leq 0.05$) setback viscosities for both cowpea varieties. The setback viscosity of Bechuana White seeds was reduced by 78.8% and 67.0% after 20 and 40 days of HTHH storage, respectively. With Agrigold, γ-irradiation reduced the setback viscosities by 51.9% and 55.7% after 20 and 40 days of storage, respectively. The decrease in peak-, breakdown- and setback viscosity in cowpea seeds after irradiation could be attributed to changes in starch molecules such as starch degradation and debranching to form simpler units (Rombo et al., 2001; Rombo et al., 2004), inducing the reduction in paste viscosities. The decrease in viscosity in cowpea seeds after γ-irradiation has been previously reported by Falade and Kolawole (2013).

Regarding the cooked, prepared cowpea pastes, varietyhad a significant effect ($P \leq 0.05$) on the peak viscosity (Table 4.2.3). Irradiation and HTHH storage had no significant effect ($P > 0.05$) on the peak viscosity of cooked, prepared cowpea pastes. There were no significant

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differences (P>0.05) in peak viscosity of cooked, prepared cowpea pastes for both varieties after HTHH storage (Table 4.2.1). Gamma-irradiation had no significant effect (P>0.05) on peak viscosity in cooked, prepared pastes for both cowpea varieties. The peak viscosity for the cooked, prepared pastes for both cowpea varieties was substantially much lower compared to the raw seeds (Figs 4.2.2 and 4.2.4). This could be due to the pregelatinization which occurred during the cooking process, leading to granule disruption (Doublier, Colonna and Mercier, 1986; Wadchararat, Thongngam and Naivikul, 2006)

Variety, HTHH storage, and irradiation had no significant effect (P>0.05) on breakdown viscosity of dehulled cooked, prepared cowpea pastes (Table 4.2.5). There were no significant differences (P>0.05) in breakdown viscosity of dehulled cooked, prepared cowpea pastes for both varieties after HTHH storage (Table 4.2.1). Gamma-irradiation had no significant effect (P>0.05) on breakdown viscosity in dehulled cooked, prepared pastes for both cowpea varieties. The method of preparation of the pastes, which involved boiling the seeds first, could have counteracted the effects of both HTHH storage and γ-irradiation.

Gamma-irradiation had a significant effect (P≤0.05) on the setback viscosity of dehulled cooked, prepared cowpea pastes. Variety and HTHH storage had no significant effect (P>0.05) on setback viscosity of dehulled cooked, prepared cowpea pastes (Table 4.2.5). There were no significant differences (P>0.05) in setback viscosity of dehulled cooked, prepared cowpea pastes for both varieties after HTHH storage (Table 4.2.1). Gamma-irradiation had no significant effect (P>0.05) on setback viscosity in dehulled cooked, prepared pastes for both cowpea varieties. This could be generally attributed to the fact that,

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the effects of both γ-irradiation and HTHH storage diminish due to the method of paste preparation, which involved boiling first, this could have influenced the irradiation effects.

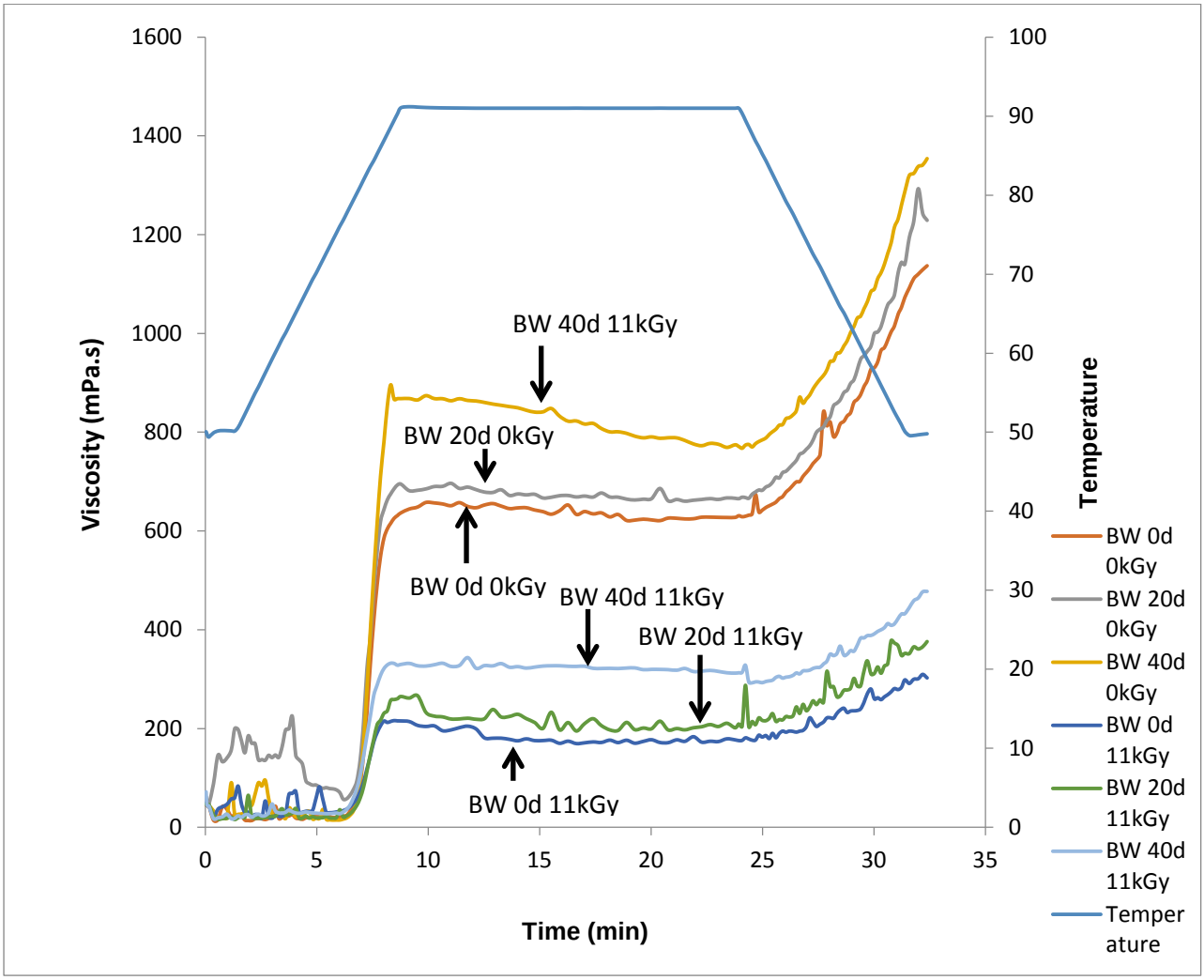

Figure 4.2.1 Effects of HTHH storage days at 40°C and 80% and γ-irradiation on the pasting properties of Bechuana White cowpea seeds. (BW-Bechuana White)

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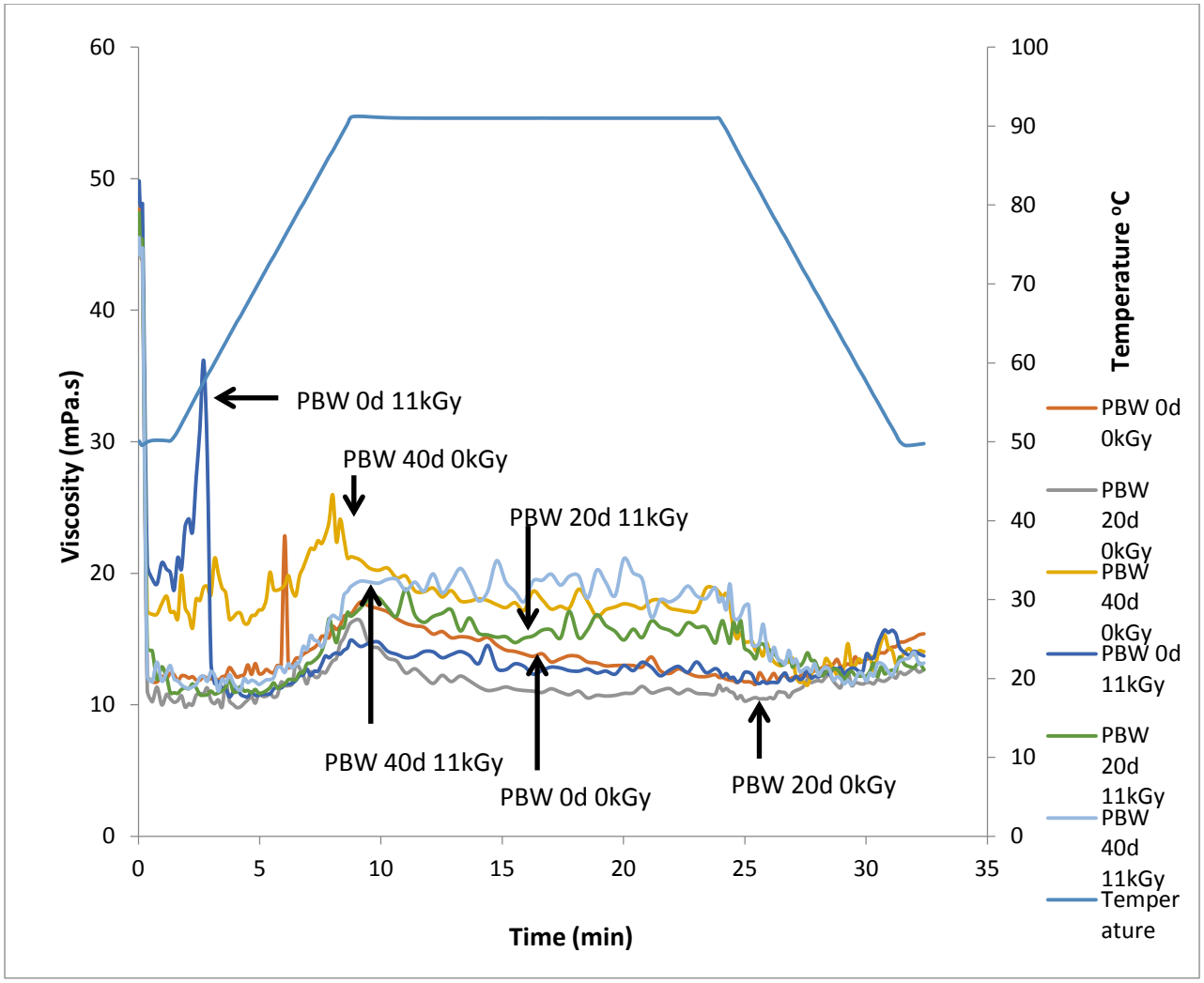

