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Micronisation and hot-air roasting as pre-treatments to control hard-to-cook phenomenon in cowpeas

by

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DECLARATION

I declare that the thesis which I hereby submit for the degree PhD Food Science at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other another tertiary institution.

SIGNATURE: …….

DATE: …….

ABSTRACT

Hard-to-cook phenomenon in cowpeas: effect of micronisation and hot-air roasting on storage quality

By

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Supervisors: Prof. A. Minnaar

Prof. M. N. Emmambux

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most important grain legume in sub-Saharan Africa. It is an important source of affordable dietary proteins for the low-income populations. Cowpeas develop hard-to-cook (HTC) defect during storage at high temperature-high humidity (HTHH) conditions experienced in these regions. Cowpeas with HTC defect are characterised by long cooking time that lead to increased energy demands that add constraints on the consumers with limited resources. The main objective of this work was to determine the effect of micronisation and hot-air roasting as strategies in the control of HTC defect development.

A preliminary study determined the effect of accelerated storage conditions of 40 °C and 80% relative humidity for 40 days on HTC defect development among 3 cowpea types. The accelerated conditions simulate the HTHH storage conditions. The 3 cowpea types (*Bechuana white*, *Agripienk* and *Mae–e–tsiliwane*) stored at these HTHH conditions developed HTC defect as shown by the increased cooking time. The increase in cooking time was not observed in the control cowpeas stored at 4 ^{o}C. The increase in cooking time differed among the 3 cowpea types and this indicated varying degree of susceptibility to HTC defect. HTC defect development at the accelerated HTHH conditions was shown to be due to increased phytase activity that resulted in decreased phytate content and decrease in water soluble pectin. This was in agreement with the

"phytase-phytate-pectin" theory. The role of lignification was not established during HTHH storage conditions since peroxidase activity and lignin content did not increase. *Agripienk* which had the highest increase in cooking time was selected for the heat pre-treatment study.

Cowpeas of the *Agripienk* type were pre-conditioned to 25% moisture before being micronised or hot-air roasted at 150 ^{o}C for 5 minutes. Phytase activity was reduced by 70% and 45% by micronisation and roasting pre-treatments respectively. After HTHH storage the cooking time increased by >430%, 243% and 92% for the control, roasted and micronised cowpeas respectively. Micronised cowpeas had the lowest increase in cooking time. Therefore, HTC defect was controlled but not prevented by the heat pre-treatments. Micronised cowpeas which had the lowest phytase activity has higher phytate and water soluble pectin contents when compared to both roasted and control cowpeas.

The mechanism of HTC defect control by the heat pre-treatments was attributed to partial phytase inactivation which decreased the rate phytate hydrolysis and liberation of divalent cations available to bind to pectin in the middle lamella. There was higher content of water soluble pectin and lower content of chelator soluble pectin in the heat pre-treated cowpeas than in the control after HTHH storage. Increase in chelator soluble pectin indicated formation of pectates that limit the rate of cell separation during cooking. Based on these findings, cell separation during cooking was investigated further. Confocal laser scanning microscopy showed that there was more cell separation in the heat pre-treated cowpeas when compared to the control after HTHH storage. The control cowpeas showed minimal cell separation even after 2 hours of cooking. The ease in cell separation in the heat pre-treated cowpeas was due to presence of more soluble pectin in the middle lamella when compared to the control that had more chelator soluble pectin. Micronised cowpeas had more cell separation when compared to the hot-air roasted cowpeas.

Differential scanning calorimetry of cowpea flours showed that HTHH did not lead to an increase in gelatinisation temperatures (T_o and T_p) or gelatinisation enthalpy(ΔH) in control, micronised and hot-air roasted cowpeas. Increase in these thermal properties is suggested to lead in increased cooking time. The pasting viscosities of the cowpea flours decreased after heat pre-treatments but HTHH conditions did not alter the viscosities of either the control or heat pre-treated cowpeas. Therefore the changes in starch thermal and pasting properties due to HTHH storage were not observed.

This study indicates that HTC defect development was dependent on phytase activity during HTHH storage. The effectiveness of micronisation and hot-air roasting in preventing HTC defect was dependent on the degree of phytase inactivation. Micronisation was more effective than hot air roasting in controlling the development of HTC defect due to a higher degree of phytase inactivation.

DEDICATION

To my late supervisor, Prof. Amanda Minnaar.

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To God for His abundant grace and provision.

I would like to sincerely thank:

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The staff members and postgraduate students of the department of Food Science for sharing your knowledge and support.

To family and friends for their support and encouragement

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION AND PROBLEM STATEMENT

Cowpea (*Vigna unguiculata* L. Walp) is a leguminous plant widely cultivated in the tropics (Chavan, Kadam & Salunkhe, 1989). The adaptation of cowpeas to low rainfall (Ehlers & Hall, 1997) and marginal soils (Hall, Cisse, Thiaw, Elawad, Ehlers, Ismail, Fery, Roberts, Kitch & Murdock, 2003) render them a nutritious legume in sub-Saharan Africa. Cowpea seeds are a rich and inexpensive source of dietary proteins (Alayande, Mustapha, Dabak & Ubom, 2012) in often low-income regions of sub-Saharan Africa with protein deficient diets. Cowpeas are important in alleviating protein energy malnutrition when complemented with cereal-based diets (Iqbal, Khalil, Ateeq & Sayyar Khan, 2006). One of the major limitations in dry whole cowpea grains utilisation is long cooking time and thus, high energy demands (Stanley & Aguilera, 1985). This limitation is further exacerbated by development of "hard-to-cook" (HTC) defect that occurs during storage at high temperature (30-35^{o}C) and high humidity (60-80%). HTC cowpea seeds are characterised by longer cooking times in attaining acceptable softening for consumption (Aguilera, 2000). HTC defect reduces the acceptability of stored legumes since prolonged cooking increases time and energy demands on the under resourced populations of sub- Saharan Africa.

Several mechanisms have been proposed to explain the development of HTC although the contribution of each mechanism to the overall process is not clear (Aguilera, Stanley & Baker, 2000). Most of the proposed mechanisms of HTC in literature implicate increased enzymatic activity during storage at high temperature and high relative humidity. The two main theories reported in literature are the "phytase-mineral and mineral-pectin interactions" (Jones & Boulter, 1983; Galiotou-Panayotou, Kyriakidis & Margaris, 2008) and "lignification mechanisms" (Hincks, McCannel & Stanley, 1987).

Refrigeration of legume seeds of low moisture content (< 10%) at low temperatures (4^{o}C) seems to be an effective way of controlling HTC defect development (Garruti & Bourne, 1985; Berrios, Swanson & Adeline Cheong, 1999) due to reduced enzyme activities. However, refrigeration is costly and may not be accessible in developing countries. Preventative techniques such as heat

pre-treatments and gamma irradiation before HTHH storage have failed to completely prevent the defect development (Plhak, Stanley, Hohlberg & Aguilera, 1987; Cunha, Sgarbieri & Damasio, 1993). Heat pre-treatments already investigated include treatments with sand roasting of beans (Aguilera & Stanley, 1985; Plhak *et al.*, 1987; Rivera, Hohlberg, Aguilera, Plhak & Stanley, 1989), microwave heating of beans (Plhak *et al.*, 1987; Cunha *et al.*, 1993) retorting of beans (Molina, Baten, Gomez-Brenes, King & Bressani, 1976) and steaming of beans and cowpeas (Molina *et al.*, 1976; Affrifah, Chinnan & Fang, 2006). However, none of the pre-treatments has totally prevented the hardening phenomenon during storage probably as a result of residual enzyme activity.

Micronisation (Infrared heating), a relatively novel way of heat processing, has been shown to reduce the generally long cooking times of legumes such as cowpeas (Mwangwela, Waniska & Minnaar, 2006) and lentils (Arntfield, Scanlon, Malcolmson, Watts, Cenkowski, Ryland & Savoie, 2001). Infrared (IR) heating is reported to be more effective than conventional heating methods due to its superior heating properties (Sakai & Hanzawa, 1994; Fasina, Tyler, Pickard, Zheng & Wang, 2001; Krishnamurthy, Khurana, Soojin, Irudayaraj & Demirci, 2008). Therefore micronisation, a technique not previously used as a pre-treatment in the prevention of HTC defect has a potential to be a more effective heat pre-treatment strategy in comparison to the already studied techniques. Affrifah (2004) observed that a shortcoming of most of the heat pre-treatment studies is that the role of inactivation of enzymes such as phytase in preventing the HTC defect was not experimentally investigated. Furthermore, the mechanisms through which the reductions in HTC defect development were achieved are not clearly reported in these studies. These studies fail to investigate the effect of the heat pre-treatments on physico-chemical characteristics suggested to lead to HTC defect development.

There is still need for strategies to prevent HTC defect development since most of the strategies used do not completely prevent development of the defect. The potential of micronisation in the prevention of HTC defect needs to be evaluated and compared to conventional heat treatments

such as hot-air roasting. It is critical to understand the prevention mechanisms, which would lead to designing of more effective strategies.

2 LITERATURE REVIEW

2.1 Physical, Structure and chemical composition of cowpeas

Cowpea seeds are small dicotyledonous seeds of different colours, shapes and sizes. The length ranges from 2 to 12 mm Chavan & Kadam (1989). The seed shape is normally globular to kidney shaped (Henshaw, McWatters, Oguntunde & Phillips, 1996). The cowpea seed colour ranges from white, brown, red, purple to black with some varieties being speckled or mottled (Taiwo, 1998; Affrifah & Chinnan, 2005; Giami, 2005). A cowpea seed has two major structural parts; the seed coat and the cotyledon seed weight fractions are 85 - 90% cotyledon and 8.5-10% seed coat (Deshpande & Damodaran, 1990).

The seed coat or testa is the outer protective layer of the cowpea seed. The seed coat is important as it is the first point of interaction between the seed and the external environment. The seed coat influences important physico-chemical properties of the cowpea seed which impacts on its utilization as human food. Some of the properties such as seed coat thickness are known to affect the rate of water uptake which in turn influences the cooking quality of the seeds (Penicela, 2011).

Like all leguminous seeds, the cowpea seed has two cotyledons which are the major parts with respect to weight and volume. The parenchyma cells are the main storage sites containing protein bodies and starch granules (Liu, McWatters & Phillips, 1992a) and are surrounded by a cell wall. The proximate composition of cowpeas (Table 2.1) shows considerable variations in proximate composition due to variations in cultivar, soil, climate and agricultural practices (Hsieh, Pomeranz & Swanson, 1992). Starch is the most abundant single carbohydrate fraction in cowpeas ranging from 26% to 48% (Longe, 1983).

2.2 Physico-chemical changes during cooking of cowpeas

Like for most legumes, cowpea seeds are mainly prepared for consumption by cooking. The cooking of whole cowpea seeds is normally done by boiling the seeds at atmospheric pressure and temperature. Boiling of the seeds can be done directly or after hydration (soaking) as

practiced in Latin America. In easy-to-cook cowpeas, the process of cooking leads to softening of the seeds until they attain a texture that is considered acceptable to consumers (Affrifah & Chinnan, 2005).

Table 2.1 Proximate composition of cowpeas

Parameter (%)	**Hsieh *et al.* (1992)**	**Taiwo (1998)**	**Phadi (2004)**	**Mwangwela (2006)**
Moisture	7.38-15.34	8.50-11.00	8.60	8.9-12.1
Ash	3.68-4.36	a	3.50	a
Protein	21.68-28.68	24.6-25.10	22.30	24.0-28.3
Lipid	0.3-1.44	2.50-5.10	1.40	a
Carbohydrate	65.92-73.17	54.20-58.60	64.20	a

a No reported values

Cowpeas like other legumes are judged to be cooked when they attain a soft texture. The time required for the seeds to attain this texture is referred to as the cooking time. In literature, wide ranges in cowpea cooking times are reported: 24 to 62 min (Jackson & Varriano-Marston, 1981), 36 to 56 min (Demooy & Demooy, 1990), 31 to 160 min (Akinyele, Onigbinde, Hussain & Omololu, 1986), 57 to 59 min (Mwangwela *et al.*, 2006) and 83 to 216 min (Penicela, 2011). During cooking, flavour development and inactivation of anti-nutritional factors also take place (Chavan & Kadam, 1989). The cooking time required to attain an acceptable soft texture is an important characteristic influencing consumer acceptability of legumes (Deshpande & Damodaran, 1990). This is because long cooking time means more energy demands, which is a challenge to scarce resourced region of sub-Sahara Africa that consume legumes as a staple.

During cooking of legumes, the seed is hydrated and heated simultaneously causing structural and physico-chemical changes. Three main transitions in macromolecules are reported during cooking: solubilisation and or degradation of middle lamella pectin, starch gelatinisation, and cytoplasmic protein denaturation (Stanley & Aguilera, 1985). Water uptake and hydration is necessary for these changes to take place. It is generally agreed among researchers that one of the main determinants of textural properties of cooked legumes is the separation of adjacent parenchyma cells at the middle lamella (Stanley & Aguilera, 1985).

The middle lamella (Figure 2.1) is the region between the double wall formed by two adjacent cells known to cement individual cells of the cotyledon (Stanley & Aguilera, 1985). The middle lamella is composed mainly of heat-labile pectin (Jackman & Stanley, 1995), which determine the physical strength of the tissue (Stanley & Aguilera, 1985). During cooking, heat induced dissolution/degradation of the pectin weakens the intercellular adhesion which permits the separation of adjacent cells (Aguilera, 2000); (Ilker & Szczesniak, 1990); (Bernal-Lugo, Parra, Portilla, Pena-Valdivia & Moreno, 1997).

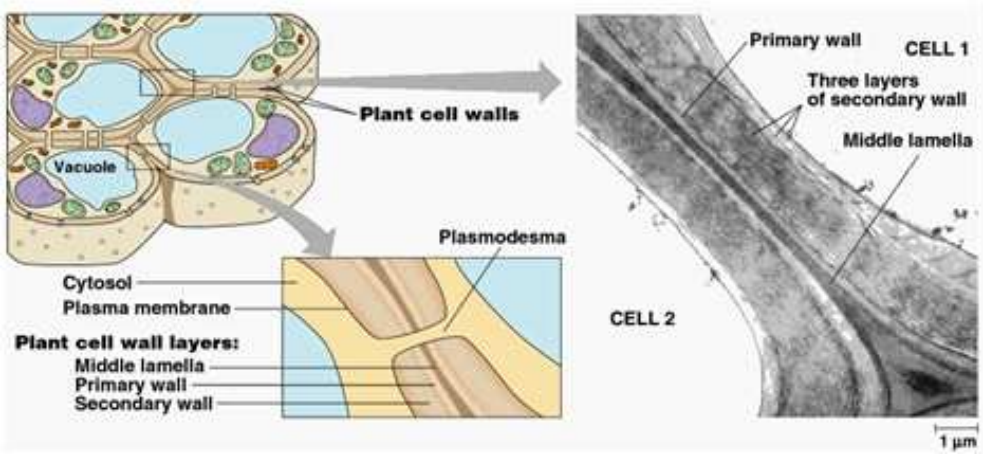


Figure 2.1 Schematic representation of a plant cell and a transmission electron micrograph of the cell wall showing the middle lamella (Chandler, 2011)

Texture softening in cooked legumes is dependent on the cells separating (Stanley & Aguilera, 1985). In cowpeas, cell separation along the middle lamella during cooking has been reported (Sefa-Dedeh, Stanley & Voisey, 1979); (Stanley & Voisey, 1978); (Liu, Hung & Phillips, 1993a). The heat induced degradation of pectin involves depolymerisation of the pectin polymer via the beta (β) elimination of the methyl esterified polygalacturonic acid (Liu, Phillips & McWatters, 1993b); (Brett & Waldron, 1996)). The β eliminative reaction (Figure 2.2) involves the breakage of glycosidic bonds adjacent to carboxyl groups (Liu *et al.*, 1993a); (Bernal-Lugo *et al.*, 1997). The solubilisation/degradation of pectin is dependent on its composition. For instance, less esterified middle lamella pectins are able to form Ca^{2+} cross links forming calcium pectates that are insoluble, thus impairing cell separation (Bhatty, 1990).