Figure 4.2.2 Effects of HTHH storage days at 40˚C and 80% and γ-irradiation on the pasting properties of Bechuana White cowpea cooked prepared pastes. (PBW-Pastes Bechuana White)

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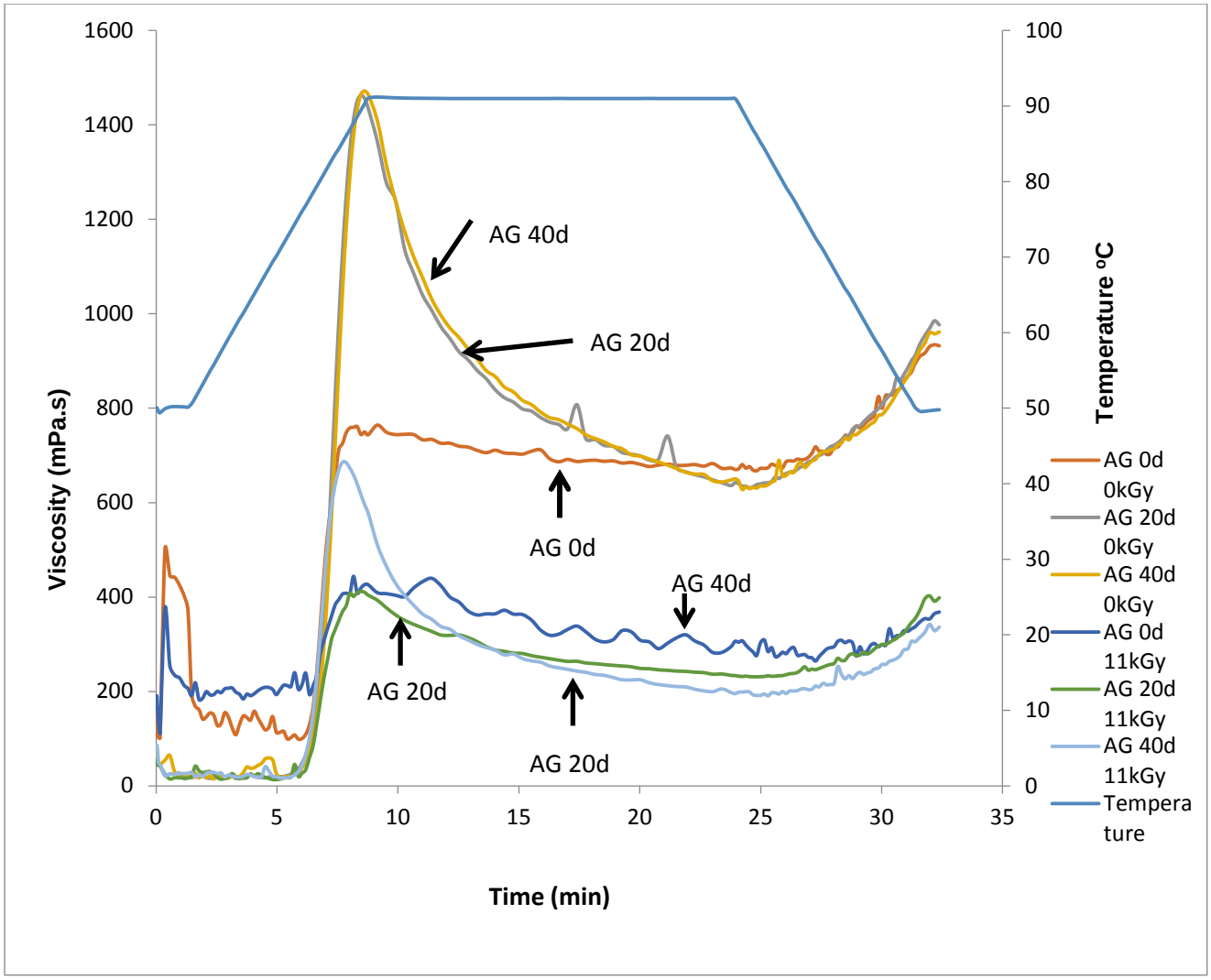

Figure 4.2.3 Effects of HTHH storage days at 40°C and 80% and γ-irradiation on the pasting properties of Agrigold cowpea seeds. (AG-Agrigold)

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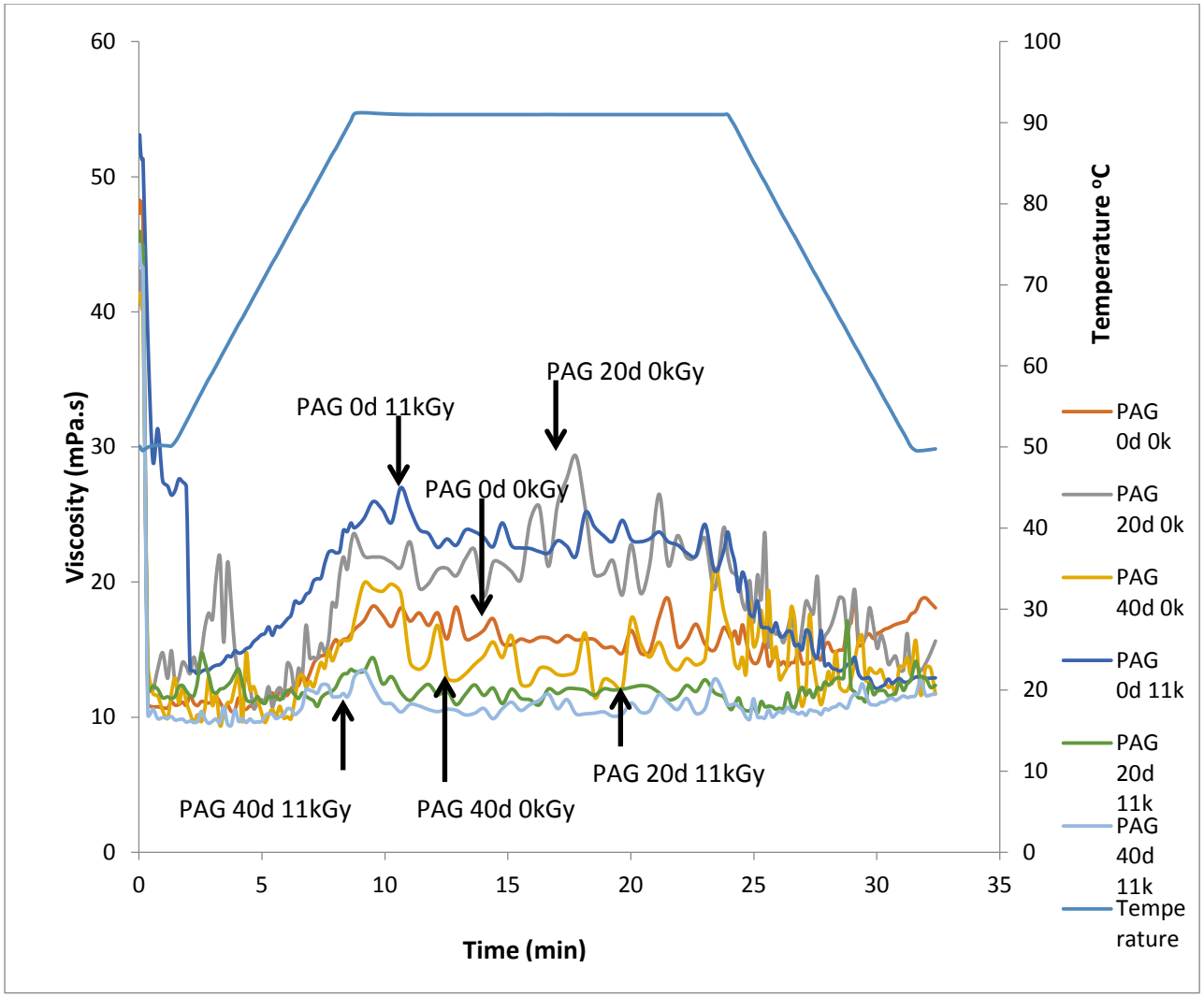

Figure 4.2.4 Effects of HTHH storage days at 40°C and 80% and γ-irradiation on the pasting properties of Agrigold cowpea cooked prepared pastes. (PAG-Pastes Agrigold)

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Table 4.2.1 Effects of HTHH storage at 40ºC and 80% RH and γ-irradiation on peak, breakdown and setback viscosities of dehulled cowpea and cooked prepared pastes of Bechuana White and Agrigold varieties

Cowpea variety[1]	Storage time	Peak Viscosity (PV) (centipoise)				Breakdown Viscosity (BV) (centipoise)				Setback Viscosity (SV) (centipoise)			
		Irradiation dose (kGy)				Irradiation dose (kGy)				Irradiation dose (kGy)			
		0		11		0		11		0		11	
			% Change due to HTHH storage		% Change due to Irradiation		% Change due to HTHH storage		% Change due to Irradiation		% Change due to HTHH storage		% Change due to Irradiation
Bechuana White Seed	0	655.5[b] (3.5)[2]		210.0[a] (8.5)	-68.0	31.5[a] (7.8)		40.5[a] (7.8)	+28.6	492.0[b] (33.9)		152.0[a] (26.9)	-69.1
	20	752.5[b] (78.5)	+14.8	284.5[a] (24.7)	-62.2	91.5[a] (77.1)	+190.4	82.0[a] (21.2)	-10.4	529.5[b] (55.9)	+7.6	112.0[a] (90.5)	-78.8
	40	922.5[c] (37.4)	+40.7	325.0[a] (26.2)	-64.8	169.5[a] (58.7)	+438.1	49.5[a] (2.1)	-70.8	548.0[b] (53.7)	+11.4	181.0[a] (5.6)	-67.0
Agrigold Seed	0	728.0[b] (50.9)		381.5[a] (88.4)	-47.6	86.5[a] (14.8)		139.5[a] (57.3)	+61.3	310.0[b] (63.6)		120.0[a] (2.7)	-61.3
	20	1464.5[c] (2.1)	+101.1	434.5[a] (34.6)	-70.3	840.0[c] (12.7)	+871.1	198.5[a] (27.6)	-76.3	341.0[b] (5.7)	+10	164.0[a] (4.2)	-51.9
	40	1515.0[c] (60.8)	+108.1	698.0[b] (17.0)	-53.9	858.0[c] (19.8)	+891.9	502.0[b] (10.0)	-41.5	327.5[b] (9.2)	+5.6	145.0[a] (0.0)	-55.7
Bechuana White Paste	0	16.5[a] (2.1)		17.5[a] (3.5)	+6.1	5.0[a] (1.4)		5.5[a] (3.5)	+10	3.0[a] (0.0)		2.0[a] (0.0)	-33.3
	20	14.5[a] (2.1)	-12.1	15.5[a] (3.5)	+6.9	4.5[a] (2.1)	-10	4.5[a] (2.1)	0	3.0[a] (0.0)	0	1.5[a] (0.7)	-50
	40	18.0[a] (4.2)	+9.1	17.5[a] (3.5)	-2.8	7.0[a] (4.2)	+40	7.0[a] (1.4)	0	2.5[a] (0.7)	-16.7	1.5[a] (0.7)	-40
Agrigold Paste	0	23.5[a] (7.8)		25.0[a] (2.8)	+6.4	10.0[a] (8.4)		14.0[a] (1.4)	+40.0	3.5[a] (0.7)		2.5[a] (2.1)	-28.5
	20	20.0[a] (2.8)	-14.9	13.0[a] (1.4)	-35.0	7.0[a] (2.8)	-30	3.0[a] (0.0)	-57.1	1.5[a] (0.7)	-57.1	1.5[a] (0.7)	0
	40	25.0[a] (2.8)	+6.4	13.5[a] (0.7)	-46.0	10.5[a] (3.5)	+5	4.0[a] (1.4)	-61.9	2.5[a] (0.7)	-28.6	2.2[a] (0.0)	-9.2

[1] For cowpea seed or paste, for each parameter, means followed by different letters in each block are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of two independent experiments.

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Table 4.2.2 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the peak viscosity of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTHH storage days	1	0.000000
Irradiation	1	0.000000
Variety x HTHH storage days	2	0.000007
Variety x Irradiation	1	0.000048
HTHH storage days x Irradiation	2	0.000008
Variety x HTHH storage days x irradiation	2	0.000038

Table 4.2.3 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the peak viscosity of dehulled cooked prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.036355
HTHH storage days	1	0.053139
Irradiation	1	0.100235
Variety x HTHH storage days	2	0.216015
Variety x Irradiation	1	0.054985
HTHH storage days x Irradiation	2	0.164913
Variety x HTHH storage days x irradiation	2	0.282010

Table 4.2.4 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the breakdown viscosity of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTHH storage days	1	0.000000
Irradiation	1	0.000000
Variety x HTHH storage days	2	0.000000
Variety x Irradiation	1	0.000001
HTHH storage days x Irradiation	2	0.000001
Variety x HTHH storage days x irradiation	2	0.000002

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Table 4.2.5 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the breakdown viscosity of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.098222
HTHH storage days	1	0.113897
Irradiation	1	0.487014
Variety x HTHH storage days	2	0.140153
Variety x Irradiation	1	0.419133
HTHH storage days x Irradiation	2	0.278438
Variety x HTHH storage days x irradiation	2	0.346074

Table 4.2.6 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the setback viscosity of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000067
HTHH storage days	1	0.340672
Irradiation	1	0.000000
Variety x HTHH storage days	2	0.374299
Variety x Irradiation	1	0.000109
HTHH storage days x Irradiation	2	0.735222
Variety x HTHH storage days x irradiation	2	0.563619

Table 4.2.7 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the setback viscosity of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	1.000000
HTHH storage days	1	0.129308
Irradiation	1	0.027915
Variety x HTHH storage days	2	0.305149
Variety x Irradiation	1	0.337049
HTHH storage days x Irradiation	2	0.939717
Variety x HTHH storage days x irradiation	2	0.655547

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4.2.4.2 Effects of HTHH storage and γ-irradiation on nitrogen solubility index (NSI)

Variety and HTHH storage had a highly significant effect ($P \leq 0.001$) on NSI of the dehulled cowpea (Table 4.2.9). Irradiation had a significant effect ($P \leq 0.05$) on NSI of cowpea seeds. As for the dehulled cooked, prepared pastes, HTHH storage had a significant effect ($P \leq 0.01$) on NSI (Table 4.2.10). Variety and irradiation had no significant effect ($P > 0.05$) on NSI of dehulled cowpea cooked prepared pastes. However, variety in combination with irradiation had a highly significant effect ($P \leq 0.001$) on NSI of the dehulled cowpea cooked prepared pastes. HTHH storage significantly decreased ($P \leq 0.05$) the NSI of Bechuana White, and Agrigold dehulled cowpea and dehulled cooked prepared pastes, with the exception of Bechuana White dehulled cooked cowpea pastes (Table 4.2.8). The NSI of Bechuana White dehulled cowpea were decreased by 18.4% and 30.0% after 20 and 40 days of storage, respectively. The NSI of Agrigold dehulled cowpea were decreased by 63.6% and 65.4% after 20 and 40 days of storage, respectively. NSI of cooked Agrigold dehulled cooked prepared pastes were decreased by 33.9% and 52.1% after 20 and 40 days of storage, respectively. The decrease in protein solubility in cowpea due to HTHH storage has been reported previously by Liu et al. (1992). The authors found a decrease in protein thermal stability and solubility during HTHH storage in cowpeas, which they suggested was as a result of protein hydrolysis and aggregation reactions. Generally, in this present research the NSI was substantially much lower in the cooked, prepared pastes, and this could be due to protein denaturation during cooking of the cowpea seeds. Gamma-irradiation caused a significant decrease ($P \leq 0.05$) in NSI of Bechuana White dehulled cowpea before storage (Table 4.2.8). However, γ-irradiation, generally, caused an insignificant ($P > 0.05$) but a consistent decrease in NSI of Agrigold dehulled cowpea, Agrigold dehulled cooked prepared pastes before storage, and Bechuana White dehulled pastes before and after storage. The decrease in NSI solubility has been previously reported in cowpeas by Abu et al. (2005). The

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decrease in solubility could be due to protein denaturation, resulting in exposure of hydrophobic groups, followed by aggregation of the unfolded protein molecules (Cheftel, Cuq and Lorent, 1985). Furthermore, the decrease in solubility during irradiation could be due to Maillard type reactions involving protein resulting in products that are less soluble, as suggested by Abu et al. (2005). Surprisingly with the more HTC variety, there were not always decreases in NSI after γ-irradiation. In fact, there were also increases that occurred particularly after HTHH storage. As highlighted earlier, since protein solubility decreases with HTC development possibly due to protein hydrolysis and aggregation reactions as suggested by Liu et al. (1992). It is probable that irradiation-induced denaturation could have resulted in burying of previously exposed hydrophobic groups consequently increasing protein solubility.