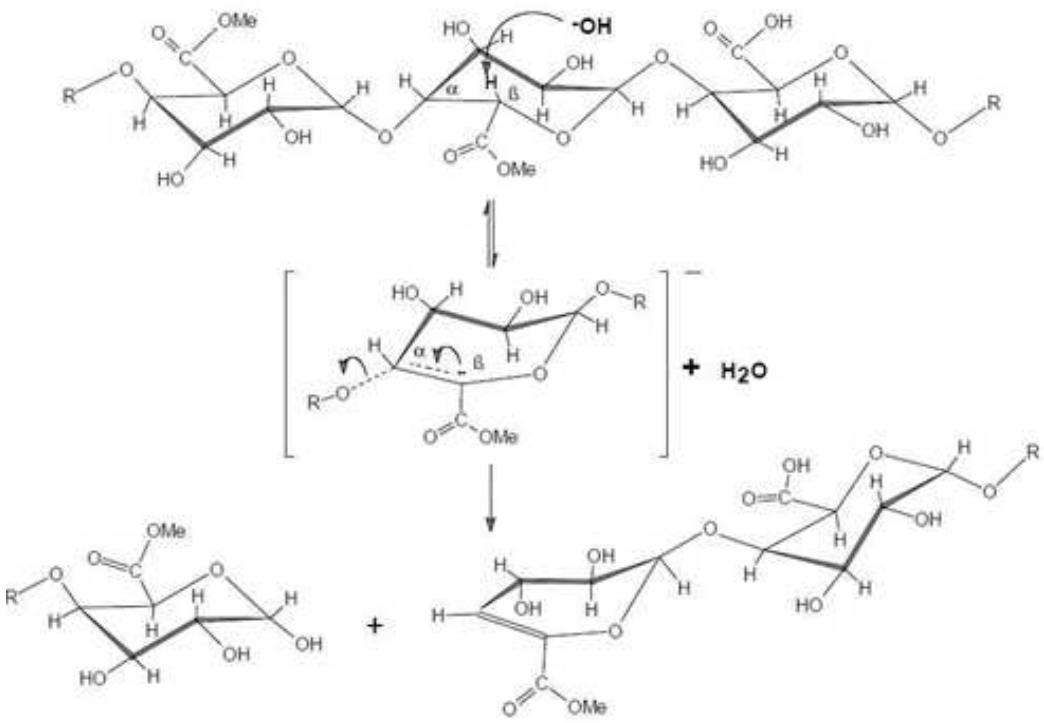


Figure 2.2 Depolymerisation of a partially esterified pectic galacturonan chain by β eliminative reaction

Starch, one of the major macromolecular constituents of cowpeas (Kerr, Ward, McWatters & Resurreccion, 2001) contributes to the soft texture of cooked legume seeds when it is gelatinised (Aguilera & Stanley, 1985); (Yousif, Kato & Deeth, 2007). Gelatinisation occurs in the presence of adequate water and heat; the starch granules absorb water, swell up and lose their crystalline order (Biliaderis, 1991). It has been suggested that the swelling of starch granules promotes cell expansion which enables the cell to separate from the adjacent ones (Jarvis, 1998). During cooking, after the entry of water via the cell wall, much of the water is absorbed by protein. Hydration of the protein thus facilitates hydration within the cotyledon cell. Cowpea protein has been shown to be relatively hydrophilic absorbing about 1.24 g of water per g of protein (Mwasaru, Muhammad, Bakar & Man, 1999). Cooking causes the denaturation of proteins, which involves the unfolding of the protein molecule possibly increasing the exposed hydrophobic sites leading to aggregation and possible gel formation (Clark, Kavanagh & Ross-Murphy, 2001). Protein thermal properties such as denaturation temperatures are suggested to influence cooking time of legumes. Starch gelatinisation and protein gelation are both water dependent processes. Starch gelatinisation temperatures are lower than the protein denaturation temperatures, therefore there is minimal

competition for water between the two processes (Yousif *et al.*, 2007). However, it has been suggested that low protein denaturation temperatures could lead to prolonged cooking time due to less water being available for starch gelatinisation (Yousif *et al.*, 2007); (Liu, McWatters & Phillips, 1992a). Since starch granules are embedded in protein matrices, low protein denaturation temperatures would mean increased water competition by the proteins therefore limiting starch gelatinisation and resulting in hard textures/long cooking time (Liu *et al.*, 1992a).

Some legumes fail to soften under normal cooking conditions, thus requiring longer cooking times to achieve tenderness. Two common textural defects associated with cooking of legumes are the hard-shell and the hard-to-cook defect (Stanley & Aguilera, 1985). The focus of the current research is on the hard-to-cook phenomenon.

2.3 Hard-shell defect

Hard-shell defect is defined as hardening of the seed coat (Stanley, 1992). Hard-shell is characterised by inability of seeds to imbibe water and hydrate and thus they do not soften during cooking (Agbo, Hosfield, Uebersax & Klomparens, 1987; Stanley, 1992; Mullin & Xu, 2001). The hydration capacity of common beans is inversely proportional to the occurrence of hard-shell defect (Antunes & Sgarbieri, 1979). Hard-shell is associated with impermeability of the seed coat and generally regarded as a seed coat defect since it is eliminated with either seed coat removal or mechanical scarification of the seeds (Shehata, 1992). Hard-shell is found in both freshly harvested and stored legume grains. The occurrence of the defect in freshly harvested seeds is influenced by the seed size, genetics, growth climatic conditions and degree of maturity (Shehata, 1992). Legume crops that experience a period of low moisture and high temperatures during the final maturation stage are more prone to the hard-shell defect (Mullin & Xu, 2001). Hard-shell is prevalent in the northern latitudes where legume grains are stored at high temperatures and low humidity (Liu, 1995).

2.4 Hard-to-cook phenomenon

Hard-to-cook defect is distinguished from hard-shell in that the seeds with HTC defect hydrate normally but fail to soften under normal cooking conditions thus leading to longer cooking times (Shehata, 1992). HTC defect occurs during storage under high temperature - high relative humidity conditions (Aguilera & Rivera, 1992a). These storage conditions,

normally experienced in tropical climates, lead to hardened beans within a few months. The defect is further accelerated by storing legumes with a high moisture of above 13% (Aguilera & Stanley, 1985). The HTC defect thus reduces the commercial acceptability of stored beans due to high energy cost required for the beans to acquire an acceptable texture to the consumers. Salvador (2007) observed that cowpeas stored under High Temperature and High Humidity (HTHH) conditions increased the cooking time from 89 to more than 270 min. Legume varieties within the same species exhibit differences in the severity of the defect development. Cowpea varieties; *Mogwe-oKgotsheng*, *Bechuana white* and *Mae-a-tsilwane* stored at 42 °C and 67% RH for 21 days had a 72%, 31% and 30% increase in cooking time respectively (Salvador, 2007). These differences in HTC defect among legume species or within varieties of the same species are due to genotypic differences and growth conditions that affect cooking characteristics. For instance, differences in phytate content due to soil mineral composition could affect the HTC defect development in beans susceptibility (Paredes-Lopez, Montes-Rivera, Reyes-Moreno & Carabez-Trejo, 1989b).

There are several mechanisms/theories proposed to explain the hard-to-cook phenomenon in legumes. These multiple mechanisms are proposed to be of enzymatic and non-enzymatic nature. The suggested mechanisms include: formation of insoluble pectates in the middle lamella (Rodriguez & Mendoza, 1990; Galiotou-Panayotou *et al.*, 2008), lignification of the middle lamella (Hincks *et al.*, 1987; Rodriguez & Mendoza, 1990; Garcia, Filisetti, Udaeta & Lajolo, 1998a), degradation of cell membranes due to lipid oxidation (Varriano-Marston & Jackson, 1981; Liu *et al.*, 1992a), pectin-phenolic acids interactions (Garcia & Lajolo, 1994; Maurer, Ozen, Mauer & Nielsen, 2004) and poor starch gelatinisation (Liu *et al.*, 1992a; Garcia *et al.*, 1998a; Yousif, Batey, Larroque, Curtin, Bekes & Deeth, 2003). However, the contribution of each mechanism to HTC is not clearly understood and still a subject of study (Aguilera, 2000). Several enzymes i.e. phytase, peroxidase, pectin esterase, lipoxygenase and protease have been implicated in HTC phenomenon as shown in Table 2.2.

The two main hypotheses reported are the “phytase-mineral and mineral-pectin interaction” and “lignification of the cell wall”. These two hypotheses will be reviewed with reference to studies conducted in cowpeas and other legumes. The role of starch, protein and phenolic acids in HTC defect development will also be reviewed.

Table 2.2 Possible enzymes implicated in HTC defect development

Enzyme	Reaction	HTC defect mechanism	References
Phytase	Hydrolysis of phytate to inositol and orthophosphate	Phytate losing its chelating ability, thus divalent cations released to bind to pectin.	Galiotou-Panayotou *et al.* (2008), Affrifah & Chinnan (2005)
Pectin esterase	Removal of methyl groups in pectin exposes carboxyl groups	Exposed carboxyl groups of pectin crosslinking with divalent cations	Liu, Phillips & Hung (1992b), Kilmer, Seib & Hoseney (1994), Jones & Boulter (1983)
Peroxidase	Polymerisation of monolignols to lignins, phenolic acids crosslinking to cell wall polysacharides e.g. pectins	Lignification of the middle lamella, phenolic acids bound to soluble pectin	Hincks & Stanley (1987), Hohlberg & Stanley (1987), Rivera *et al.* (1989)
Lipoxygenase	Conversion of lipids to polar, oxygenated polymers	Degradation of cell membranes, solute leakage	Richardson & Stanley (1991), Shewfelt & Erickson (1991)
Proteases	Hydrolysis of storage proteins	Free aromatic amino acids that are lignin precursors	Hohlberg & Stanley (1987)

2.4.1 Phytase-mineral and mineral-pectin interaction

Bean cooking quality is partially determined by the rate of dissolution of the pectin in the cotyledon middle lamella during cooking (Hincks & Stanley, 1986; Liu *et al.*, 1993a). There is agreement in literature that the "phytase-mineral and mineral-pectin interaction" theory offers one of the most plausible mechanisms in HTC defect development. According to this theory, during storage of legumes in high humidity and high temperature, phytase activity is stimulated (Galiotou-Panayotou *et al.*, 2008) leading to hydrolysis of phytate. Phytate is the salt form of phytic acid with mono- and divalent cations (Reddy, 1989). According to Galiotou-Panayotou *et al.* (2008), phytate chelates divalent cations such as Ca^{2+} and Mg^{2+} preferentially over the weak carboxylic groups in pectin due to the six strong phosphate groups in phytate. On hydrolysis, the chelation potential of phytate is lowered and phytate releases the bound cations. The cations migrate to the middle lamella and crosslink with the free carboxyl groups in the pectin molecule forming covalent bonds (Galiotou-Panayotou *et al.*, 2008). The cation binding mechanism of pectins is theorised as the "egg-box" model (Figure 2.3)

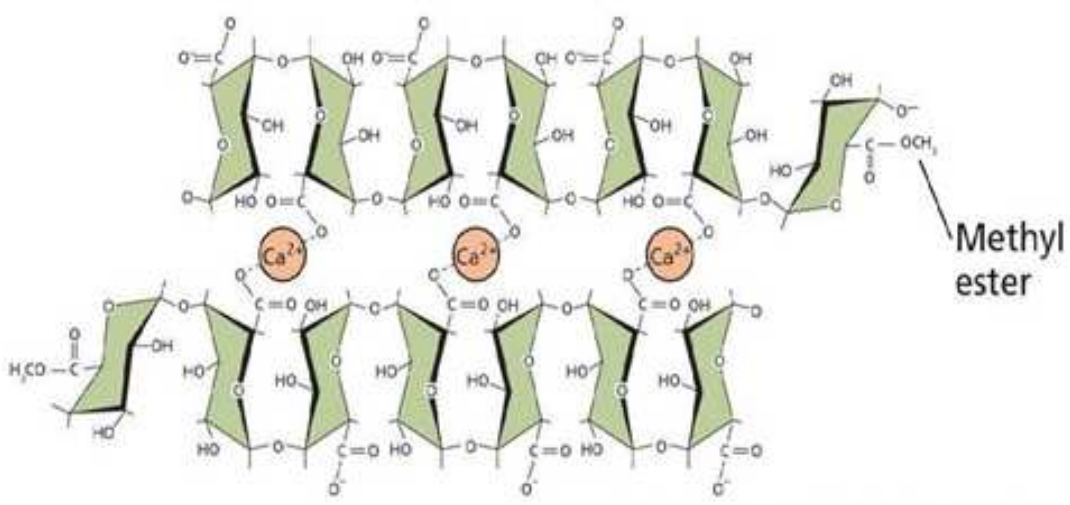


Figure 2.3 Ionic bonding of pectin network by calcium (Cosgrove, 2006)

Pectin in the middle lamella acts as an adhesive between the parenchyma cells in the cotyledon (Yousif *et al.*, 2007) and therefore its solubility is important in determining the cooking quality and softening of the cotyledon. The pectin which holds adjacent cells

together exists mainly in water soluble form (Galiotou-Panayotou *et al.*, 2008). However, on cross-linking with the divalent cations, insoluble pectates, e.g. calcium or magnesium pectates, are formed. These pectates are not easily soluble on heating, therefore, restricting cell separation during cooking (Hentges, Weaver & Nielsen, 1991).

2.4.1.1 Phytic acid/phytate

Phytic acid (*myo*-inositol 1,2,3,4,5,6, hexakisphosphate) is the primary source of inositol and the chief storage form of phosphorus in plant seeds (Lott, Ockenden, Raboy & Batten, 2007). Phytic acid is an important mineral storage compound in plant seeds due to its strong chelating characteristic due to the multiple negatively charged phosphate groups. Phytic acid binds polyvalent cations more strongly than monovalent cations (Graf, 1986). The phytic acid molecule consists of an inositol ring with 6 phosphate groups attached (

Figure **2.4**).