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Table 4.2.8 Effects of HTHH storage days at 40°C and 80% RH and γ-irradiation on the nitrogen solubility index of dehulled cowpea and cooked prepared pastes in Bechuana White and Agrigold cowpeas

Cowpea Variety	Storage time (days)	Nitrogen Solubility Index (NSI)				
		Irradiation dose (kGy)				
		0		11		
			% Change due to HTHH storage		% Change due to HTHH storage	% Change due to Irradiation
	Seeds					
Bechuana White	0	48.3^{c} (0.6)[2]		37.2^{b} (2.5)		-23.0
	20	39.4^{b} (3.6)	-18.4	34.2^{ab} (0.1)	-8.1	-13.2
	40	33.8^{ab} (2.5)	-30.0	28.0^{a} (0.7)	-24.7	-17.1
Agrigold	0	43.4^{c} (3.9)		37.2^{bc} (3.4)		-14.3
	20	15.8^{a} (0.3)	-63.6	30.1^{b} (0.4)	-19.1	+90.5
	40	15.0^{a} (1.6)	-65.4	13.0^{a} (0.7)	-65.1	-13.3
	Pastes					
Bechuana White	0	13.6^{a} (3.9)		9.9^{a} (0.2)		-27.2
	20	11.0^{a} (1.1)	-19.1	8.9^{a} (2.2)	-10.1	-19.1
	40	10.8^{a} (1.3)	-20.6	7.5^{a} (0.1)	-24.2	-30.1
Agrigold	0	12.1^{cd} (0.0)		10.7^{cd} (0.2)		-11.6
	20	8.0^{b} (0.2)	-33.9	12.7^{d} (0.0)	+18.7	+58.8
	40	5.8^{a} (0.1)	-52.1	10.1^{c} (1.2)	-5.6	+74.1

[1] For cowpea seed or paste, means followed by different letters in a block across are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of two independent experiments.

Table 4.2.9 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the nitrogen solubility index of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTHH storage days	1	0.000000
Irradiation	1	0.011292
Variety x HTHH storage days	2	0.000061
Variety x Irradiation	1	0.000191
HTHH storage days x Irradiation	2	0.000192
Variety x HTHH storage days x irradiation	2	0.006454

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Table 4.2.10 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the nitrogen solubility index of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.554969
HTHH storage days	1	0.004499
Irradiation	1	0.705441
Variety x HTHH storage days	2	0.559356
Variety x Irradiation	1	0.000568
HTHH storage days x Irradiation	2	0.049379
Variety x HTHH storage days x irradiation	2	0.196225

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Table 4.2.11 Effects of HTHH storage at 40ºC and 80% RH and γ-irradiation on functional properties of dehulled Bechuana White and Agrigold cowpea and cooked prepared pastes

Cowpea variety[1]	Storage Time	Water absorption capacity (WAC) (%)					Swelling power (SP)					Water solubility Index (WSI)				
		Irradiation dose (kGy) 0		11			Irradiation dose (kGy) 0		11			Irradiation dose (kGy) 0		11		
			% Change due to HTHH storage		% Change due to HTHH storage	% Change due to Irradiation		% Change due to HTHH storage		% Change due to HTHH storage	% Change due to Irradiation		% Change due to HTHH storage		% Change due to HTHH storage	% Change due to Irradiation
Bechuana White seeds	0	86.1^{a} (1.5)[2]		85.4^{a} (2.8)		-0.8	8.3^{bc} (0.2)		7.1^{a} (0.1)		-14.5	71.2^{ab} (6.5)		61.1^{a} (1.0)		-14.2
	20	91.7^{ab} (3.6)	+6.5	100.3^{bc} (3.4)	+17.4	+9.4	7.8^{ab} (0.6)	-6	6.8^{a} (0.2)	-4.2	-12.8	76.2^{b} (2.0)	+7	67.2^{ab} (1.8)	+10	-11.8
	40	92.2^{ab} (2.0)	+7.1	108.1^{c} (1.3)	+26.6	+17.2	9.3^{c} (0.2)	+12	6.7^{a} (0.0)	-5.6	-39.8	72.1^{ab} (2.4)	+1.3	69.5^{ab} (1.6)	+13.7	-2.3
Agrigold Seeds	0	84.7a (0.8)		97.4^{b} (1.6)		+15	8.1^{bc} (0.1)		6.6^{a} (0.1)		-18.5	74.2^{a} (3.4)		63.6^{a} (2.7)		-14.3
	20	125.2d (3.6)	47.8	112.1^{c} (0.1)	+15.1	-10.5	8.9^{c} (0.2)	+9.9	7.0^{ab} (0.3)	6.1	-21.3	76.4^{a} (0.3)	+3	64.6^{a} (4.3)	+1.6	-15.4
	40	146.6^{e} (0.9)	73.1	149.7^{e} (4.2)	+53.7	+2.1	9.1^{c} (0.6)	+12.3	7.2^{ab} (0.0)	9.1	-20.9	78.6^{a} (8.2)	+5.9	67.6^{a} (2.0)	+6.3	-14
Bechuana White Paste	0	153.0^{ab} (6.0)		166.9^{b} (6.8)		+9.1	5.3^{a} (0.0)		5.5^{a} (0.3)		+3.8	84.2^{a} (1.4)		85.4^{a} (1.2)		+1.4
	20	148.5^{ab} (2.9)	-2.9	154.2^{ab} (10.7)	-7.6	+3.8	5.5^{a} (0.6)	+3.8	5.2^{a} (0.0)	-5.5	5.5	87.0^{a} (0.9)	+3.3	84.2^{a} (0.3)	-1.4	-3.2
	40	140.0^{a} (1.8)	-8.5	138.9^{a} (7.4)	-16.8	-0.8	5.2^{a} (0.2)	-1.9	5.1^{a} (0.2)	-7.3	-1.9	86.7^{a} (1.4)	-3	87.0^{a} (1.4)	+1.9	+0.3
Agrigold Paste	0	160.0^{cd} (8.5)		163.9^{d} (1.9)		+2.4	5.8^{b} (0.2)		5.4^{ab} (0.0)		-6.9	85.7^{d} (0.7)		84.7^{a} (0.4)		-1.2
	20	129.3^{b} (0.1)	-19.2	145.4^{c} (1.7)	-11.3	+12.5	4.7^{a} (0.3)	-19	4.9^{a} (0.1)	-9.2	+4.3	84.3^{a} (4.4)	-1.6	80.3^{a} (0.1)	-5.2	-4.7
	40	111.5^{a} (0.5)	-30.3	164.9^{d} (0.5)	+0.6	+47.9	4.6^{a} (0.3)	-20.7	5.2^{ab} (0.2)	-3.7	+13	86.3^{a} (2.5)	+0.6	83.6^{a} (4.6)	-1.3	-3.1

[1] For cowpea seed or paste, for each parameter, means followed by different letters in a block are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of two independent experiments.

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Table 4.2.12 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the water absorption capacities of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000000
HTHH storage days	1	0.000000
Irradiation	1	0.000994
Variety x HTHH storage days	2	0.000000
Variety x Irradiation	1	0.004823
HTHH storage days x Irradiation	2	0.001609
Variety x HTHH storage days x irradiation	2	0.000041

Table 4.2.13 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the water absorption capacities of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.065378
HTHH storage days	1	0.000007
Irradiation	1	0.000015
Variety x HTHH storage days	2	0.026400
Variety x Irradiation	1	0.001253
HTHH storage days x Irradiation	2	0.013939
Variety x HTHH storage days x irradiation	2	0.000192

Table 4.2.14 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the swelling power of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.274126
HTHH storage days	1	0.006772
Irradiation	1	0.000000
Variety x HTHH storage days	2	0.010020
Variety x Irradiation	1	0.510159
HTHH storage days x Irradiation	2	0.016241
Variety x HTHH storage days x irradiation	2	0.046955

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Table 4.2.15 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the swelling power of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.027210
HTHH storage days	1	0.000911
Irradiation	1	0.804778
Variety x HTHH storage days	2	0.011568
Variety x Irradiation	1	0.327591
HTHH storage days x Irradiation	2	0.183182
Variety x HTHH storage days x irradiation	2	0.009175

Table 4.2.16 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the water solubility index of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.415151
HTHH storage days	1	0.080522
Irradiation	1	0.000059
Variety x HTHH storage days	2	0.539318
Variety x Irradiation	1	0.218390
HTHH storage days x Irradiation	2	0.547542
Variety x HTHH storage days x irradiation	2	0.570637

Table 4.2.17 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation dose on the water solubility index of dehulled cooked, prepared pastes from Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.087848
HTHH storage days	1	0.240939
Irradiation	1	0.105353
Variety x HTHH storage days	2	0.259448
Variety x Irradiation	1	0.246473
HTHH storage days x Irradiation	2	0.294395
Variety x HTHH storage days x irradiation	2	0.919730

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4.2.4.3 Effects of HTHH storage and γ-irradiation on water absorption capacity (WAC)

Variety, HTHH storage, and irradiation all had a highly significant effect ($P \leq 0.001$) on WAC of dehulled cowpea (Table 4.2.12). HTHH storage significantly increased ($P \leq 0.05$) the WAC of the more HTC susceptible Agrigold dehulled cowpea (Table 4.2.11). In contrast, HTHH storage had no significant effect ($P > 0.05$) on WAC of Bechuana White dehulled cowpea. HTHH storage substantially increased WAC of Agrigold dehulled cowpea by 47.8% and 73.1% after 20 and 40 days of storage, respectively. The high water holding capacity of HTC dehulled cowpea seed supports the increase in paste viscosity observed with the more HTC variety. The increase in water absorption capacity could also probably have been due to interactions of Ca^{2+} with pectin and starch. Pectin dispersions with added Ca^{2+} and pectin and added sucrose have been found to have higher complex viscosities (Norziah et al., 2001). Gamma-irradiation significantly increased ($P \leq 0.05$) the WAC of HTC Bechuana White. In Agrigold dehulled cowpea, irradiation increased WAC before HTHH storage, with no substantial effect after storage.