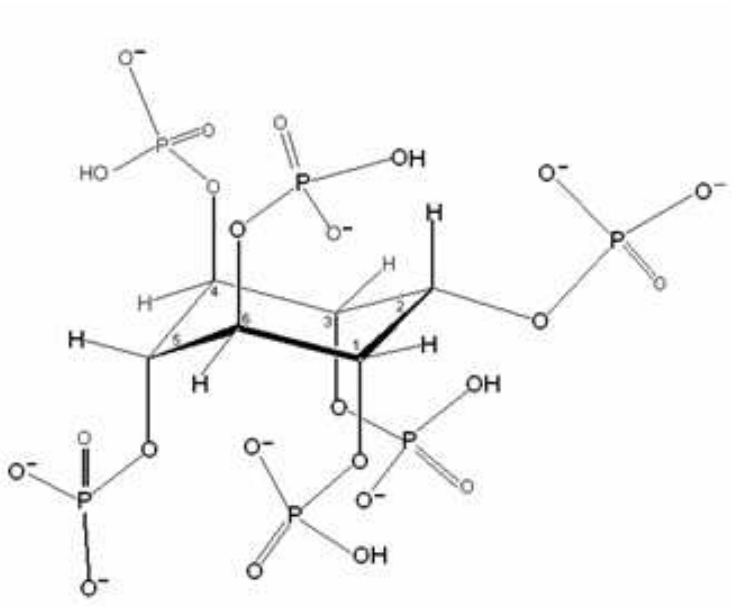


Figure 2.4 Structure of phytic acid -myo-inositol-1,2,3,4,5,6-hexakis phosphate (Iqbal *et al.*, 2006; Schlemmer, Frølich, Prieto & Grases, 2009)

In mature seeds, phytic acid occurs primarily as a complex salt of mono- and divalent cations, therefore the term phytate (Reddy, 1989). Variations in phytate content of seeds are influenced

by cultivar/variety, climatic conditions, and season of growth, location, soil type, fertiliser application and irrigation. In dicotyledonous seeds, most of the phytates are located in the cotyledons and not in the seed coat (Reddy, 1989). In most of the reported works in the literature, there is a relationship between the development of HTC defect and phytate levels. Several researchers have reported an increase in cooking time with decreasing levels of phytate (Bhatty, 1990; Liu, 1995; Reyes-Moreno, Okamura-Esparza, Armienta-Rodelo, Gomez-Garza & Milan-Carrillo, 2000a; Coelho, de Mattos Bellato, Santos, Ortega & Tsai, 2007). Legume grains with higher phytate content have a shorter cooking time when compared to those of a lower content (Aguilera & Stanley, 1985; Vindiola, Seib & Hoseney, 1986; Reddy, 1989). It is postulated that the higher content of phytate promotes more chelation of divalent cations that would otherwise bind to pectin in the middle lamella (Van Buggenhout, Sila, Duvetter, Van Loey & Hendrickx, 2009). Therefore, higher phytate content promotes easier dissolution of pectic substances at the cotyledon cell walls leading to softening. During accelerated storage of cowpeas at high temperature (42°C) and high relative humidity (67 % RH) phytate content of cowpeas was significantly lower than those kept at 4 ^{o}C (Salvador, 2007). These findings were consistent with those of (Reyes-Moreno *et al.*, 2000a) who observed a significant decrease in phytate content during storage of chickpeas at high temperature (25 °C) and high relative humidity (65% RH). (Kon & Sanshuck, 1981) reported a one-thirds loss of phytic acid content in common beans stored at high temperature (32 ^{o}C) and high moisture conditions (16%) for 10 months in comparison to storage at low temperature (22 ^{o}C) and low moisture (10.5 %). Bhatty & Slinkard (1989) reported a 91% increase in lentil hardness after 5 weeks storage at 50 ^{o}C and 95% relative humidity with 38% decrease in phytate content. The decrease in phytate content is suggested to occur due to phytate hydrolysis by phytase.

2.4.1.2 Phytase

Phytases catalyse the hydrolysis of phytate into myo-inositol, cations and inorganic phosphate (Viveros, Centeno, Brenes, Canales & Lozano, 2000) as shown on Figure 2.5. Plant phytases of seeds of higher plants are generally of the 6-phytases (EC 3.1.3.26) (Konietzny & Greiner, 2002). These types of phytase preferentially initiate the phytate dephosphorylation at the C6 carbon. The characterisation and *in vivo* function of phytases is not fully known (Konietzny & Greiner, 2002). The classification of phytases is based on the in vitro capability of these enzymes to release phosphate from phytate (Konietzny & Greiner, 2002). The knowledge on legume

phytases is limited and only a few phytases from soybean seeds, faba beans, lupin seeds mung bean seeds and scallion leaves appear to have been purified to homogeneity and characterised (Greiner, 2002). In general most isolated plant phytases activity are optimum at an acidic pH (4.0 to 5.6) (Dvořáková, 1998) and moderate temperature (around 55 ^{o}C) (Greiner, 2002).

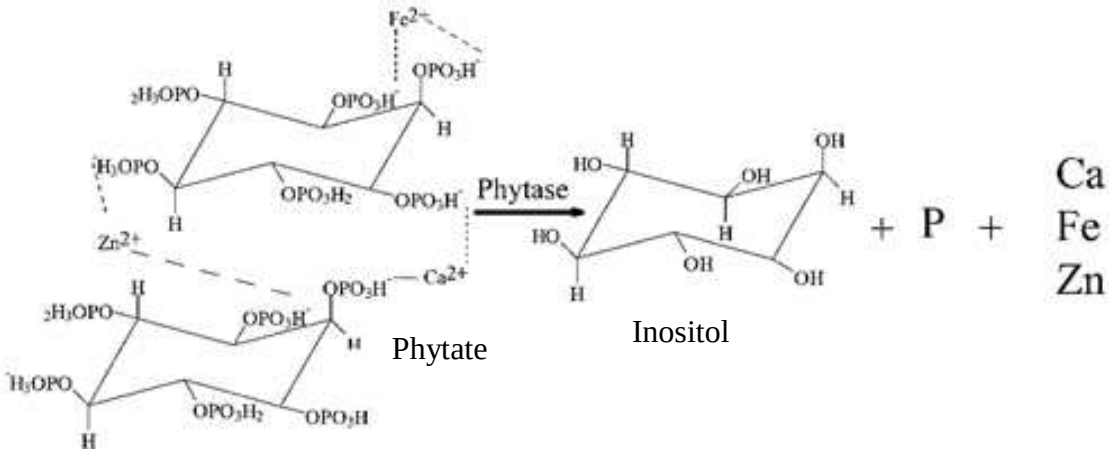


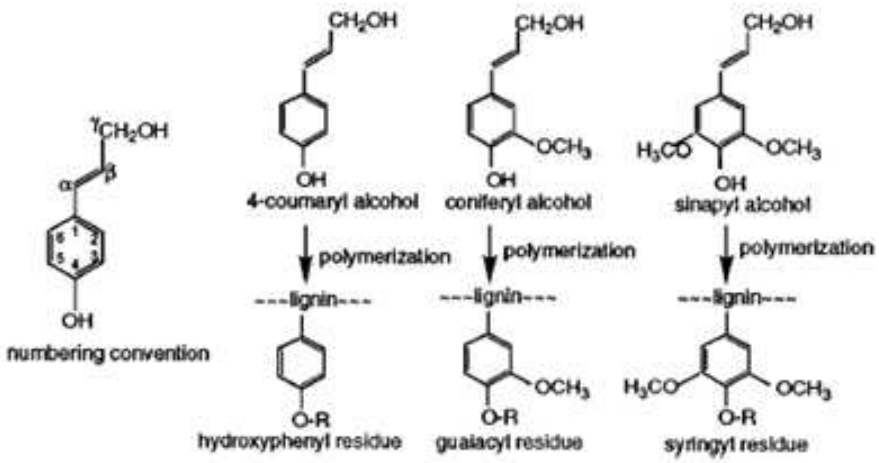
Figure 2.5 Phytate hydrolysis by the enzyme phytase into inositol, phosphorus, and other divalent elements (Lei & Porres, 2003)

According to Maga (1982), phytase appears to have little effect on phytate in dry or dormant seed. However, during HTHH conditions increased phytase activity with corresponding decrease in phytate content was observed (Kyriakidis, Galiotou-Panayotou, Stavropoulou & Athanasopoulos, 1998; Hotz & Gibson, 2007). The cause of the increased activity of phytase is not certain but suggested to be due to *de novo* synthesis, activation of intrinsic enzyme or both (Liu, Rafiq, Tzeng & Rob, 1998; Hotz & Gibson, 2007). Increased phytase activity during HTHH storage has been reported in cowpeas (Affrifah & Chinnan, 2005) and common beans (Mafuleka, Ott, Hosfield & Uebersax, 1993). However, phytase hydrolyses phytate into myo-inositol hence reducing its chelating potential with subsequent release of bound cations (Yousif *et al.*, 2007). In HTC development it is suggested that the released divalent cations diffuse to the cell wall forming insoluble pectates that reduce cell separation thus, contributing to increased cooking time.

2.4.2 Lignification in legumes during High Temperature and High Humidity (HTHH) storage

Lignification of the cell wall during storage under HTHH conditions is one of the mechanisms postulated to cause HTC defect in legume seeds (Hincks *et al.*, 1987; Del Valle & Stanley, 1995). Deposition of lignin-like material in cotyledon cell walls could lead to HTC defect development by restricting water movement within the cotyledons or by increased rigidity of the cell wall thus impairing swelling and separation (Hincks *et al.*, 1987). The Lignin matrix around the polysaccharide components could also make the cell wall hydrophobic and water impermeable (Medoua & Mbofung, 2006). Post-harvest lignin formation could occur as a stress response to adverse environmental conditions (Maldonado, Molina-Garcia, Sanchez-Ballesta, Escribano & Merodio, 2002). It is postulated that the HTHH storage conditions trigger lignin synthesis by the seed as a stress response to the adverse conditions (Hincks & Stanley, 1987; Martín-Cabrejas, Esteban, Perez, Maina & Waldron, 1997).

According to Ros Barcelo (1997) lignins are complex cell wall phenolic heteropolymers covalently associated with both polysaccharides and proteins. Lignins comprise of polyphenolic polymers built from the oxidative polymerisation of the three cinnamyl alcohols



(monolignols); p-courmaryl, coniferyl and sinapyl alcohol via the phenylalanine pathway (Luo, Xu & Yan, 2008). This leads to formation of hydroxyphenyl (H), guaiacyl (G), syringyl(S) units respectively within the lignin (Ros Barcelo, 1997) as shown in
Figure **2.6**.

Figure 2.6 Structures of the three monolignols and the residues derived from them. The numbering convention used is shown on the left (Whetten, MacKay & Sederoff, 1998)

The characteristics and structures of lignins are highly variable and differ among cell walls, cell type, tissues, plant organs and species (Ros Barcelo, 1997; Grabber, Ralph, Lapierre & Barrière, 2004). Lignin formation has been reported to involve three enzymes; phenyalalanine ammonia lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), and peroxidase (POD) (Whetten, MacKay & Sederoff, 1998). Lignification is thought to be controlled by PAL, which catalyses the deamination of phenylalanine to transcinnamate. CAD catalyses the conversion of coniferaldehyde into coniferyl alcohol and POD catalyses the polymerisation of monolignols to lignins (Imberty, Goldberg & Catesson, 1985). However, the actual extent of regulation of the lignification pathways by each of these enzymes are different in different tissues and presently not clear even in legumes (Luo *et al.*, 2008). The lignification hypothesis was first proposed by (Varriano-Marston & Jackson, 1981). Mafuleka *et al.* (1993) observed that there was minimal quantitative data critically investigating the role of lignification in HTC defect development. Most studies postulate lignification as a possible mechanism rather than providing actual experimental observation. In the lignification theory, storage of legumes under HTHH causes degradation of proteins thus yielding aromatic amino acids and small polypeptides (Hohlberg & Stanley, 1987; Martín-Cabrejas *et al.*, 1997). The aromatic amino acids then migrate to the middle lamella where the lignification process takes place. Hohlberg & Stanley (1987) reported an increase in free aromatic amino acids in HTC black beans (*Phaseolus vulgaris*) as a result of hydrolysis of storage proteins. Significant amounts of these amino acids specifically phenylalanine and tyrosine could lead to lignin formation in the cell wall (Hohlberg & Stanley, 1987). Deamination of phenylalanine or tyrosine by PAL or tyrosine ammonia lyase respectively would lead to formation of trans-cinnamic and trans-p-coumaric acids (Goodwin & Mercer, 1983; Garcia *et al.*, 1998a; Whetten *et al.*, 1998). These lignin precursors (monolignols) could then be polymerised into lignins in the presence of POD in the cell wall (Hohlberg & Stanley, 1987).

Qualitatively, Hincks & Stanley (1987) investigated the presence of lignin by staining extracted cell wall material of black bean cotyledons with potassium permanganate. Lignin presence was observed using a transmission electron microscope based on the reduction of potassium permanganate by lignin to manganese dioxide. They found a heavier deposition of manganese dioxide in cell corners, secondary walls and middle lamella of hard beans indicating lignification. Using a similar staining technique Bhatty (1990) reported darker and regular staining of the middle lamella of HTC lentils and lignin content was significantly higher for the HTC lentils. Garcia *et al.* (1998a) observed thickened cell wall junctions of cell wall isolates of HTC black beans using scanning electron micrographs. However the phlorogluconical -HCl staining test for lignin was negative.

Quantitative determination of lignin by Srisuma, Hammerschmidt, Uebersax, Ruengsakulrach, Bennink & Hosfield (1989) however showed no significant difference in lignin content in both seed coat and cotyledon cells in Navy beans. Mafuleka *et al.* (1993) reported increased lignin content of both decorticated white and red beans (*Phaseolus vulgaris*) during storage for four months at high temperature and humidity. However there was a decrease in lignin content when the storage time was increased from 4 to 8 months which was attributed to possible lignin-protein cross linking. Red beans had higher lignin content and higher hardness values compared to white beans indicating possible differences in phenolic contents due to genotype differences. Nasar-Abbas, Plummer, Siddique, White, Harris & Dods (2008) observed a 3 fold increase in lignin content during storage of faba beans at 50^{o}C for 12 months. Bhatty (1990) differed with the suggestion that partial lignification of the cell wall could hinder water permeation across cells since the hydration coefficient of both HTC lentils and control was generally similar. Although lignification is proposed as one of the main mechanism leading to HTC defect development, experimental studies investigating this theory are few. Further research work is required to understand the role of lignin in HTC defect development.

2.4.2.1 Peroxidase

Although both qualitative and quantitative tests have identified presence of lignin in HTC legume grains, studies of peroxidase enzyme leading to lignification have been contradictory. For instance, Paredes-Lopez *et al.* (1989b) observed the activity of peroxidases (POD) in HTC beans (stored at 30 ^{o}C and 85% RH) to be lower by 30-50% than that of control beans (stored at 15 ^{o}C and 35%). Plhak *et al.* (1987) had reported earlier that high levels of POD activity were not observed to relate to increased hardening or the rate of bean hardening. Hohlberg & Stanley (1987) also reported no difference in peroxidase activity in control bean samples stored at low temperature/humidity (15 ^{o}C, 35% RH) and high temperature/humidity (30 ^{o}C, 85% RH) conditions. Although peroxidases are proposed to catalyse the polymerisation of monolignols to lignins, no studies have shown a specific POD isoenzyme to be involved in lignin formation (Ostergaard, Teilum, Mirza, Mattsson, Petersen, Welinder, Mundy, Gajhede & Henriksen, 2000). The observation that lignin biosynthesis pathways and actual extent of regulation by enzymes is complex and presently not known (Ros Barcelo, 1997; Luo *et al.*, 2008), would explain the inconsistencies in correlating enzyme activity to lignin formation.