HTHH storage and irradiation had a highly significant effect ($P \leq 0.001$) on WAC of dehulled cooked, prepared cowpea pastes (Table 4.2.13). Variety had no significant effect ($P > 0.05$) on the WAC of cooked dehulled cooked cowpea pastes. However, variety in combination with irradiation had a significant effect ($P \leq 0.01$) on WAC of cooked, prepared cowpea pastes. HTHH storage caused an insignificant decrease ($P > 0.05$) in WAC of dehulled cooked, prepared cowpea pastes from Bechuana White cowpeas (Table 4.2.11). The WAC of Agrigold dehulled cooked prepared pastes were significantly decreased ($P \leq 0.05$) with HTHH storage. Since the NSI in dehulled cooked, prepared cowpea pastes were lower than in the

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raw cowpea seeds (Table 4.2.8) presumably due to protein denaturation,this could have possibly affected WAC of the cooked, prepared pastes since protein also plays a role in water absorption in legumes (Du, Jiang, Yu and Jane, 2014). Gamma-irradiation significantly increased ($P \leq 0.05$) the WAC of Agrigold dehulled cooked prepared pastes after HTHH storage (Table 4.2.11). However, γ-irradiation had no significant effect ($P > 0.05$) on WAC of Bechuana White dehulled cooked prepared pastes after HTHH storage. This was probably due to irradiation-induced unfolding which could have exposed non-polar protein sites reducing the availability of polar amino groups for water binding (Zayas, 1997). Furthermore, the differences in the WAC of the two varieties after γ-irradiation could be due to differences in the protein structure and amount of polar amino acids in the proteins of the two varieties (Yi-Shen, Shuai, and FitzGerald, 2018).

4.2.4.4 Effects of HTHH storage and γ-irradiation on Swelling power (SP)

Irradiation had a highly significant effect ($P \leq 0.001$) on SP of dehulled cowpea (Table 4.2.14). HTHH storage had a significant effect ($P \leq 0.01$) on SP of dehulled cowpea. Variety had no significant effect ($P > 0.05$) on SP of dehulled cowpea. However, variety in combination with HTHH storage had a significant effect ($P \leq 0.05$) on SP of dehulled cowpea. HTHH storage did not have a significant effect ($P > 0.05$), but a consistent increase in SP of both Agrigold and Bechuana White dehulled cowpeas (Table 4.2.11). The increase in SP probably contributed to the increase in peak viscosity observed (Table 4.2.1). Generally for both cowpea varieties, SP significantly decreased ($P \leq 0.05$) after γ-irradiation (Table 4.2.11). Since swelling behaviour is attributed to the amylopectin fraction (Tester and Morrison, 1990), the reduction in swelling capacities could be due to the depolymerisation of

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amylopectin fraction. The irradiation-induced partial depolymerisation of amylopectin was probably the cause of the reduction in paste viscosity (Table 4.2.1).

HTHH storage had a highly significant effect ($P \leq 0.001$) on SP of cowpea dehulled cooked prepared pastes (Table 4.2.15). Variety had a significant effect ($P \leq 0.05$) on SP of dehulled cowpea cooked prepared pastes. However, irradiation had no significant effect ($P > 0.05$) on SP of dehulled cowpea cooked prepared pastes. HTHH storage caused a significant decrease ($P \leq 0.05$) in SP of Agrigold dehulled cowpea (Table 4.2.11). However, HTHH storage had no significant effect ($P > 0.05$) on SP of Bechuana White dehulled cooked prepared pastes. The SP of Agrigold dehulled cooked prepared pastes were decreased by 19.0% and 20.7% after 20 and 40 days of storage, respectively. The decrease in SP could be due to additional interactions which might have occurred such as between amylose-amylose and amylose-amylopectin chains (Gunaratne and Hoover, 2002). The associations that may have formed during starch retrogradation could have probably limited swelling potential during the subsequent gelatinisation, as suggested by Mwangela, Waniska, McDonough and Minnaar, 2007). A decrease in SP has also been associated with the formation of protein-amylose complex in native starches and flours (Pomeranz, 1991). Furthermore, the difference in swelling between HTC Agrigold and Bechuana White dehulled cooked prepared pastes could be attributed to varietal differences in the molecular structure of the starch within the granule.

4.2.4.5 Effects of HTHH storage and γ-irradiation on water solubility index

Irradiation had a highly significant effect ($P \leq 0.001$) on the WSI of the dehulled cowpeas (Table 4.2.16). HTHH storage and variety had no significant effect ($P > 0.05$) on the WSI of

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the dehulled cowpea. Generally, there were no significant differences (P>0.05) in the WSI of dehulled cowpea for both varieties after HTHH storage (Table 4.2.11). This could probably be due to the formation of protein-amylose complex in native starches and flours (Pomeranz, 1991). Gamma-irradiation caused an insignificant (P>0.05) but a consistent decrease in WSI of dehulled cowpea seeds for both cowpea varieties. Since WSI was analysed at a higher temperature 95°C, the starch probably could be surrounded by the hydrophobic protein, which could have contributed to the decrease in WSI.

Variety, HTHH storage, and irradiation had no significant effect (P>0.05) on WSI of dehulled cooked, prepared cowpea pastes (Table 4.2.17). There were also no significant differences (P>0.05) in WSI of dehulled cooked, prepared cowpea pastes for both varieties after HTHH storage (Table 4.2.11). Gamma-irradiation also had no significant effect (P>0.05) on the WSI of dehulled cooked, prepared pastes for both cowpea varieties. This was probably due to the method of preparation of dehulled cooked, prepared cowpea pastes which resulted in pre-gelatinisation of starch. Furthermore, the method of preparation of the pastes most probably resulted in the loss of the water soluble fraction (Fasasi, Adeyemi, and Fagbenro, 2007).

4.2.4.6 Effects of HTHH storage days and γ-irradiation on the thermal properties

Variety had significant effects (P≤0.01) on the transition onset (T_o), transition peak (T_p) and gelatinisation enthalpy of dehulled cowpea (Tables 4.2.19, 4.2.20, 4.2.22). However, HTHH storage and irradiation had no significant effect (P>0.05) on the transition onset (T_o), transition peak (T_p) and gelatinisation enthalpy of dehulled cowpea. Furthermore, variety,

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HTHH storage, and irradiation had no significant effect (P>0.05) on the transition endset (T_c) of dehulled cowpea (Table 4.2.21). HTHH storage and γ-irradiation had no significant effect (P>0.05) on the onset, peak, endset and gelatinisation enthalpy for both dehulled cowpeas (Table 4.2.18). The transition onset (T_o) and peak (T_p) temperature in both varieties were indicative of endothermic transitions associated with starch gelatinisation in cowpea. The values are generally in agreement with those in the literature, which show values for cowpea starch to be between 67 to 78ºC (Abu et al., 2006b; Mwangela et al., 2006). Despite the fact that Agrigold was the more HTC variety, intrinsically the starch gelatinised at a lower temperature and had a lower gelatinisation enthalpy compared to the less HTC Bechuana White. This strongly supports the concept that changes in starch are a consequence of the defect, and starch does not play a role in the development of the HTC defect in legumes . As for the cooked, prepared cowpea pastes, they did not show any peaks for endothermic transitions, associated with starch gelatinisation. This almost certainly is due to the cooking process exposed to the cowpea seeds during paste preparation, which resulted in the pre-gelatinisation of starch. Furthermore, they were no observable protein thermal transitions.

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Table 4.2.18 Effects of HTHH storage days at 40°C and 80% RH and for γ-irradiation on the thermal properties of dehulled Bechuana White and Agrigold cowpeas

Cowpea variety[1]	Storage time (days)	Onset T_o°C		Peak T_p°C		Endset T_c°C		ΔH(J/G)	
		Irradiation dose (kGy)		Irradiation dose (kGy)		Irradiation dose (kGy)		Irradiation dose (kGy)	
		0	11	0	11	0	11	0	11
Bechuana White	0	73.3[a] (1.1)[2]	72.8[a] (0.2)	76.4[a] (0.5)	76.6[a] (0.4)	78.6[a] (0.6)	79.6[a] (1.5)	17.2[a] (10.5)	29.5[a] (2.0)
	20	72.2[a] (0.2)	72.9[a] (0.6)	77.4[a] (1.2)	76.5[a] (0.0)	80.0[a] (0.8)	78.6[a] (3.1)	26.5[a] (10.7)	23.3[a] (9.9)
	40	73.3[a] (1.6)	72.9[a] (0.1)	77.9[a] (1.4)	76.4[a] (0.1)	78.7[a] (3.8)	79.0[a] (1.3)	29.6[a] (8.5)	22.3[a] (9.5)
Agrigold	0	70.4[a] (3.0)	70.6[a] (2.5)	75.8[a] (2.4)	75.7[a] (2.6)	80.3[a] (1.4)	79.8[a] (1.9)	9.6[a] (7.7)	10.5[a] (3.3)
	20	68.2[a] (0.6)	70.7[a] (0.5)	72.7[a] (0.2)	73.8[a] (0.3)	77.4[a] (2.3)	77.0[a] (1.0)	5.7[a] (0.9)	10.1[a] (7.3)
	40	69.9[a] (0.3)	71.2[a] (2.2)	74.1[a] (1.3)	74.4[a] (2.6)	78.3[a] (3.3)	78.9[a] (1.2)	8.6[a] (9.8)	7.3[a] (1.2)

[1] For each parameter, means followed by different letters in each block are significantly different at $P \leq 0.05$.
[2] Means (±) standard deviations of two independent experiments.