2.4.3 Involvement of phenolic compounds in the middle lamella

The "phytase-mineral and mineral-pectin interaction" and "lignification" theories in HTC defect in legumes suggest formation of new interactions or bonds in the middle lamella polymers that make cell separation difficult during cooking. Researchers have proposed that phenolic compounds other than lignin in the middle lamella may also form insoluble complexes with pectin and proteins that could impair cell separation (Srisuma *et al.*, 1989). Srisuma *et al.* (1989) observed increased free hydroxycinnamic acids during HTHH storage of beans with no increase in lignin content. The increase in free hydroxycinnamic acids was associated with increased hardening (Srisuma *et al.*, 1989). The free hydroxycinnamic acids could have been synthesized *de novo* from free aromatic amino acids liberated from hydrolysis of proteins during HTHH storage (Srisuma *et al.*, 1989). Hohlberg & Stanley (1987) reported an increase of aromatic amino acids as a result of storage proteins hydrolysis during HTHH storage of beans. Aromatic amino acids such as phenylalanine and tyrosine are immediate precursors of hydroxycinnamic acids (C_6-C_3 molecules) biosynthesis via phenylalanine and

tyrosine ammonia lyases (Whetten *et al.*, 1998). It is suggested that the increase in free phenolic acids observed during HTHH storage could promote protein-phenol interaction in the middle lamella therefore resulting in increased protein hydrophobicity (Srisuma *et al.*, 1989). Free phenolic acids have a high affinity for interacting with proteins. The increased protein hydrophobicity could inhibit water imbibition restricting water uptake and impairing cell separation during cooking (Srisuma *et al.*, 1989; Machado, Ferruzzi & Nielsen, 2008; Pirhayati, Soltanizadeh & Kadivar, 2011). It is proposed that free phenolic acids provides phenolic compounds for cross-linking to pectin in middle lamella and/or proteins that could result in HTC defect development (Srisuma *et al.*, 1989; Garcia *et al.*, 1998a). However, there seem to be no experimental study investigating the role of phenol-protein interaction in HTC defect development.

Garcia *et al.* (1998a) observed that phenolic acids bound to the water soluble pectin fraction were three times higher in HTC beans (*Phaseolus vulgaris*) than in control beans. Maurer *et al.* (2004) also observed more phenolic compounds in the soluble pectin fraction of HTC beans when compared to control. The observation of more ferulic acid bound to soluble pectin in HTC beans could inhibit cell separation during cooking as a result of crosslinking (Garcia *et al.*, 1998). Ferulic acid has been implicated in cross-linking cell wall polysaccharides leading to increased inter cell adhesion (Brett & Waldron, 1996)). Hydroxycinnamic acids are reported to cross-link plant cell wall polymers, especially polysaccharides and lignin (Ralph, Bunzel, Marita, Hatfield, Lu, Kim, Schatz, Grabber & Steinhart, 2004). Ferulic acids esterified to pectin can form diphenyl or ether bonds between the hydroxyl groups of phenolic compounds and the hydroxyl groups on polysaccharides (Shiga, Cordenunsi & Lajolo, 2011). According to Garcia, Filisetti, Udaeta & Lajolo (1998b), presence of more ferulic acid bound to soluble pectin, if involved in cross-links with other polysaccharides could ultimately lead to changes in cell adherence therefore leading to HTC defect by imparing cell separation upon cooking. As reported, resistance to softening even in the presence of chelating agents e.g. EDTA, suggests that crosslinking of pectic polymers via calcium ions is not the only factor limiting cell cell separation during thermal treatment of plant parenchyma cells (Parker & Waldron, 1995; Waldron, Ng, Parker & Parr, 1997; Marry, Roberts, Jopson, Huxham, Jarvis, Corsar, Robertson & McCann, 2006). According to Waldron *et al.* (1997), lack of thermally induced cell separation in plant

tissues often suggests "secondary thickening and associated lignification". However in non-lignified, thin walled plant tissues, failure to soften or extremely slow softening during cooking of parenchyma-rich plant tissues has been reported (Waldron *et al.*, 1997). The mechanism leading to thermal stability of these tissues has been suggested to be linked to the presence of ferulic acid dimers that crosslink cell wall polymers such as pectin (Parker & Waldron, 1995; Waldron *et al.*, 1997). This crosslinking is suggested to result from the activity of cell wall peroxidase (Biggs & Fry, 1987; Wallace & Fry, 1995; Brett & Waldron, 1996). Although increased phenolic acid bound to pectin has been observed during HTHH storage of beans (Garcia *et al.*, 1998b; Maurer *et al.*, 2004), there are no studies showing the proposed possible crosslinks with other polysaccharides in the middle lamella.

2.4.4 The role of starch and protein in HTC development

According to Hentges *et al.* (1991) starch could contribute to HTC phenomena because of alterations observed in starch. In a study to investigate possible changes on starch as a result of the HTC phenomena in common beans (*Phaseolus vulgaris*), Garcia & Lajolo (1994) observed more birefringence in starch granules of HTC beans. Differential scanning calorimetry (DSC) thermograms showed a 10% increase in starch gelatinisation temperature between the control (64.2 ^{o}C) and HTC beans (72.9 ^{o}C). Such an increase could be attributed to an increase in starch granule crystallinity or lower water availability which is necessary for gelatinization (Yousif *et al.*, 2007). Chemically hardened kidney beans were shown to exhibit high transition temperatures and enthalpy of gelatinisation of starch (Kaur & Singh, 2007). However, Hohlberg & Stanley (1987) found no differences for melt temperature, gelatinisation energy in isolated starch as a result of storage time or conditions. In the parenchyma cells in the cotyledon of cowpea seed, starch granules are embedded in a proteinaceous matrix (Sefa-Dedeh & Stanley, 1979). It has been suggested that during storage at high temperature and relative humidity there is a decrease in the solubility and thermal stability of protein (Hohlberg & Stanley, 1987; Liu *et al.*, 1992a). These could be due to enzymatic (proteases) hydrolysis (Liu *et al.*, 1992a). Thus, during cooking, protein coagulation/gelation is expected to occur before starch gelatinisation due to the reduction in the protein thermal transition temperature of stored cowpea seeds. This would lead to formation of a protein network around the starch granules, which would act as a water

barrier thus leading to reduction of water available for starch gelatinization (Yousif *et al.*, 2007). Liu *et al.* (1992a) observed that the thermal denaturation temperature (T_m) of unstored cowpea seeds was higher than 100 ^oC with no coagulation observed while after 18 months of storage, the T_m was 56 ^oC. A significant decrease in the protein denaturation enthalpy of common black beans has been observed on HTC beans (Garcia-Vela & Stanley, 1989).

2.5 Prevention of HTC defect

This section will review pre-treatment strategies in prevention of HTC defect in legumes. However, the main focus will be on heat pre-treatment techniques applied prior to storage of legumes.

2.5.1 Appropriate storage conditions

HTC defect develops during storage at high temperature and high relative humidity. Therefore, conditions under which cowpeas are stored are important in preventing the HTC defect development. The moisture content of the cowpeas, storage temperature, time and humidity are the main factors that need to be controlled (Molina *et al.*, 1976; Hohlberg & Stanley, 1987; Reyes-Moreno & Paredes-Lopez, 1993). Low temperature storage would reduce the enzymatic processes reported to lead to HTC. Studies have shown no practical change in texture during low temperature storage (Garruti & Bourne, 1985; Berrios *et al.*, 1999). Berrios *et al.* (1999) demonstrated that even after 2 years of storage at 4.5 ^oC and between 30 to 50% RH, common beans had good cooking characteristics. Common beans stored under refrigeration temperatures (0 to 5 ^oC) had minimal changes in hardness (Molina *et al.*, 1976; Affrifah & Chinnan, 2005). Therefore, low temperature storage of legumes is a practical way of preventing the HTC defect development. However, in tropical climates experienced in the developing countries, where legumes are a staple food, the refrigeration interventions may prove to be a challenge due to scarcity of resources. Therefore, there is need for alternative ways of preventing the defect.

2.5.2 Gamma irradiation

Gamma irradiation is an ionising radiation, no-heat process that has been investigated in the control of

HTC defect development in beans. Aguilera & Stanley (1985) reported that beans irradiated at 0.5 kGy had a reduced incidence of HTC when compared to the control after 10 months of storage at 22 ^{o}C. Cunha *et al.* (1993) later demonstrated that γ-irradiation at 2.0 kGy reduced the intial cooking time of beans by 30 minutes and a 50% reduction in cooking time when compared to the control after 6 months of storage at 30 ^{o}C, 75 % RH. Although these researchers did not provide the mechanisms through which the cooking time was improved, γ-irradiation are known to penetrate plant cells causing changes in structural features and split chemical bonds. For instance softening in fruits after γ-irradiation has been associated with the disintegration of the middle lamella due to dissolution of pectin (Kovacs, Keresztes & Kovacs, 1988) and increase in water soluble pectin (Gunes, Hotchkiss & Watkins, 2001). This is because irradiation causes random hydrolytic fissuring of the glycosidic bonds along the pectin molecule (Skinner & Kertesz, 1960; Sánchez Orozco, Balderas Hernández, Flores Ramírez, Roa Morales, Saucedo Luna & Castro Montoya, 2012). Therefore it is possible that the observed improvement in cooking time was due to the dissolution of the middle lamella pectin.

2.5.3 Heat pre-treatments

These pre-treatments aim at thermal inactivation of enzymes responsible for HTC development prior to storage of legumes. Practically, in designing a heat pre-treatment procedure, a balance between achieving a sufficient degree of enzyme inactivation and retention of physicochemical characteristics is essential. Dry and moist heat treatments have been applied as pre-treatments to legumes with varying levels of success. Heat pre-treatments have been demonstrated to reduce the HTC phenomenon in beans but total prevention of the phenomenon has not been achieved by these methods. This section will review the various studies focusing on heat pre-treatment techniques as strategies to prevent HTC defect development. The use of micronisation as a potential pre-heat treatment technique will also be reviewed.

2.5.3.1 Steaming

The earliest study in HTC defect prevention in literature seems to be that of Molina *et al.* (1976). In this study retorting (15 psi, 121 ^{o}C) and steaming (98 ^{o}C) were used as heat pre-

treatments. After treatment, the beans were stored at 25 ^{o}C, 70% RH for 9 months while the control was kept at 4^{o}C. Bean hardness was measured by a puncture test after cooking. The heat treatments decreased the hardness of the black beans when compared to the untreated when stored at 25^{o}C and 70% RH. It was interesting to note that the effectiveness of the heat-treatments in reducing hardness decreased with longer treatment times. However, no significant difference ($P > 0.05$) was found between the hardness of cooked beans treated at the shortest time (2 min at 121°C or 10 min at 98°C) and the control kept at 4 ^{o}C. This was in contrast with other studies which report increasing effectiveness in the prevention of HTC defect development with increased heating temperature and time (Aguilera & Stanley, 1985; Affrifah *et al.*, 2006). The mechanisms by which the heat treatments reduced hardness of beans stored under the adverse conditions were not clearly shown. The germinating capacity of the beans decreased as the thermal treatment increased indicating possible inactivation of enzymes responsible for the pathways of the defect development (Molina *et al.*, 1976). Although, the differences in germinating capacity indicate possible enzyme inactivation, no particular enzyme was investigated in this study.

Affrifah *et al.* (2006) investigated the possibility of preventing the HTC defect in cowpeas by using steam prior to storage (42°C, 80%RH). The goal of the steam treatment was to inactivate phytase. This appears to be the first study that investigated the effect of phytase inactivation in the control of the HTC defect (Affrifah *et al.*, 2006). The investigation into inactivation of phytase in cowpea flour revealed that it was dependent on initial moisture content, heating temperature and time (Affrifah & Chinnan, 2005). It was also noted that the plot of residual phytase activity as a function of heating time was nonlinear irrespective of the heating temperature or initial moisture content (**Figure 2.7**).

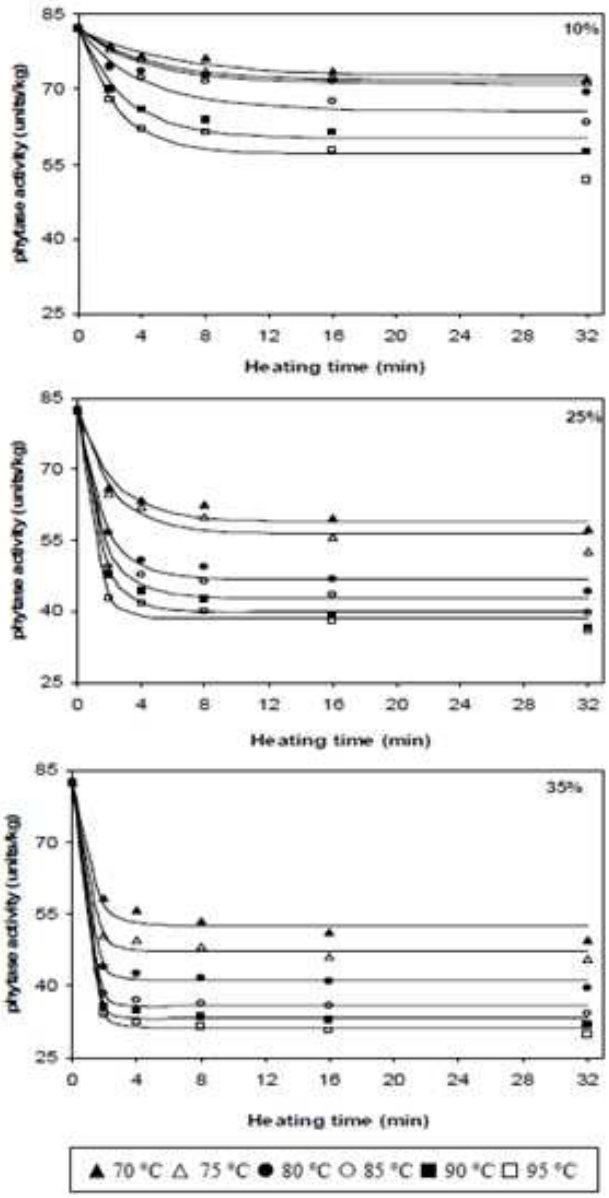


Figure 2.7 Thermal inactivation of phytase in cowpea flour as a function of heating time at various temperatures and moistures (Affrifah *et al.*, 2005)

Increased moisture content caused an increased degree of phytase inactivation. This was because water promoted unfolding of the protein during heat denaturation (Affrifah & Chinnan, 2005). In cowpea seeds, Affrifah *et al.* (2006) observed that higher steaming temperature (121°C) resulted in more phytase inactivation when compared to a relatively lower temperature (100 ^{o}C). During storage, the factors investigated were the initial moisture content (13, 20%), steaming temperature (100, 121°C), and steaming time (2, 4, 6 min). It was observed that only steaming for 4 or 6

minutes at 121°C was effective in controlling the defect development during HTHH storage for 9 weeks (Affrifah, 2004). The cooked texture of the steam treated cowpeas was comparable to that of the control samples before storage. Steaming at 121°C reduced the phytase activity by up to 64% of the original activity. However, an increase in phytase activity during HTHH storage was reported (Affrifah *et al.*, 2006). Interestingly, the main chemical constituents reported to contribute to HTC defect did not seem to have an influence on cooked texture of the cowpeas after the heat preatments (Affrifah, 2004). There was no significant correlation between the cooked texture and the measured indices such as phytase activity, phytate content and pectin loss (Affrifah, 2004). According to these researchers, the changes in the cooked hardness following pre-treatments could have occurred through different pathways than those associated with HTC defect development (Affrifah, 2004).

2.5.3.2 Roasting

Roasting has been used in prevention of HTC defect in beans. Roasting involved the use of dry roasters that consistent of rotating chambers that had either pre-heated sand (Aguilera & Stanley, 1985; Plhak *et al.*, 1987; Rivera *et al.*, 1989) or ceramic beads (Aguilera, Lusas, Uebersax & Zabik, 1982). The mechanism of heat transfer in was via conduction since it was a particle-to-particle (solid-to-solid) heat transfer (Aguilera, Roman & Hohlberg, 1987). Heating occurred when the beans were in contact with the heating medium. Earlier roasting studies indicated that the effectiveness of roasting was as a result of moisture loss due to drying (Aguilera, Hau & Villablanca, 1986) and did not investigate the role of enzyme inactivation. Therefore, after roasting, storage of beans in moisture impermeable packaging was necessary to prevent re-entry of water during HTHH storage (Rivera *et al.*, 1989). This was considered an expensive procedure that would not be practical in tropical and subtropical bean producing regions (Rivera *et al.*, 1989). Later studies demonstrated that the effectiveness of roasting dependent on enzyme inactivation and not just moisture reduction (Plhak *et al.*, 1987; Rivera *et al.*, 1989).

Aguilera & Stanley (1985) used high temperature-short time (HTST- 105 ^{o}C, 3min) and medium temperature-long time (MTLT-70 ^{o}C, 60 min) roasting as pre-treatments before

storage. The beans were dry heat treated and stored in a sealed polyethylene bag at 22 ^{o}C for 10 months. The RH conditions were not stated but the beans moisture content was 12%. The hardness was determined by puncture test after cooking (autoclaving for 15 or 20 minutes at 121^{o}C). In comparison to the control at day 0, both HTST and MTLT samples increased hardness by 2.5 times while the control had a 2.9 times increment after storage. The mechanism of HTC defect reduction was not clearly explained though reduced germination capacity of the heat-treated seeds suggested some degree of enzyme inactivation. The roasting temperatures were low and thus it was probable that enzyme inactivation was not effective. It has been suggested that low heating temperatures during the pre-treatments cause a less inactivation effect on the enzymes that could subsequently recover during storage and initiate the hardening process (Affrifah, Chinnan & Phillips, 2005).

Plhak *et al.* (1987) demonstrated that sand roasting black beans to an internal temperature of 80 ^{o}C in 2 min did not prevent HTC defect at 30°C/80% RH for 12 months. Peroxidase (POD) was not inactivated at these conditions and showed increased activity between 4-7 months of storage. This was associated with increased hardness of cooked beans. Rivera *et al.* (1989) used sand roasting at 200 ^{o}C as a pre-treatment technique. The beans were pre-conditioned at 16, 25, 30, and 52 % moisture content before roasting. The beans were heated to three different temperatures per each moisture level (Table 2.3). The heating time per period was however not stated. After roasting, the beans were adjusted to 11% moisture, sealed in polyethylene-aluminium foil bags and stored for 337 days at 32 ^{o}C. The effect of roasting on POD activity and hardness of cooked beans after storage is shown in Table 2.3. It was observed that in each moisture group the residual POD activity and hardness of cooked beans were a function of the temperature during roasting (Table 2.3). Lower temperatures favored higher POD activities and hardening ($P \leq 0.01$). It was also observed among the groups, that higher equilibrium temperatures were necessary to inactivate POD at lower bean moistures and subsequently achieve less hardening.

Table 2.3 Effect of heat treatment on bean hardening (Rivera *et al.*, 1989)).

Initial Moisture (%)	Temperature (^oC)	Final Moisture (%)	Relative Hardness (F_{337}/F_0)	POD Activity (Δabs/min/g)
52	77	46	2.39	43
52	89	42	1.84	0
52	100	39	1.66	0
30	94	22	2.01	40
30	105	20	2.07	0
30	109	18	1.69	0
25	93	14	2.29	0
25	103	15	1.89	0
25	110	11	1.59	0
16	96	12	2.27	113
16	102	12	2.46	59
16	118	7	1.55	0

Interestingly, the researchers noted that while the samples with temperatures above 102 ^oC had no POD activity (Table 2.3) and had acceptable hardness (< 2, relative hardness), increasing equilibrium temperatures above 105 ^oC continued to reduce the hardness. The researchers however did not explain what caused the reduction in hardness with increasing equilibrium temperatures. The researchers only studied the effect of heat treatments on hardening and POD inactivation. They did not investigate other physico-chemical changes associated with hardening therefore it would be difficult to explain probable mechanisms that led to reduction in hardness with increased temperature.

2.5.3.3 Microwave

Cunha *et al.* (1993) studied the use of microwave heating (105^oC, 2min) and gamma irradiation prior to storage as means to prevent the HTC defect. The beans which had about 10% moisture were not pre-conditioned. It was noted that while irradiation reduced the initial cooking time, microwave heating increased the cooking time by 16 minutes (Table 2.4). Microwave heating

for longer than 2 minutes was increased the initial cooking time exponentially. Low moisture content during heating may lead to hardening and contraction of the bean seed coat referred to as "case hardening" that reduces the ability of the seed to hydrate during cooking (Aguilera & Stanley, 1985). This leads to an increased cooking time. After 6 months of storage at HTHH, microwaved beans had 7.4 times increase in cooking time when compared to the control which increased the cooking time by 5.1 times (Table 2:4). Complete inactivation of POD by microwave heating did not prevent or reduce the HTC defect development. Although phytase activity was not investigated, microwaved beans were observed to have lower phytate content when compared to the control after storage for 6 months at HTHH.

Table 2.4 Changes in cooking time (min) of dry beans stored for 6 months under refrigeration (4 °C) or at 30 °C, 75 % RH (Cunha *et al.*, 1993)

Storage time (months)	Storage conditions	Treatment*	
		control	microwaved (2min)
0		47^{b}	63^{c}
2	Refrigeration	46^{bA}	49^{bA}
	30 °C, 75 % RH	65^{aB}	122^{bB}
4	Refrigeration	42^{bA}	45^{bA}
	30 °C, 75 % RH	233^{bB}	224^{bB}
6	Refrigeration	41^{bA}	46^{bA}
	30 °C, 75 % RH	292^{bB}	412^{cB}

*a-c (rows) indicate statistically significant differences ($P \leq 0.05$), among treatments for each condition and time of storage. A, B (columns) indicate statistically significant differences ($P \leq 0.05$) between storage conditions at each storage time.

2.5.3.4 Micronisation

Micronisation (Infrared (IR)) heat processing involves heating with radiant electromagnetic energy in the wavelenth range of 1.8 to 3.4 µm (Fasina, Tyler, Pickard & Zheng, 1999). IR heating has been shown to be superior over conventional heating under similar heating conditions (Sandu, 1986; Hebbar & Rastogi, 2001) and is gaining increased use in food processing (Fasina *et al.*,

2001). IR heating is advantageous due to its ability to be absorbed and it penetrates the product causing internal heating (Krishnamurthy *et al.*, 2008). This leads to a fast increase in temperature within the product (Andrejko, Rydzak, Slaska-Grzywna, Gozdziewska & Kobus, 2008). It also has high heat intensity, efficient heat transfer and uniform heating of the material is achieved (Sakai & Hanzawa, 1994; Fasina *et al.*, 2001; Krishnamurthy *et al.*, 2008). Conventional heating processes require a longer processing time compared to radiant electromagnetic heating such as micronisation and microwave heating. Microwave heating has similar advantages to micronisation (faster heating rates, high energy efficiencies and shorter processing times) over conventional heating (Fakhouri & Ramaswamy, 1993; Oliveira & Franca, 2002). The heat intensity of IR radiation is reportedly higher by about 20-100% as compared to convection heat intensity (Ginzburg, 1969).

The aim of most studies of micronisation of legumes was in reduction of cooking time. Legumes are known to take a considerable time to get cooked and thus pretreatment with micronisation reduces the cooking time required for legumes to attain a texture soft enough for consumption (Arntfield, Scanlon, Malcolmson, Watts, Ryland & Savoie, 1997). Micronisation has been shown to reduce the cooking time of tempered cowpeas by 44 % (Mwangwela *et al.*, 2006). The cooking time of lentils was reported to be reduced by 50 % on being micronized (Cenkowski & Sosulski, 1997). The mechanisms by which micronisation reduces cooking time are not fully understood. However, physico-chemical changes during micronisation such as improved water uptake due to fissure formation in the seed coat and cotyledon, pre-gelatinisation of starch (Arntfield *et al.*, 1997; Bellido, Arntfield, Cenkowski & Scanlon, 2006; Mwangwela *et al.*, 2006) and denaturation of protein (Cenkowski & Sosulski, 1997; Arntfield *et al.*, 2001) are suggested to promote the reduction in cooking time. Therefore, the effectiveness of micronisation is dependent on the processing parameters (temperature-time-moisture) that promote these physico-chemical changes (Scanlon, Malcolmson, Arntfield, Watts, Ryland & Prokopowich, 1998).

For micronisation to be effective, it is critical that the legume seeds which are generally of low moisture content are first tempered (pre-conditioned) to a higher moisture level. The generation of super-heated steam from the tempering moisture is critical since the physico-chemical changes such as partial starch gelatinisation and protein denaturation are dependent on the moisture

content. The observed fissures (Mwangwela *et al.*, 2006), fracture lines (Arntfield *et al.*, 2001) and increased porosity in micronised legumes (Scanlon, Cenkowski, Segall & Arntfield, 2005; Mwangwela *et al.*, 2006), which improve water uptake during cooking are also due to the increased vapour pressure. Studies indicate that there is a possible hardening that occurs when legume seeds are micronised with limited moisture. Arntfield *et al.* (1997) observed that micronised lentils with moisture contents of < 20% failed to attain an acceptable softness after cooking. Abdul-Kadir, Bargman & Rupnow (1990) reported an increased cooking time of micronised pinto beans that had 17 % moisture content.

Micronisation temperatures ranging from 69 ^{o}C (Sarantinos & Black, 1996) to 180 ^{o}C (Arntfield *et al.*, 2001; Phadi, 2004) have been used in legumes. Legumes micronised at high temperatures (> 160 ^{o}C) are shown to have a longer cooking time (Mwangwela *et al.*, 2006) or harder (Arntfield *et al.*, 2001) when compared to those micronised at relatively low temperatures (about 150 ^{o}C). The reason for longer cooking time or harder textures observed with high temperature micronisation is not clearly known. Mwangwela, Waniska, McDonough & Minnaar (2007) suggested that starch depolymerisation and/or amylose associated crosslinking due to the high micronisation temperature (170 ^{o}C) could contribute to longer cooking times. Phadi (2004) suggested that reduced starch gelatinisation of cowpeas due to denaturation of the protein matrix surrounding the starch granules which would limit hydration of starch granules and thus prolong the cooking time.

2.5.4 The use of micronisation in preventing HTC

Micronisation has not been used in the prevention of HTC defect in legumes. However, Salvador (2007) used micronisation to improve the cooking time of cowpeas after HTC defect had developed in Cowpeas. It was demonstrated that the cooking time of HTC cowpeas was decreased significantly when pre-conditioning with either water or in a solution with monovalent (Na+) cations was combined with micronization. Interestingly, although micronisation increased pectin solubility, it was not coincident with reduction in the cooking times. The improvement in cooking time could have been due to pre-gelatinisation of starch and denaturation of proteins that occurs during micronisation of legumes (Mwangwela *et al.*, 2006).

The better heating properties of micronisation could have potential in reducing HTC defect development in cowpeas (legumes). The aim of micronisation would be to inactivate enzymes such as phytase and peroxidase which are reported to catalyse reactions leading to defect development. The effectiveness of the heat pretreatments appears to be dependent on temperature, time and moisture conditions of the cowpeas. Temperatures of about 100^{o}C are shown to completely inactivate peroxidase (Rivera *et al.*, 1989). However, cowpea phytase appears to be heat stable (Affrifah *et al.*, 2006). Severe heat treatments such as increasing the temperature (170 ^{o}C) and time could result in increased inactivation of phytase. However, these severe conditions such as high temperatures (170 ^{o}C) are observed to increase cooking times in cowpeas (Mwangwela *et al.*, 2006) and increased hardness in lentils (Arntfield *et al.*, 2001). Therefore, a balance between enzyme inactivation and not negatively changing the physico-chemical properties of the seed is crucial.

Pre-conditioning is crucial the cowpeas have a low moisture content. However, the relatively high moisture (40%) pre-conditioning applied by Mwangwela *et al.* (2006) and Salvador (2007) might not be suitable for seeds that are to undergo storage. Micronising (150 ^{o}C) cowpeas pre-conditioned to 40 % moisture content yielded a final moisture content of 15%(Mwangwela *et al.*, 2006). Temperatures above 13% seem to promote HTC defect development (Aguilera & Rivera, 1990). Therefore, it could be necessary to dry the seeds to lower moisture content if the final moisture content after micronisation is still high. High pre-conditioning moisture would also cause seed cracking or fissuring (Mwangwela *et al.*, 2006) due to increased vapour pressure within the seed. Although not studied, the fissuring along the cells could have the potentiation effect by bringing the reactants and substrates leading to the HTC defect together.

2.6 Gaps in knowledge

Storage of legumes at high temperature and relative humidity leads to a HTC defect. Legumes with this defect are characterised by long cooking time in attaining the acceptable soft texture. Cowpeas, as other legumes are prone to the hard-to-cook (HTC) phenomenon. Several hypotheses have been proposed to explain the cause of HTC, but the mechanisms are still unknown. The “phytase-phytate-pectin” hypothesis and the lignification hypothesis are among the main

hypotheses suggested to lead to HTC defect. Heat pre-treatments are some of the strategies utilised in possible prevention of HTC defect development. The aim of these heat pre-treatments is to inactivate enzymes implicated in the development of the HTC phenomenon. Complete prevention of the HTC defect development has not been achieved through the use of heat pre-treatments although reduction in the defect development has been achieved. Micronisation which is IR heating treatment of pre-conditioned seeds has not been utilised as a heat pre-treatment strategy in the prevention of HTC defect development. The superior heating properties of micronisation over conventional heating could lead to more effective inactivation of these enzymes. Therefore, there is need to explore its effectiveness in the control of HTC defect in comparison to conventional heating techniques such as hot-air roasting.

The current review of literature demonstrates that most heat pre-treatment studies report on reduced incidence of HTC without explanation on the mechanisms via which the heat pre-treatment improved the cooking time of the legumes. There is therefore a need for studies to investigate the effect of the heat pre-treatments on the physico-chemical changes known to lead to HTC phenomenon.

2.9 Hypotheses

Storage of cowpea seeds at high temperature - high relative humidity conditions will result in the development of HTC defect due to the increased enzymatic activities of phytase (Galiotou-Panayotou *et al.*, 2008) and peroxidase. Increased phytase activity will hydrolyze phytate resulting in release of divalent cations that will bind with pectin in the middle lamella to form pectates. This will lead to a decrease in water-soluble pectin and subsequent increase in cooking time. Peroxidase promotes lignification of the cell walls therefore imparting toughness and a barrier to water penetration during cooking (Hincks *et al.*, 1987) therefore resulting increased cooking time.