Table 4.2.19 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation dose on the transition onset (T_o) of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000548
HTHH storage days	1	0.452444
Irradiation	1	0.309026
Variety x HTHH storage days	2	0.933555
Variety x Irradiation	1	0.268056
HTHH storage days x Irradiation	2	0.497965
Variety x HTHH storage days x irradiation	2	0.918938

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Table 4.2.20 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the transition peak (T_p) of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.001211
HTHH storage days	1	0.377151
Irradiation	1	0.822414
Variety x HTHH storage days	2	0.152895
Variety x Irradiation	1	0.327678
HTHH storage days x Irradiation	2	0.860606
Variety x HTHH storage days x irradiation	2	0.691895

Table 4.2.21 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the transition endset (T_c) of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.625552
HTHH storage days	1	0.449015
Irradiation	1	0.941543
Variety x HTHH storage days	2	0.365443
Variety x Irradiation	1	0.984042
HTHH storage days x Irradiation	2	0.786461
Variety x HTHH storage days x irradiation	2	0.831678

Table 4.2.22 Multifactor ANOVA of the effect of cowpea variety and in combination with HTHH storage days and Irradiation on the gelatinisation enthalpy (ΔH) of dehulled Bechuana White and Agrigold cowpeas

Treatment	Degrees of freedom	*P* value
Variety	1	0.000483
HTHH storage days	1	0.991447
Irradiation	1	0.781698
Variety x HTHH storage days	2	0.839143
Variety x Irradiation	1	0.912721
HTHH storage days x Irradiation	2	0.450020
Variety x HTHH storage days x irradiation	2	0.475701

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4.2.5 Conclusions

Application of γ-irradiation to HTC cowpea seeds increases water absorption capacity (WAC), and decreases water solubility index (WSI), swelling power (SP) and pasting properties. The effects of γ-irradiation were mainly related to starch-related functional properties, decreasing WSI, SP, peak-, breakdown and setback viscosities for the HTC highly and less susceptible HTC defect type cowpea, presumably due to starch depolymerisation. However, γ-irradiation does not affect thermal properties in HTC cowpeas. With irradiation, the WAC of dehulled cowpea was affected differently, with increases associated with less HTC susceptible variety. Since γ-irradiation affects all starch related functional properties with the exception of thermal properties, the non-reversal of the HTC defect by γ-irradiation, suggests that changes in starch during HTC development are a consequence and not a cause of the HTC defect. Furthermore, they were no differences in the functional properties of pastes from both varieties after γ-irradiation; hence dehulling, cooking, and preparation of HTC cowpea pastes eradicate differences in the functional properties of cowpea pastes. Thus, the HTC cowpea flour rather than seeds can be utilised in the preparation of flour-based products

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4.2.6 References

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5 GENERAL DISCUSSION

This general discussion is divided into four sections. The first is a critical review of the experimental design and methodologies applied in this research project. The second section focuses on the main research findings and concerns the proposed mechanisms involved in HTC development and building on these to develop a model with findings from this present research as to how the HTC defect develops in cowpeas. The third section concerns future research arising from the findings of this project. The final section focuses on the conclusions and recommendations based on the findings from the study.

5.1 A CRITICAL REVIEW OF EXPERIMENTAL DESIGN AND METHODOLOGIES

The main purpose of the study was to apply γ-irradiation to two cowpea varieties with different susceptibility to HTC defect to further elucidate the mechanisms responsible for HTC defect development.

The two cowpea varieties were selected based on their susceptibility to HTC defect (unpublished data). Bechuana White cowpea was the variety less susceptible to development of the HTC defect and Agrigold cowpeas more susceptible to development of the defect. The HTC defect was induced in the cowpea varieties by storing them at high temperature (40°C) and high relative humidity (80%). The HTC defect occurs when legumes are stored under tropical conditions (Hägg, Ahvenainen, Evers and Tiilikkala, 1999). In the current study, accelerated storage was used to mimic tropical conditions. Jackson and Varriano-Marston (1981) demonstrated the ability to achieve HTC defect in common beans in a relatively short period of two weeks, with results comparable to those obtained when stored under tropical conditions for 1 year. In the current study, the two cowpea varieties were stored for 20 and 40

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days under accelerated tropical-type conditions, and the period was sufficient to induce the HTC defect as indicated by the substantial increase in cooking time to greater than 10 hours in the more HTC variety.

5.1.1 Cooking time

The cooking quality of legumes is a significant parameter that can influence consumer acceptance. The cooking quality characteristics of legumes that are considered important include cooking time, cooked texture, appearance and flavour (Tiwari and Singh, 2012). Cooking time is the most commonly used characteristic for evaluating the cooking quality of legumes (Ehlers and Hall, 1997). Legumes are usually deemed cooked when they have reached an acceptable softness for consumption (Proctor and Watts, 1987). Based on the length of cooking time, pulses can thus be classified as easy-to-cook or hard-to-cook. The most common method used to evaluate cooking time of dry legumes is using the Mattson bean cooker (MBC) (Arruda, Guidolin, Coimbra and Battilana, 2012). It is a simple method, which provides objective data of cooking times of pulses. In the present study, cooking time was determined using a MBC as modified by Jackson and Varriano-Marston (1981) with 25 plungers. The MBC has drawbacks. For example, careful attention has to be paid to place the plunger pins on the surface of the seeds. Furthermore, careful uninterrupted attention is required by the operator to monitor when the rods penetrate seeds during cooking (Carvalho et al., 2017). This task can be very tedious especially when cooking HTC seeds which, as observed in this study, have cooking times greater than 10 hours. In addition, it is difficult to count accurately when several plungers fall at once (Wang and Daun, 2005). To overcome these drawbacks of the traditional MBC, an automated MBC can be utilised. The automated MBC does not require uninterrupted attention as a computer monitors the cooking process

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and stops once the test parameters have been reached (Miller and Wang, 2013). Furthermore, the computer records data of each plunger drop on a spreadsheet, allowing the data to be easily organised to charts, graphs and other forms of statistical analysis. The automated MBC can accurately record plunger drops even when several occur simultaneously. However, although the automated MBC addresses most of the challenges associated with the traditional MBC, it is relatively expensive. Furthermore, although both MBC types provide objective data, the cooking times of easy-to-cook, HTHH stored and irradiated cowpeas obtained in this study most probably do not reflect the actual cooking time by the consumer. A more representative cooking time for the cowpea seeds could have been obtained by correlation of cooking time to consumer sensory analysis. Notwithstanding this, the MBC provided an objective quantitative basis for comparison of cooking times for the two cowpea varieties.

5.1.2 Phytate

The levels of phytate in the cowpea seeds were determined using an indirect quantitative analysis method, as described by Frubeck et al. (1995). Anion exchange chromatography was applied to remove inorganic phosphate, followed by colorimetric determination of phosphate at 500 nm based on the pink colour of Wade reagent. Anion exchange methods are some of the most widely used for phytate determination (Preedy, 2016). The method used in this study is suitable for phytate determination especially for legumes, which was the plant material that was focused on when the method was developed. The method is relatively simple, precise and reproducible for quantitatively assessing total phytate (Frubeck et al., 1995). However, the drawbacks to this method are that it is relatively time consuming due to the anion exchange chromatographic separation of phytate and it can overestimate the quantity of phytate since it includes partially dephosphorylated isomers of phytic acid (Wu, Tian, Walker

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and Wang, 2009). In addition, it does not determine the lower inositol esters such as penta- and tetra-phosphates (Sandberg, 1995). Phytate determination could have been improved by the use of high performance anion exchange (HPAE) chromatography. HPAE inositol phosphate separation can provide rapid (~10 min) sample phytate analysis capabilities (Thavarajah and Thavarajah, 2014). HPAE can also separate inositol phosphates based on the number of phosphate groups, including different isomeric forms (except enantiomers) (Thavarajah and Thavarajah, 2014). Although HPAE may be superior to traditional methods of phytate analysis, the technique is relatively expensive and requires experience to set up and operate (Wu et al., 2009).

5.1.3 Pectin

Pectin plays a significant role in determining the cooking behaviour of pulses. In the present study, the pectin fractions estimated were CWSP, HWSP, and CSP. These three fractions indicated changes occurring to pectin with HTC development, such as a decrease in solubility. However, more detailed analysis of the fractions could have helped understand the changes in characteristics and composition of the pectin fractions in the two HTC cowpea varieties, and/or probable interactions of pectin with other constituents of the cotyledon cell walls.

Firstly, other cotyledon cell wall fractions which include alkali-soluble pectins and hemicellulose could have been extracted and characterised to better understand changes which could have influenced limited cell separation. The pectin fraction that is soluble in Na_2CO_3, could have been extracted and characterised, since it has been shown to increase during HTC defect development (Shiga et al., 2009). This pectin fraction is predominantly

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linked to the cell wall polysaccharides through covalent ester bonds (Renard and Ginies, 2009). Furthermore, plant materials with a lower water soluble polymer fraction and higher non-starch polysaccharide (NSP) fraction have a longer cooking time due to the insensitivity of the NSP to the beta-elimination process, as described by Sila, Smout, Elliot,Van Loey and Hendrickx (2006) and Shiga et al. (2004). Hence, the Na_2CO_3 soluble pectin fraction is important to characterise. In addition, the hemicellulose fractions could also have been isolated and characterised. The Na_2CO_3 soluble fraction also contains residual pectins that are strongly bound to cellulose or hemicellulose (Sila et al., 2006).