Micronisation and hot-air pre-treatments of pre-conditioned cowpeas will prevent/reduce the development of HTC defect in cowpeas during storage at HTHH conditions due to inactivation of phytase and peroxidase. Heat pre-treated cowpeas will have a shorter cooking time due to less formation of pectates in the middle lamella thereby leading to easier separation of parenchyma

cells during cooking.

Changes in the physico-chemical characteristics of starch due to the heat pre-treatments and storage under HTHH conditions will influence the cooking time of the cowpeas. Heat moisture treatments are reported to pre-gelatinise starch (Islam, Shimizu & Kimura, 2002; da Rosa Zavareze & Dias, 2011) therefore reducing the energy and time required in cooking of starchy foods (Islam *et al.*, 2002). Micronisation has been observed to pre-gelatinise starch in legumes thereby contributing to the reduction in the cooking time of these legumes (Cenkowski & Sosulski, 1997; Arntfield *et al.*, 2001; Mwangwela *et al.*, 2007). On the other hand, storage of legumes under HTHH conditions is proposed to increase starch granule crystallinity (Hohlberg & Stanley, 1987; Liu *et al.*, 1992a) as demonstrated by increase in starch gelatinisation temperature and gelatinisation enthalpy (Garcia & Lajolo, 1994; Reyes-Moreno, Rouzaud-Sandez, Milan-Carrillo & Garzon-Tiznado, 2001). Therefore, more thermal energy and longer cooking times will be required to gelatinise the starch.

2.10 Objectives

The primary objective of this research was to determine the effect of micronisation and hot-air roasting on prevention of the hard-to-cook phenomenon during HTHH storage of cowpeas.

The specific objectives were to:

1. Determine the effect of accelerated storage conditions of HTHH on the development of HTC defect in different cowpea types.

2. Determine the effects of micronisation and hot-air roasting of pre-conditioned cowpeas on enzymatic activity, physico-chemical and microstructural characteristics of cowpeas during accelerated HTHH conditions.

3. Determine the effects of micronisation and hot-air roasting of pre-conditioned cowpea seeds on the thermal and pasting properties of cowpea flour and starches after HTHH storage with the aim of understanding scientific mechanism of HTC defect prevention.

3 RESEARCH

The research work comprised three phases. The first phase (section 3.1) investigated the role of HTHH storage on the development of HTC defect in three cowpea types and selecting a cowpea type that was more prone to HTC defect. The second phase (section 3.2) involved heat pre-treatment of the selected cowpea type (pre-conditioned) with micronisation and hot-air roasting in prevention of HTC defect. The aim of the pre-treatments was to inactivate enzymes involved in HTC defect development. The third phase (section 3.3) focused on exploring further structural changes and physico-chemical changes that contributed to prevention of HTC defect by the heat pre-treatments.

3.1 Hard-to-cook phenomenon in cowpeas: effect of accelerated storage on cooking and physico-chemical characteristics of cowpeas

Abstract

Storage of legumes at accelerated conditions of high temperature and high relative humidity (HTHH) similar to tropical conditions leads to development of the hard-to-cook (HTC) phenomenon. Cowpeas with the HTC defect are characterised by long cooking time. The objective of this study was to determine the effect of accelerated storage conditions (40 ^{o}C and 80% RH for 40 days) that simulate tropical conditions on the development of HTC defect development in cowpeas. Three cowpea types were used (*Bechuana white*, *Agripienk* and *Mae–e–tsiliwane*). The hard-to-cook (HTC) defect developed in cowpeas stored under HTHH conditions but not in those kept under refrigeration conditions (4 ^{o}C) as indicated by increased cooking time. The accelerated conditions of HTHH storage used in this study were effective in inducing the hard–to-cook (HTC) defect. The cowpeas differed in their susceptibility to HTC defect, with *Bechuana white* being the least susceptible to the defect. The increased cooking time in HTHH stored legumes was associated with decreased phytate content and decreased water soluble pectin content which was in support of the phytase-phytate-pectin theory. However, the role of lignification in the development of HTC defect could not be established as both peroxidase activity and lignin content did not increase during HTHH storage.

3.1.1 Introduction

Legumes such as cowpeas play an important role in the diets of the people in sub-Saharan Africa. Cowpeas are important source of affordable protein and complement the mainly carbohydrate-based diets in this region (Phillips, McWatters, Chinnan, Hung, Beuchat, Sefa-Dedeh, Sakyi-Dawson, Ngoddy, Nnanyelugo & Enwere, 2003). After harvesting, legumes are generally stored for long periods before consumption. Storage of legumes at low temperatures seems to have minimal or no significant effect on the cooking time of legumes (Hentges, Weaver & Nielsen, 1990; Berrios *et al.*, 1999). However, storage under adverse conditions of high humidity and high temperature (HTHH) prevalent in tropical regions leads to hard-to-cook

(HTC) defect (Coelho *et al.*, 2007). Cowpeas with HTC defect are characterized by long cooking times since they do not soften enough for consumption during cooking. Differences in the degree of HTC defect development during storage at HTHH are reported among varieties of the same legume species (Reyes□Moreno, Rouzaud□Sandez, Milán□Carrillo, Garzón□Tiznado & Camacho□Hernández, 2001; Coelho *et al.*, 2007). This is because the physico-chemical characteristics that affect the cooking time of legumes are influenced by either genotype differences in varieties and/or agronomic practices (Coelho *et al.*, 2007). For instance differences in phytate content among common bean varieties has been shown to affect their susceptibility to HTC defect (Coelho *et al.*, 2007). On the other hand agronomic practices such as addition of phosphorus in the soil could lead to increased phytate content (Coelho *et al.*, 2007).

The two main hypotheses proposed to explain HTC defect development are the phytase-phytate - pectin theory (Hincks *et al.*, 1987; Galiotou-Panayotou *et al.*, 2008) and lignification theory (Hincks *et al.*, 1987). The "phytase-phytate-pectin" theory proposes insolubilisation of the middle lamella pectin due to crosslinks with divalent cations such as calcium. These cations results from the hydrolysis of phytate due to increased phytase activity at HTHH (Reyes-Moreno *et al.*, 2000a). During cooking, the insoluble pectates in the middle lamella hinder hydration of the cell and restrict cell separation which is essential for softening (Galiotou-Panayotou *et al.*, 2008). The second theory proposes lignification of the cell wall/middle lamella due to potential catalysation by increased peroxidase activity (Hincks & Stanley, 1987). Strengthening of cell walls due to lignin deposition in the cell wall or middle lamella would results in poor water penetration into the parenchyma cells during cooking (Hincks & Stanley, 1987). It is also suggested that strengthening of cell walls reduces swelling of the cell during cooking therefore restricting cell separation (Stanley, 1992).

There is lack of an integrated study investigating the dual theories of lignification and "phytase-phytate-pectin" in cowpeas. Most studies focus on the "phytase-phytate-pectin" theory and not the lignification theory. Therefore there is need to investigate these two theories with respect to the suggested mechanisms i.e. the enzymes involved and their influence on physico-chemical characteristics.

The objective of this study was to determine the effect of storing different cowpea types under accelerated conditions of HTHH that simulate tropical conditions on the development of the hard-to-cook defect. The changes in the physico-chemical characteristics of the cowpeas associated with the proposed phytase-phytate-pectin and lignification theory were also investigated.

3.1.2 Materials and methods

3.1.2.1 Raw materials

Eleven cowpea types were sourced from Botswana and South Africa for this study. However, 8 types were found to have excessively long cooking times (> 270 minutes) which indicated they already had the HTC defect. Therefore only three types were selected for the study : *Bechuana white* (Agricol, Potchefstroom, South Africa), *Agripienk* (Agricol, Potchefstroom, South Africa) and *Mae–e–tsiliwane* (Botswana). The cowpeas were cleaned to remove any foreign material, broken and shriveled seeds. The cleaned seeds were packed in air-tight buckets and stored at 4 °C until used.

3.1.2.2 Inducement of HTC defect

HTC defect was induced by incubating cowpea seeds at 40 ^{o}C and 80% RH for 40 days. Cowpea samples were placed on wire mesh tray (single layer) at three different levels, with each variety on its own tray. These were placed inside a 45cm x 30 cm x 40 cm air tight plastic container containing saturated potassium chloride (KCl) solution at the bottom. The KCl solution was to maintain constant humidity conditions (80%) as described in ASTM E 104-02 (ASTM International E104-02). The container was then placed in a growth chamber at 40 °C (Labcon, Johannesburg, South Africa). The temperature and relative humidity were maintained at 40 °C and 80% RH throughout as monitored using a humidity - temperature logger (ThermaData™, ETI ltd, West Sussex, UK).

3.1.2.3 Flour preparation for assays

Cowpea seeds were finely ground to pass through a 0.8 mm screen using a laboratory hammer mill 3100 (Falling number, Sweden) and packaged in zip lock polyethylene bags. Analyses were conducted at day 0, storage at 4 ^{o}C for 40 days and storage at 40^{o}C and 80% RH for 40 days.

3.1.2.4 Moisture content

Moisture content was determined according to AACC Method 44-15A (American Association of Cereal Chemists 1995). Moisture tins were dried in a hot-air oven at 103 °C and cooled in a desiccator. 5g of the cowpea flour was weighed into the weighed dried moisture tins. The tins were then covered in foil and dried in a hot-air oven at 103 °C for 4h. The samples were then cooled in a desiccator and weighed. The moisture content was then calculated as:

$$\% \text{ moisture content} = \frac{\text{moisture loss(g)}}{\text{intial sample weight(g)}} \times 100$$

3.1.2.5 Cooking time

Cooking time was determined by a Mattson bean cooker as described by Mwangwela *et al.* (2006). For each treatment, 25 cowpea seeds were positioned in the perforations of the cooker, placed in an aluminium pot with 1.5 L of deionised water and boiled. The cooking time of the cowpeas was recorded as the time when 80% of the 50 g pins had penetrated the cowpea seeds and plunged through the hole at the base.

3.1.2.6 pH

pH was determined as described by mixing 5g of cowpea flour with 100 mL of deionised water and stirred for 45 min at room temperature. The pH of the solution was expressed as cowpea tissue pH (Liu *et al.*, 1992a).

3.1.2.7 Water activity

Flour water activity was measured using a Pawkit water activity meter (Decagon Devices, Inc., Pullman, Washington, USA).

3.1.2.8 Phytate

Cowpea flour was extracted in hydrochloric acid (0.66 M) and neutralised by addition of 0.75M sodium hydroxide. The neutralised sample extract was used in the enzymatic de-phosphorylation reaction assay using a phytic acid (phytate)/total phosphorus analysis kit (K-PHYT) of Megazyme (Megazyme International, 2010). For the free phosphorus, 0.62 mL of distilled water, 0.20 mL of sodium acetate buffer and 0.05 mL of sample extract were pipetted into a 1.5 mL microfuge tube. The mixture was vortexed and incubated in a water bath set at 40^{o}C for 10 min. After incubation, 0.02 mL of distilled water and 0.2 mL of glycine buffer were added. The mixture was vortexed and incubated in a water bath at set at 40^{o}C for 15 min. The reaction was stopped by addition of 0.30 mL of trichloroacetic acid (TCA) (50 % w/v). The solution was then centrifuged at 13000 rpm for 10 min. The supernatant was pippeted for the colorimetric determination of phosphorus. For the total phosphorus, 0.60 mL of distilled water, 0.20 mL of sodium, acetate buffer, 0.05 mL of sample extract and 0.02 mL phytase suspension were pipetted into a 1.5 mL microfuge tube. The mixture was vortexed and incubated in a water bath set at 40^{o}C for 10 min. After incubation, 0.2 mL of glycine buffer and 0.02 mL of alkaline phosphatase suspension were added. The mixture was vortexed and incubate din a water bath at set at 40^{o}C for 15 min. The reaction was stopped by addition of 0.30 mL of trichloroacetic acid (50 % w/v). The solution was then centrifuged at 13,000 rpm for 10 min. The supernant was pippeted for the colorimetric determination of phosphorus.

Phosphorous content was determined calorimetrically using ammonium molybdate. The absorbance was read at in a spectrophotometer (Lambda EZ150 Inc, Perkin Elmer, Waltham Massachusetts, USA) 655 nm within 3 hours. Phytate content was calculated using Megazyme's Mega-Calc^{TM} for phytic acid (phytate)/total phosphorus determination.

3.1.2.9 Phytase activity and free inorganic phosphorus (Pi)

Phytase activity was measured using the direct incubation method with some modifications (Shen, Yin, Chavez & Fan, 2005). To calibrate for background P contributions in the samples, 15 mL of TCA stop solution (0.92 M) was added to 1 g of sample in a 250 mL Erlenmeyer flask. This was followed by addition of 85 mL of sodium acetate buffer at pH 5.5. The flask was swirled and 1 mL of the mixture was transferred to a 15 mL centrifuge tube for the measurement of non-specific P contributions at time zero. Free inorganic phosphate (P*i*) before incubation was expressed as sample blank before incubation.

One gram of cowpea flour was incubated with 100 mL of sodium phytate substrate (1.5 M) at 37 ^{o}C for 30 min. After 30 min, 2 mL of the incubated mixture was transferred into a 15 mL centrifuge tube containing 1 mL of TCA stop solution. The mixture was vortexed and then centrifuged at 2000*g* for 15 min. One mL of the supernatant was added to 1 mL of colour reagent. Liberated phosphate was measured by the ammonium molybdate method (Eeckhout & De Paepe, 1994). The absorbance was read at 700 nm in a spectrophotometer (Lambda EZ150) within 3 hours after centrifugation at 10000 *g* for 3 min. Calibration standards were prepared from potassium dihydrogen phosphate. Phytase activity was measured as phytase units (FTU). FTU was the amount of phytase that catalyses the release of micromoles of inorganic phosphate per minute from 1 kg of dry matter (DM) samples.

3.1.2.10 Peroxidase activity

Peroxidase activity was determined as described by Plhak *et al.* (1987). Five grams of cowpea flour was extracted with 50 mL of 0.1 M citrate phosphate buffer (pH 6.0) containing small amount of polyvinylpolypyrrolidone (PVPP) for 30 min at 4 ^{o}C under continuous shaking. The solution was then centrifuged at 6000 *g* at 4 ^{o}C for 15 min. 1 mL of the supernatant (crude peroxidase extract) was pipetted into a reaction mixture containing 2 mL of 0.05 M citrate phosphate buffer (pH 6.0) and 2mL of 0.4 mM guaicol. The reaction was intilialised by addition of 2 mL of 3% hydrogen peroxide and the change in absorbance measured in a

spectrophotometer (Lambda EZ150 Inc, Perkin Elmer, USA for 5 min at 470 nm. Results were expressed as ΔA/ min/g.

3.1.2.11 Water soluble pectin extraction and determination

Water-soluble pectin was determined in raw cowpeas. Five g of cowpea flour was extracted 3 times with 95% ethanol to remove soluble sugars.. The alcohol insoluble solids (AIS) pellet was vacuum dried at room temperature and stored in a desiccator.

One g of AIS was extracted with 10 mL deionised water for 10 min at 300 rpm in an orbital shaker (Grant-bio, Grant instruments ltd., Cambridgeshire, UK) and allowed to stand for 10 min. The mixture was centrifuged and the supernatant collected into a 25 mL volumetric flask. The extraction was repeated on the residue and the supernatant was added to the same volumetric flask. The supernatant solution was diluted to the 25 mL mark and filtered using a Whatman No. 41 ashless filter paper. The filtered cold water soluble pectin extract was stored (<24 h) at 4 °C until analysis. Pectin content of each extraction was determined by using *m*-hydroxydiphenyl method.