Secondly, with regard to characterisation of the pectin fractions (CWSP, HWSP and CSP), the molar mass distribution could have been determined by high performance size exclusion chromatography. This would have provided more information on the changes that occur to the pectin polymers with HTC development. This is because other authors such as Shiga et al. (2009) found a decrease in the water soluble fraction, with a corresponding increase in the water-insoluble fraction. Furthermore, common beans treated with $CaCl_2$ have been found to show an increase in molar mass of the polymers coupled with limited cell separation (Njoroge, Kinyanjui, Chigwedere, Christiaens, Makokha, Sila and Hendrickx, 2016). Other properties of the pectin fractions that could have been analysed include sugar composition and degree of methylesterification (DM). Legume cotyledon cell walls are generally indicated to be rich in arabinan (Shiga et al., 2009). The neutral sugar composition could have helped understand the potential interactions of pectin and other cell wall polymers. This is because water-insoluble polymers of poor cooking beans have higher amounts of arabinose, and arabinan insolubility is associated with HTHH stored beans (Shiga et al., 2004; Shiga et al., 2009). Hence, measuring the changes in this sugar composition with HTC development could also have helped further understand the mechanisms of HTC development. Knowing the DM

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of pectin would also have been useful since it influences the interaction of the pectin with divalent ions in the cotyledon cell wall (Tucker and Mitchell, 1993). Low methylated pectin is easily cross-linked via Ca^{2+} bridges between two carboxylates from different chains (Alexos and Thibault, 1991). Knowing the DM of the pectin could probably also have helped explain the differences in susceptibility to HTC of the two cowpea varieties. However, the pectin fractions that were analysed in this study which include CWSP, HWSP and CSP were adequate in that they managed to indicate decrease in pectin solubility, and varietal differences that occur in pectin with HTC development.

5.1.4 Protein

From the research Chapter 4.2, it was evident that protein may be involved in the HTC development since its solubility decreased under HTHH conditions. This finding agrees with findings of the study by Liu et al. (1992) which showed a decrease in protein solubility with HTC development in common beans. However, NSI alone, which was measured in the present study, did not provide adequate information on the changes which occurred to the protein component of the HTC cowpea varieties. To address this, more detailed analyses, such as SDS-PAGE and size exclusion chromatography could have been used to investigate the effects of HTC and γ-irradiation on the molecular weight and subunit distribution of the cowpea proteins and formation of disulphide crosslinks. Furthermore, FT-IR spectroscopy could have been used to study the secondary protein structure evaluation. Pamar et al. (2017) used SDS-PAGE to study the protein in HTC common beans and found an increase in accumulation of 27 kDa polypeptides compared to their counterpart easy-to-cook common beans. Also, using size exclusion chromatography they found an increase in small polypeptide chains and a higher proportion of ß-sheets and α-helix, and a lower proportion of

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anti-parallel ß-sheets and ß-turns as compared to their counterpart easy-to-cook was observed using FT-IR. Protein solubility is used mostly as an indicator of other functional properties. It can be used to predict the performance of protein in emulsions, foams and gels (Zayas 1997). NSI specifically is one of the most widely used quick tests for predicting protein functionality. In this particular study, NSI revealed that changes occur to protein during HTC development.

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5.2 SUMMARY OF THE MAIN RESEARCH FINDINGS

In this study, the application of γ-irradiation to understand the mechanisms involved in HTC development led to a number of significant research findings (Tables 5.1 and 5.2).

Table 5.1. Summary of the main research findings on effects of γ-irradiation on cotyledon cell separation and pectin solubilisation in HTC cowpeas from research Chapter 4.1

Summarised findings for research Chapter 4.1
Gamma-irradiation decreased cotyledon cell wall thickness of the more HTC and less HTC varieties.
Gamma-irradiation increased the intercellular spaces of the more HTC and less HTC varieties.
Gamma-irradiation increased cell size in the more HTC and less HTC varieties.
Gamma-irradiation increased the size of starch granules in less HTC variety.
Gamma-irradiation did not increase the size of starch granules in more HTC variety.
Gamma-irradiation increased the cold water soluble pectin in the more HTC and less HTC varieties, with more solubilisation in the less HTC variety.
Gamma-irradiation increased hot water soluble pectin of less HTC variety.
Gamma-irradiation did not affect hot water soluble pectin of more HTC variety.
Gamma-irradiation did not have an effect on chelator soluble pectin fraction in both cowpea varieties.

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Table 5.2. Summary of the main research findings on effects of γ-irradiation on the functional and thermal properties of induced HTC dehulled cowpea seeds and cooked prepared pastes from research Chapter 4.2.

Summarised findings for research Chapter 4.2
Gamma-irradiation reduced the peak-, breakdown- and setback viscosities of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the peak-, breakdown- and setback viscosities of cooked prepared pastes of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the nitrogen solubility index of the more HTC and less HTC varieties.
Gamma-irradiation had no an effect on the nitrogen solubility index of the cooked prepared pastes of the more HTC and less HTC varieties.
Gamma-irradiation increased the water absorption capacity of the less HTC variety.
Gamma-irradiation had no effect on the water absorption capacity of the more HTC variety.
Gamma-irradiation had no effect on the water absorption capacity of cooked prepared pastes of the less HTC variety.
Gamma-irradiation increased the water absorption capacity of cooked prepared pastes of the more HTC variety.
Gamma-irradiation decreased the swelling power of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the swelling power of the cooked prepared pastes of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the water solubility index of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the water solubility index of the cooked prepared pastes of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the gelatinisation temperatures of the more HTC and less HTC varieties.
Gamma-irradiation had no effect on the gelatinisation enthalpy of the more HTC and less HTC varieties.

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5.3 PROPOSED MECHANISMS FOR HTC DEVELOPMENT BASED ON FINDINGS ON THE EFFECTS OF γ-IRRADIATION

Application of γ-irradiation to cowpeas resulted in an increase in cell size, increase in intercellular spaces and a reduction in cell wall thickness in cotyledon cells (Table 5.1). This was accompanied by an increase in cell wall pectin solubility, which indicates partial disintegration of the middle lamella. In fact, it has been found that γ-irradiation hydrolyses glycosidic bonds in pectin, resulting in greater pectin solubility (Cho et al., 2003). Partial depolymerisation of the middle lamella could have led to a greater uptake of water by cotyledon cells, leading to cell expansion. Since the major effects of irradiation which resulted in a decrease in cooking time in Bechuana White (the less HTC variety) after HTHH storage were mainly related to pectin modification, this supports the involvement of the 'phytase-phytate-pectin mechanism' in HTC development. However, given that γ-irradiation did not reduce HTC defect in the more Agrigold (more HTC variety), this suggests the involvement of other mechanisms in HTC development. Figure 5.1 proposes a possible modified phytase-phytate-pectin mechanism based on findings of effects of γ-irradiation on cell separation, pectin and functional properties of HTC cowpeas. As shown in figure 5.1, γ-irradiation depolymerised pectin in HTC cowpea resulting in improved cell separation and greater water uptake by the cotyledon in less HTC variety. Furthermore, it depolymerised starch and formed dextrins that were more water soluble resulting in a shorter time required for full starch gelatinisation, consequently reducing the cooking time of the cowpea in the less HTC variety. However, in the more HTC cowpea, HWSP pectin and cellular separation did not increase after irradiation, suggesting involvement of other crosslinking mechanism such as those involving the more difficult to extract alkaline-soluble, ester-bonded pectins. The involvement of alkali-soluble pectin in HTC development in pulses is consistent with the

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findings of Shiga et al. (2004) and Njoroge et al. (2015) whom both found that alkaline-soluble, ester- bonded pectin increased after HTC development.

Furthermore, since it has been found that during HTC development, divalent ions move and concentrate around the cell wall area in cowpeas seeds (Kruger et al., 2015), it is probable that the calcium ions could also be interacting with the cowpea protein, leading to a decrease in solubility. In addition, the denatured cowpea protein could be interacting with the methyl groups of pectin, resulting in greater crosslinking and a reduction in protein solubility. Importantly, the HTC phenomenon diminished with milling of HTC cowpea seeds, which to an extent supports crosslinking mechanisms being responsible for the development of the HTC defect in cowpea (Table 5.2). It is possible that protein and starch in the presence of calcium ions interactions could be limiting the softening of cooked HTC cowpea seeds after γ-irradiation. Proteins have been shown to bind to carbohydrates in the presence of calcium ions (Sharon, 1993). This might explain why depolymerisation of starch failed to soften the cooked HTC seeds and consequently the stability of starch against irradiation. Moreover, HTHH storage and irradiation did not influence the functional properties of dehulled cooked, prepared cowpea pastes (Fig 5.1). In addition, paste thermal properties showed no changes in initial and peak gelatinisation temperature and gelatinisation enthalpy in the HTC cowpea seeds (Chapter 4.2). Furthermore, the starch from both cowpea varieties had differences in gelatinisation enthalpy, which suggests that differences could be due to intrinsic properties of the starch. This, in turn, suggests that starch is not part of the mechanism responsible for HTC defect development. Since protein decreased under HTHH storage, it is probable that it also plays a role in mechanisms involved in HTC development.