A calibration series of galucturonic acid (20, 40, 60, 80 and 100 µg/mL) was prepared to be used as a standard curve. For the samples and the standards three tubes were used: two tubes for sample/standard + one tube for blank determination. The assay reagent consisted of sodium tetraborate in sulphuric acid (0.0125 M solution), 0.5% sodium hydroxide and *m*-hydroxydiphenyl solution (0.15%). One mL of sample or standard was pipetted into each of three test tubes in an ice bath. After few minutes to allow cooling, 6.0 mL of the tetraborate solution was added to each test tube and mixed thoroughly using a vortex mixer. The tubes were transferred in a boiling water bath for exactly 6 min. The tubes were then cooled in an ice bath. After cooling, 0.1 mL of *m*-hydroxydiphenyl was added to the first two tubes to develop colour and mixed thoroughly using a vortex mixer. For the blank, 0.1 mL of 0.5% sodium hydroxide was added to the third tube and mixed thoroughly. All the tubes were allowed to stand for 15-20 min at room temperature to allow any bubbles formed to dissipate. The absorbance was read at

520 nm in a spectrophotometer (Lambda EZ150) by reading sample or standard against its corresponding blank.

3.1.2.12 Determination of acid detergent lignin (ADL)

Acid detergent lignin (ADL) content was determined gravimetrically by the Goering & Van Soest method (Goering & Van Soest, 1970) using a Foss Analytical FibreCap™ 2021/2023 system (FOSS Analytical ABB, Höganäs, Sweden). ADL was determined gravimetrically by first obtaining the acid detergent fibre, and then extracting it with 72% H_2SO_4 (w/w) and ashing to determine percent ADL. The sample was contained in FiberCap capsules (polypropylene containers with a snap lids).

Lignin was calculated as follows:

$$C = \frac{\text{blank capsule weight after extractions}}{\text{initial blank capsule weight}}$$

$$\%\ \text{ADL (Acid Determined Lignin)} = \frac{(W_3 - (W_1 \times C) - (W_5 - W_4 - D)) \times 100 \times 100}{W_2 \times \%\ \text{db}}$$

Where: W_1 = initial capsule weight, W_2 = sample weight, W_3 = capsule + residue weight, W_4 = crucible weight, W_5 = ash + crucible weight, C = blank correction for capsule solubility, D = capsule ash weight

3.1.3 Statistical analyses

The experiment was done in duplicate. The effect of cowpea type and storage conditions as well as their interactions on the physico-chemical characteristics were evaluated using analysis of variance (ANOVA) based on a 5% level of significance. Correlation coefficients (*r*) were determined to establish relationships between physico-chemical characteristics. Principal component analysis (PCA) of the cowpeas and physico-chemical characteristics was conducted

using a correlation matrix. Statistica Version 8.0 (Statsoft, Tulsa, OK, USA) was used for data analysis.

3.1.4 Results

Cooking time before storage of cowpeas was similar for *Bechuana white* and *Agripienk* which was about an hour (Table 3.1). *Mae-a-tsilwane* had a relatively longer cooking time of about 2 h. Storage at 4^{o}C for 40 days did not significantly ($P > 0.05$) affect the cooking time of the cowpeas except for *Mae-a-tsilwane*. However, the cooking times of all samples increased significantly ($P < 0.05$) during storage at HTHH conditions (40 ^{o}C and 80% RH). *Agripienk* had a higher increase (382%) compared to *Bechuana white* (51% increase). The fresh cowpeas had similar initial pH (Table 3.1). The pH of the cowpeas was slightly higher or equal to day 0 after storage at 4^{o}C. A significant ($P < 0.05$) decrease in pH was observed in all cowpea samples after storage. *Agripienk* and *Mae-a-tsilwane* had about 0.4 decreases in pH whereas Bechauna white had a 0.2 decrease under HTHH storage conditions. Initial moisture content of the cowpeas ranged from 8.3% to 9.7% (Table 3.1). During storage at 4 ^{o}C, only *Agripienk* showed a significant ($P < 0.05$) increase in moisture content. During HTHH storage, the moisture content increased by 2.4%, 1.1% and 2.2% for the *Bechuana white*, *Agripienk* and *Mae-a-tsilwane*, respectively. Water activity (a_w) decreased slightly during storage at 4 ^{o}C for all the cowpea types (Table 3.1). During HTHH storage there was a significant ($P < 0.05$) increase in water activity in all samples (Table 3.1).

Phytase activity was not significantly ($P > 0.05$) affected during storage at 4^{o}C in all samples (Table 3.2). After storage in HTHH conditions phytase activity decreased significantly ($P < 0.05$). Free inorganic phosphate content was highest in Agripienk and least in Mae-a-tsilwane on day 0 (Table 3.2). This amount did not change significantly ($P > 0.05$) during storage at 4 ^{o}C in all samples. However, during HTHH storage there was a significant ($P < 0.05$) increase in inorganic phosphate. There was an 86, 137 and 124% increase in inorganic phosphate content in Bechuana white, Agripienk and Mae-a-tsilwane respectively, after HTHH storage. Phytate content ranged from 4.7 g/kg to 10.3 g/kg. As noted in the other parameters tested, storage at 4

^{o}C did not affect phytate content significantly ($P > 0.05$). Storage under HTHH conditions reduced the phytate content significantly ($P < 0.05$). There was a 13, 22 and 28 % decrease in phytate content in *Bechuana white*, *Agripienk* and *Mae –a-tsilwane* respectively, after HTHH storage.

Table 3.1 Effect of storage conditions on the cooking time, pH, a_W and moisture content of cowpea types under different storage conditions*

Cowpea type	Treatment (storage)	Cooking time (min)	pH	Water activity (a_w)	Moisture content (%)
Bechuana white	Day 0	$55^{a,b} \pm 6$	$6.58^{c} \pm 0.04$	$0.40^{b} \pm 0.01$	$8.3^{a} \pm 0.1$
	Day 40 at 4 °C	$62^{b} \pm 4$	$6.66^{d,e} \pm 0.01$	$0.37^{a} \pm 0.01$	$8.2^{a} \pm 0.2$
	Day 40 at HTHH#	$83^{c} \pm 3$	$6.38^{b} \pm 0.01$	$0.53^{e} \pm 0.02$	$10.7^{d} \pm 0.1$
Agripienk	Day 0	$56^{a,b} \pm 3$	$6.59^{c} \pm 0.04$	$0.46^{d} \pm 0.02$	$8.3^{a} \pm 0.2$
	Day 40 at 4 °C	$47^{a} \pm 6$	$6.67^{e} \pm 0.01$	$0.43^{c} \pm 0.01$	$9.2^{b} \pm 0.3$
	Day 40 at HTHH	$> 270^{f}$	$6.20^{a} \pm 0.01$	$0.54^{e} \pm 0.01$	$9.4^{b,c} \pm 0.1$
Mae-a-tsilwane	Day 0	$117^{d} \pm 7$	$6.59^{c} \pm 0.02$	$0.52^{e} \pm 0.02$	$9.7^{b,c} \pm 0.1$
	Day 40 at 4 °C	$131^{e} \pm 12$	$6.62^{c,d} \pm 0.01$	$0.48^{d} \pm 0.01$	$9.9^{c} \pm 0.6$
	Day 40 at HTHH	$> 270^{f}$	$6.21^{a} \pm 0.02$	$0.53^{e} \pm 0.01$	$11.9^{e} \pm 0.1$

*Different superscript letters within a column indicate statistically significant differences (n = 2) ($P < 0.05$)

HTHH refers to High Temperature (40 °C) High Humidity (80%)

Table 3.2 Effect of storage conditions on the released P*i*, phytase activity, phytate and water soluble pectin of cowpea types stored under different storage conditions[*]

Cowpea type	Treatment (storage)	Released P*i* (g/kg)	Phytase (FTU[†]/kg)	Phytate (g/kg)	Water soluble pectin (g/kg)
Bechuana white	Day 0	$1.31^{b} \pm 0.01$	$379^{d,e} \pm 6$	$7.22^{c,d} \pm 0.29$	$2.00^{e} \pm 0.05$
	Day 40 at 4 °C	$1.32^{b} \pm 0.01$	$412^{e,f} \pm 17$	$7.51^{d} \pm 0.48$	$1.97^{e} \pm 0.03$
	Day 40 at HTHH[‡]	$2.43^{e} \pm 0.01$	$242^{a} \pm 22$	$6.25^{c} \pm 0.14$	$1.82^{d} \pm 0.01$
Agripienk	Day 0	$1.63^{d} \pm 0.01$	$336^{c,d} \pm 16$	$10.34^{e} \pm 0.64$	$1.52^{c} \pm 0.01$
	Day 40 at 4 °C	$1.43^{c} \pm 0.01$	$324^{b,c} \pm 25$	$11.19^{e} \pm 0.98$	$1.54^{c} \pm 0.02$
	Day 40 at HTHH	$3.89^{g} \pm 0.01$	$284^{b} \pm 2$	$7.98^{d} \pm 0.30$	$1.25^{b} \pm 0.00$
Mae-a-tsilwane	Day 0	$1.16^{a} \pm 0.01$	$440^{f} \pm 51$	$4.66^{b} \pm 0.19$	$1.31^{b} \pm 0.09$
	Day 40 at 4 °C	$1.29^{b} \pm 0.01$	$420^{e,f} \pm 10$	$4.53^{b} \pm 0.12$	$1.30^{b} \pm 0.01$
	Day 40 at HTHH	$2.60^{f} \pm 0.01$	$327^{b,c} \pm 6$	$3.33^{a} \pm 0.06$	$1.09^{a} \pm 0.01$

[*]Different superscript letters within a column indicate statistically significant differences (n=2) ($P < 0.05$)

[†] FTU(one phytase unit): amount of enzyme that liberates 1 µM of P*i*/min from 0.0051 mol/l sodium phytate at 37 °C and pH 5.5

[‡] HTHH refers to High Temperature (40 °C) High Humidity (80%)

Initial water soluble pectin content was highest in *Bechuana white* and lowest in *Mae-a-tsilwane* (Table 3.2). There was no significant ($P > 0.05$) change in water soluble pectin content of the cowpea samples stored at 4 °C. HTHH storage significantly reduced water soluble pectin content in all samples. The lowest decrease was in *Bechuana white* whereas *Agripienk* and *Mae-a-tsilwane* had decreases of about 17 %.

Peroxidase activity ranged from 0.39 to 0.86 Δ/mL/min (Table 3.3). In all cowpea type the peroxidase (POD) activity did not change significantly ($P > 0.05$) during storage except for *Agripienk* which decreased significantly ($P < 0.05$) during HTHH storage. There was no change in lignin content in all cowpea types during storage at either 4 ^{o}C or HTHH.

Table 3.3 Effect of storage conditions on peroxidase activity and lignin of cowpeas types stored under different storage conditions[*]

Cowpea type	Treatment (storage)	Peroxidase (Δ/mL/min)	Lignin (g/kg)
Bechuana white	Day 0	$0.61^{c} \pm 0.01$	$27.0^{a} \pm 0.1$
	Day 40 at 4 ^{o}C	$0.59^{b,c} \pm 0.02$	$27.9^{a} \pm 0.2$
	Day 40 at HTHH[#]	$0.57^{b,c} \pm 0.08$	$29.2^{a} \pm 0.1$
Agripienk	Day 0	$0.76^{d} \pm 0.01$	$30.2^{a} \pm 0.1$
	Day 40 at 4 ^{o}C	$0.86^{d} \pm 0.03$	$31.3^{a} \pm 0.5$
	Day 40 at HTHH	$0.50^{a,b,c} \pm 0.11$	$33.6^{a} \pm 0.1$
Mae-a-tsilwane	Day 0	$0.48^{a,b} \pm 0.08$	$33.9^{a} \pm 0.1$
	Day 40 at 4 ^{o}C	$0.39^{a} \pm 0.01$	$33.1^{a} \pm 0.1$
	Day 40 at HTHH	$0.50^{a,bc} \pm 0.03$	$33.3^{a} \pm 0.1$

[*]Different superscript letters within a column indicate statistically significant differences (n=2) ($P < 0.05$)

[#] HTHH refers to High Temperature (40 ^{o} C) High Humidity (80%)

Table 3.4 shows the p-values related to the physico-chemical characteristics of the cowpeas. Cowpea type had a significant ($P < 0.05$) effect on all physico-chemical characteristics. Storage effect was significant ($P < 0.05$) on all characteristics except lignin. The cowpea type × storage interaction was significant ($P < 0.05$) in all characteristics except phytate, water soluble pectin and lignin.

PCA was used to better understand the potential relationships between the independent variables (cowpeas stored under different conditions) and the dependent variables (physico-chemical characteristics) that differed significantly. The first two principal components described 83% of the variation in physico-chemical characteristics of the cowpeas.

Table 3.4 P-values to test for the effects of cowpea type and storage

Physico-chemical attribute	Cowpea type p value	Storage p value	Cowpea type × Storage p value
Cooking time	0.001	0.001	0.001
Released phosphate	0.001	0.001	0.001
Phytase	0.001	0.001	0.026
Phytate	0.001	0.001	0.060
water soluble pectin	0.001	0.001	0.206
Peroxidase	0.001	0.024	0.002
Lignin	0.001	0.271	0.620
Moisture	0.001	0.001	0.002
pH	0.001	0.001	0.001
a_W	0.001	0.001	0.001

Figure 3.1a shows the projection of the loadings of the physico-chemical characteristics and Figure 3.2 b illustrates the projection of scores of the cowpeas. PC 1 explained 66 % of the variance in the data. Cowpeas on the right of the plot were associated with long cooking times, higher moisture content, higher water activity and higher released phosphate content. These characteristics were associated with cowpeas that had undergone HTHH storage. The cowpeas on the left of the map were day 0 and those that had been stored at 4 ^{o}C, these were characterised with higher phytate content, higher water soluble pectin content and high pH. PC 2 added 16% to the explanation of variation and separated cowpeas with high released phosphate such as *Agripienk 40d HTHH*. In addition, PC 2 separated the cowpea types with higher phytate content (*Agripienk* samples*)* and those of a lower phytate content (*Mae-a tsilwane* samples). PC 3 (Figure 3.2) explained an additional 9 % (making a total of 92%). PC 3 explained the higher water soluble pectin content in *Bechuana* samples. It was interesting to note that in all cowpea types, day 0 samples and those stored at 4^{o}C were clustered together indicating similar physico-chemical characteristics.

Cooking time was significantly ($P < 0.05$) positively correlated with released phosphate ($r = 0.77$), water activity ($r = 0.69$) and moisture content ($r = 0.62$) (Table 3.5). pH ($r = 0.87$), water soluble pectin ($r = 0.75$) and phytate ($r = 0.51$) were significantly ($P < 0.05$) negatively correlated with cooking time (Table 3.5).