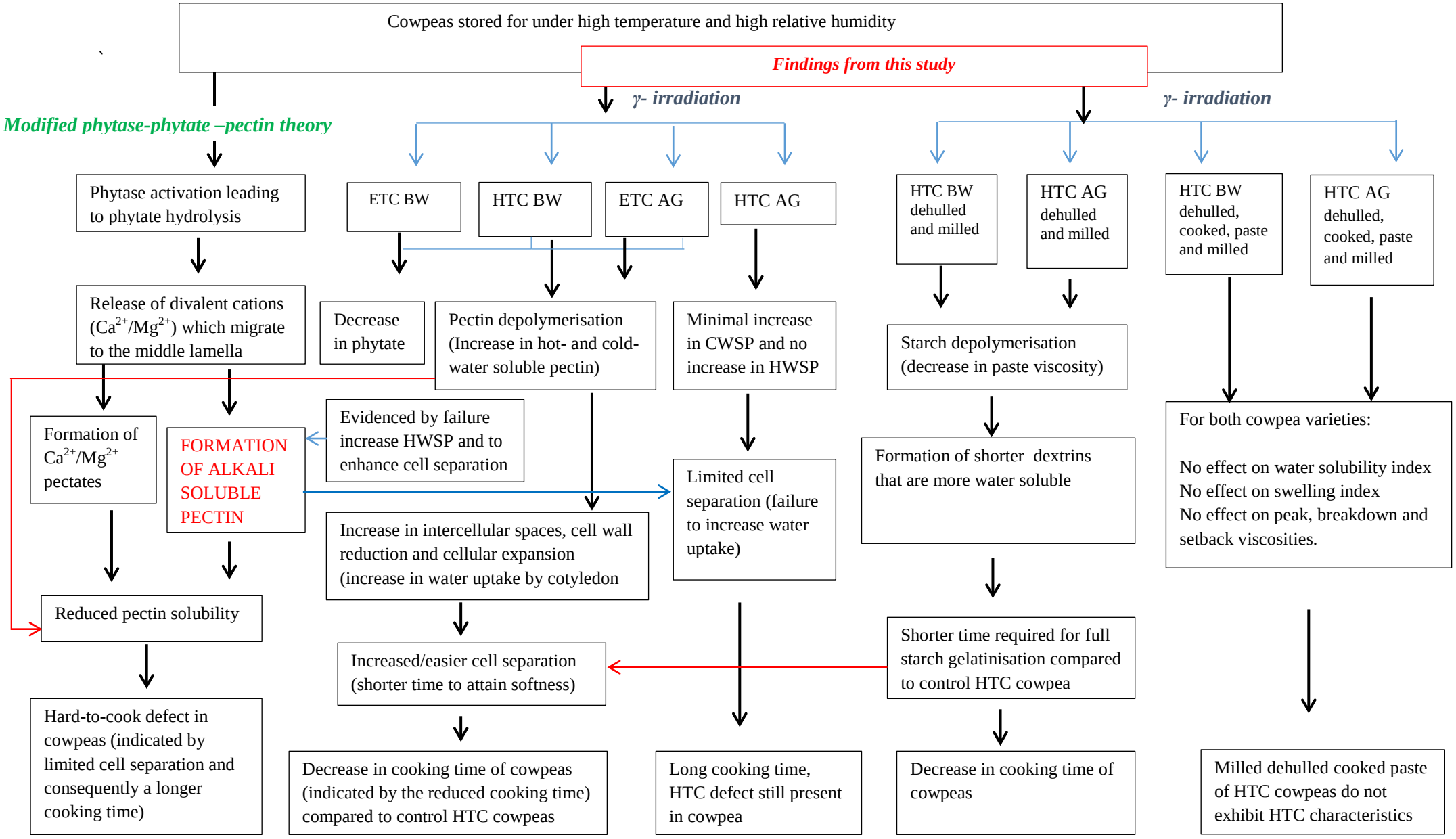

Figure 5.1. Proposed modified phytase-phytate-pectin mechanism of HTC defect development based on findings on the effects of γ-irradiation on cell separation, pectin and functional properties in HTC cowpeas which influence HTC defect. (BW-Bechuana White, AG-Agrigold, ETC-Easy to cook)

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5.4 FUTURE RESEARCH

The current research investigated the application of γ-irradiation on two cowpea varieties with different susceptibility to HTC defect in order to help better understand the mechanisms responsible for the defect. Additional research is needed to characterise the pectin composition in HTC cowpeas. This is because the changes which occur during HTC development may also be influenced by the degree of pectin methylation (DM) and neutral sugar composition. Moreover, it would be of interest to determine the molecular weight and functional groups present in the pectin after HTC development. This will probably help to identify the possible interactions of pectin in the cotyledon cell wall. This may help explain the differences in susceptibility to HTC defect in cowpeas. In addition, there is also a need to specifically characterise other cell wall polymers such as alkali-soluble pectin and possibly hemicellulose fractions. In the present study, the more HTC variety had an extended cooking time of greater than 10 hours, which is indicative of stronger cell wall interactions.

Through measurement of NSI, there was also an indication that cowpea protein changes with HTC development. Additional work is needed to characterise the amino acid composition and functional properties of the proteins in HTC cowpeas. Since the solubility of the protein in the more HTC susceptible variety decreased more substantially compared to the less HTC variety, this information about the protein could help understand differences in HTC susceptibility. More detailed information about the effects on proteins can be obtained using techniques such as SDS-PAGE and size exclusion chromatography. These techniques can be used to investigate the molecular weight and subunit distribution of the cowpea proteins, and/or formation of disulphide crosslinks, which are all important in understanding the protein changes in HTC cowpeas. Furthermore, since there could be possible interactions

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between starch and protein, determining the effects of γ-irradiation on molecular weights of the amylopectin could help provide more information on possible cellular interactions.

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6 CONCLUSIONS AND RECOMMENDATIONS

The application of γ-irradiation has helped to better understand the mechanisms by which the HTC defect prevents cotyledon cell wall separation in cowpeas during cooking. Gamma-irradiation reduces cotyledon cell wall thickness, increases cell size and intercellular spaces in both highly and less susceptible HTC defect type cowpeas. It does not, however, reverse the HTC defect in cowpeas that are very susceptible to the HTC defect. The effects of γ-irradiation are as a result of hydrolysis of pectin fractions in the cell walls. The cold water soluble pectin (CWSP), hot water soluble pectin (HWSP) and chelator soluble pectin (CSP) are affected differently, with large increases in CWSP and HWSP particularly in the cowpeas that are less susceptible to HTC development but essentially no change in CSP. The fact that the cell wall changes brought about by γ-irradiation were associated with pectin solubilisation supports the phytate-phytase pectin theory to some extent. However, the non-reversal of the HTC defect in HTC susceptible cowpeas and the absence of an effect on CSP indicate that other mechanisms are also involved in the HTC development in cowpeas, possibly the formation of alkali‑soluble, ester bonded pectins.

In addition, application of γ-irradiation to HTC cowpea increases water absorption capacity (WAC), and decreases water solubility index (WSI), swelling power (SP) and pasting properties. The effects of γ-irradiation are predominately related to starch functional properties, decreasing WSI, SI, peak-, breakdown- and setback viscosities for highly and less susceptible HTC defect type cowpeas, presumably due to starch depolymerisation. With irradiation, the WAC of dehulled cowpeas increases with less HTC susceptible variety. Since γ-irradiation affects all starch related functional properties with the exception of thermal properties, the non-reversal of the HTC defect by γ-irradiation suggests that changes in starch during HTC development are a consequence and not a cause of the HTC defect.

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Furthermore, there were no differences in the functional properties of pastes from both varieties after γ-irradiation; hence dehulling, cooking, and preparation of HTC cowpea pastes eradicate differences in the functional properties of cowpea pastes. Thus, preparation of dehulled cooked, prepared pastes eliminates HTC phenomenon in cowpeas.

This study has shown that dehulling, milling and the preparation of cooked pastes alleviates the HTC phenomenon in cowpea, the following recommendations were made. Firstly, the HTC dehulled cowpea should be used in cowpea flour-based products. This would also greatly assist households to save on the amount of time and fuel needed to cook HTC cowpea seeds. Consequently, this will help protect an already overburdened environment with regards to reducing the number of trees for firewood. Secondly, HTC cowpea seeds should be used for preparation of dehulled cooked prepared pastes. It is also recommended that further research be conducted by sensory studies to determine the effect of dehulled HTC cowpea on sensory characteristics and possible consumer acceptance of cowpea flour-based products. It is also recommended that sensory studies be conducted to determine sensory characteristics and possible consumer acceptance of the dehulled cooked prepared cowpea pastes. Further work involving characterisation of pectin composition is needed better understand HTC mechanisms in HTC development in cowpeas. In addition, further research to characterise the amino acid composition and functional properties of HTC protein is also important to better understand HTC mechanisms.

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8 PUBLICATIONS, PRESENTATIONS AND POSTERS BASED ON THIS RESEARCH

Jombo, T.Z., Minnaar, A. and Taylor, J.R.N., 2018. Effects of gamma-irradiation on cotyledon cell separation and pectin solubilisation in hard-to-cook cowpeas. *Journal of the Science of Food and Agriculture*, 98, 1725-1733.