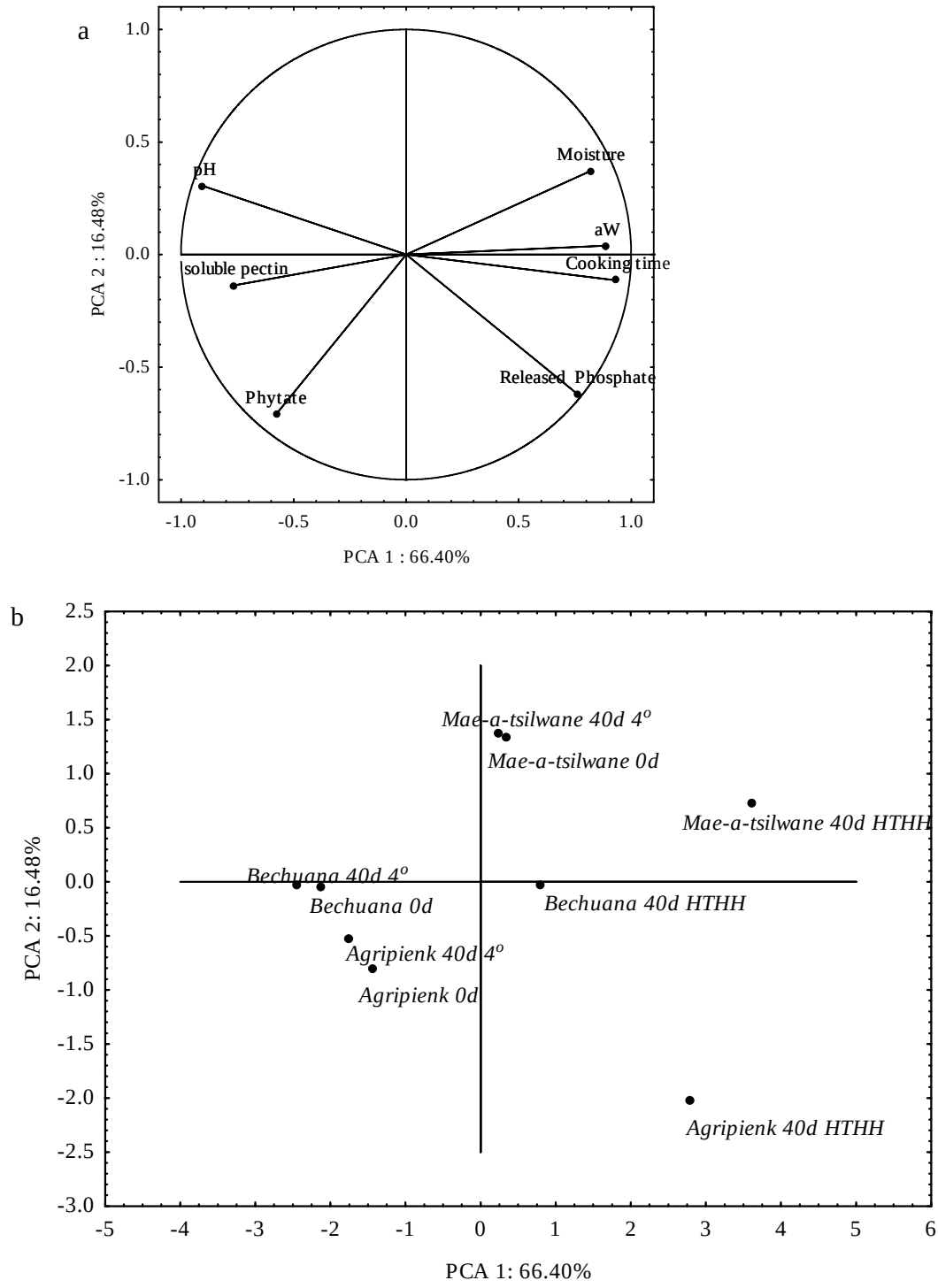


Figure 3.1 Principal component analysis (PCA) of cowpea types during storage (a) Plot of first two principal component loading vectors of physico-chemical characteristics, (b) Plot of the first two principal component scores of the cowpea types

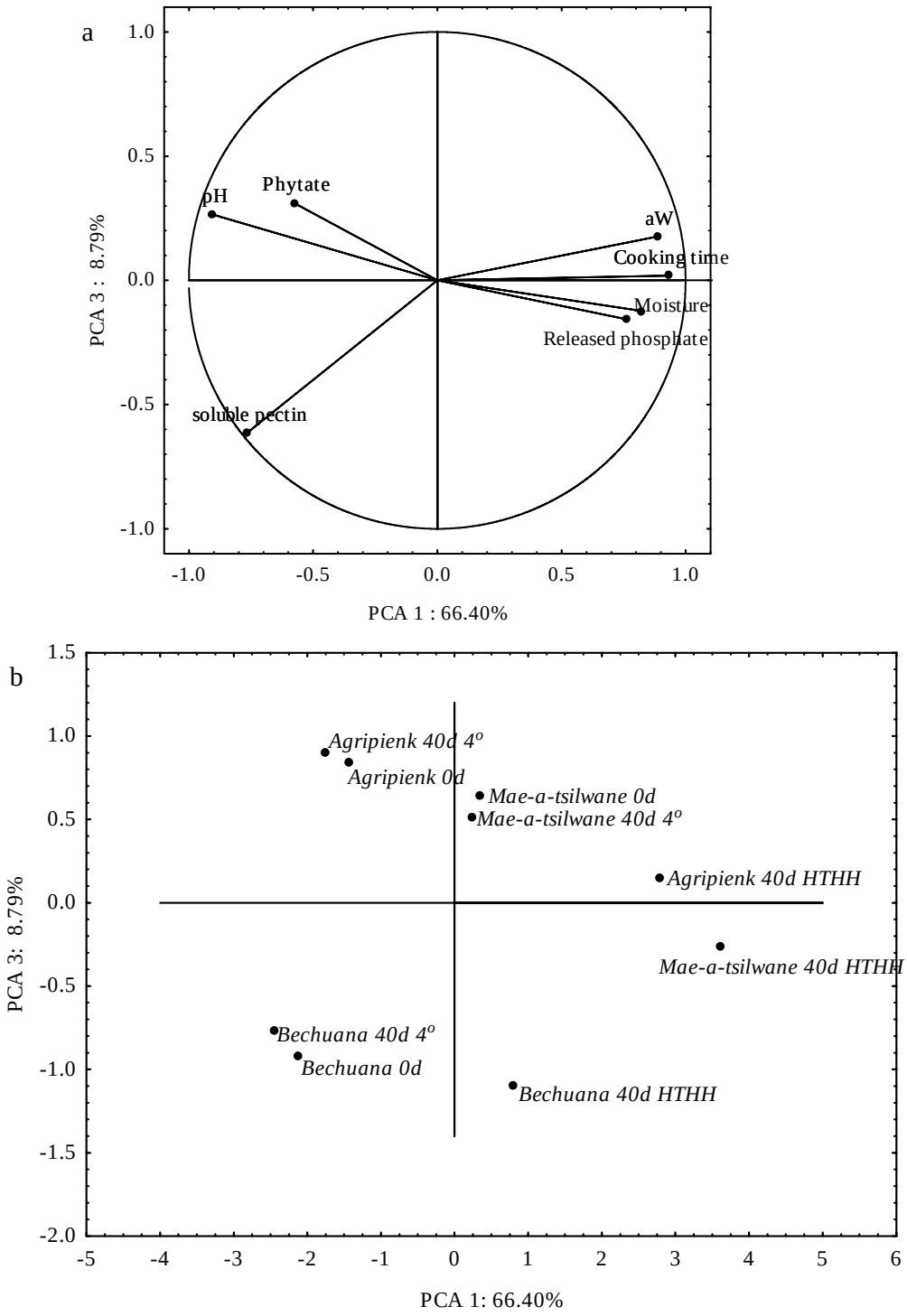


Figure 3.2 Principal component analysis (PCA) of cowpea types during storage (a) Plot of first and third principal component loading vectors of physico-chemical characteristics, (b) Plot of the first and third principal component scores of the cowpea types

Table 3.5 Correlations coefficients between physico-chemical characteristics of cowpeas at level $P < 0.05$[a]

	Cooking time	Released P*i*	Phytase	Phytate	Water soluble pectin	Moisture	pH	aW
Cooking time	1.00	0.77	-0.24	-0.51	-0.75	0.62	-0.87	0.69
Released P*i*		1.00	-0.71	-0.06	-0.40	0.41	-0.91	0.62
Phytase			1.00	-0.27	0.03	-0.34	0.60	-0.38
Phytate				1.00	0.35	-0.67	0.37	-0.45
Water soluble pectin					1.00	-0.58	0.49	-0.72
Moisture						1.00	-0.66	0.71
pH							1.00	-0.73
a_W								1.00

[a] Significant correlations are indicated in bold

3.1.5 Discussion

The initial cooking times of the cowpeas (55 to 117 min) were simmilar to the range reported for cowpeas (35 to 120 min) (Olapade, Okafor, Ozumba & Olatunji, 2002). The differences in cooking time of day 0 cowpeas could have been due to genotypic differences in variety and growth conditions (Akinyele *et al.*, 1986; Demooy & Demooy, 1990) which influence physico-chemical characteristics of the seeds. These physico-chemical characteristics such as phytate content and water soluble pectin content are known to influence cooking time (Coelho *et al.*, 2007). Studies report that legumes stored under refrigeration conditions do not develop the HTC defect (Cunha *et al.*, 1993; Berrios *et al.*, 1999; Coelho *et al.*, 2007). Beans stored under refrigerated conditions of about 0-5 ^{o}C are shown to have a simillar cooking time with fresh beans even when stored for a long time (Berrios *et al.*, 1999). Since enzymes are implicated in HTC development, refrigeration temperatures would slow down enzyme activity, therefore controlling HTC defect development. The increases in cooking time due to HTHH storage in all types indicate that these conditions promoted the HTC defect development. Under HTHH conditions, it was interesting to note that *Bechuana white* cooking time increased by 28 min whereas *Agripienk* and *Mae-a-tsilwane* had increases of over 214 min and over 153 min respectively. These results suggest that the cowpea types had different susceptibilities to the development of HTC defect. This is in agreement with several legume studies which report differences in proneness to HTC defect development among different types of legumes of the same species (Reyes-Moreno & Paredes-Lopez, 1993; Bernal-Lugo *et al.*, 1997; Coelho *et al.*, 2007).

The decrease in pH during HTHH storage was in agreement with reported decreases in pH due to HTHH storage (Liu *et al.*, 1992a). Flour slurry pH as used in this study gives an indication of cowpea tissue pH (Liu *et al.*, 1992a). The decreased pH suggests tissue acidification reported to be as a result of lipid hydrolysis (Liu *et al.*, 1992a). Hydrolysis of lipids into fatty acids and oxidation of these acids into organic acids are observed to contribute to decrease in pH (Liu *et al.*, 1992a). The initial moisture content of the cowpeas were within the recommended ≤ 10 % for maintenance of textural quality during storage (Berrios *et al.*, 1999). Moisture contents of ≥ 13 % are reported to promote the HTC defect during storage under adverse conditions (Antunes & Sgarbieri, 1979). The increase in moisture content and water activity of the cowpeas during

HTHH conditions is due to seed absorbing moisture from the surrounding (Nahar, Ali, Amin & Hasanuzzaman, 2009). Seed moisture content is directly related to the relative humidity of the surrounding air (Nahar *et al.*, 2009). Therefore water vapour will move from the surrounding air to the seed until hygroscopic equilibrium is established (Nahar *et al.*, 2009).

The increase in free inorganic phosphate (P*i*) during HTHH storage conditions indicated hydrolysis of phytate by activated phytase activity since phytase hydrolyzes phytate releasing inorganic phosphate (Mafuleka *et al.*, 1993; Galiotou-Panayotou *et al.*, 2008). The HTHH conditions enhanced phytate degradation by providing optimal conditions for phytase activity. The lack of change in phytase activity in all samples during storage at 4^{o}C indicated that enzyme activity was low. Enzyme activity is temperature dependent and at refrigeration temperatures such as 4^{0}C, low activity was expected. Although the increased content in inorganic phosphate at day 40 during HTHH storage indicated increased phytase activity, determined phyatse activity was lower than the control. The decreased phytase activity seemed to be related to the methodology used in determination. Phytase activity was measured by quantifying the amount of inorganic phosphate (Pi) ions liberated from added phytate during incubation. This was measured against the background or free inorganic phosphate present in the seed before the incubation in the assay. The background inorganic phosphate was quite high and could have resulted in the low values observed.

Phytate reduction is observed during HTHH storage and results from the action of phytase on phytate (Reyes-Moreno *et al.*, 2000a; Affrifah & Chinnan, 2005; Galiotou-Panayotou *et al.*, 2008). This corroborates the observation that inorganic phosphate increased due to hydrolysis of phytate by phytase. The extent of HTC defect has been negatively correlated with phytate content (Reyes-Moreno, Romero-Urias, Milan-Carrillo & Gomez-Garza, 2000c; Galiotou-Panayotou *et al.*, 2008)}.

The decrease in water soluble pectin during HTHH storage was in agreement with researchers who reported decreases in water solubility of pectin after storage in HTHH conditions (Reyes-Moreno *et al.*, 2000a; Galiotou-Panayotou *et al.*, 2008; Nyakuni, Kikafunda, Muyonga, Kyamuhangire, Nakimbugwe & Ugen, 2008). The decrease in water soluble pectin indicated

formation of pectates (Coelho *et al.*, 2007; Galiotou-Panayotou *et al.*, 2008). The hydrolysis of phytate by phytase seemed to release divalent cations such as calcium that are known to form ionic cross-bridges with pectin to form insoluble calcium pectates in the middle lamella (Reyes-Moreno *et al.*, 2000a; Galiotou-Panayotou *et al.*, 2008).

Although peroxidase activity was expected to increase during HTHH storage it did not change significantly for *Bechuana white* and *Mae-a-tsilwane*. There was a significant ($P < 0.05$) decrease in *Agripienk*'s peroxidise activity after HTHH storage. It was expected that storage of cowpea seeds at HTHH could increase peroxidase activity and thus lignin synthesis (Hincks & Stanley, 1987). Researchers seem to differ on the role of peroxidase in HTC defect development. Paredes-Lopez *et al.* (1989b) reported 30-50 % lower peroxidase activity of beans stored at HTHH (30 ^{o}C and 85% RH) when compared to control beans stored at low temperature and low relative humidity (15 ^{o}C and 35 % RH). Lack of increase in POD activity during storage at HTHH was also reported by Hohlberg & Stanley (1987). The lignification theory postulates polymerisation of hydrocinammic acids into lignin in the cell wall due to increased POD activity. It was noted that there is no experimental work in literature correlating increased POD activity to increased lignin content of legumes during HTC defect development. Storage conditions did not have a significant ($P > 0.05$) effect on lignin content. Researchers have shown conflicting results on the change in lignin content during HTHH storage of legumes. Jones & Boulter (1983) and Nasar-Abbas *et al.* (2008) reported increased lignin content in black beans and faba beans stored at HTHH conditions respectively. In contrast, Srisuma *et al.* (1989) and Mafuleka *et al.* (1993) observed no significant change in the lignin content of beans stored under HTHH conditions. Studies on the effect of lignin on HTC defect development are few and therefore more research is necessary to understand the lignification theory.

The physico-chemical characteristics of the day 0 cowpeas (control) and cowpeas stored at 4 ^{o}C were similar as demonstrated through their close proximity in the PCA indicating that refrigeration temperatures prevent the development of HTC (Figure 3.2). This is in agreement with researchers who demonstrated that low temperature storage prevent the development of the HTC defect. The PCA and correlation studies showed that higher cooking times were associated

with increased inorganic phosphate (increased phytase activity), decreased phytate content and decreased pectin solubility. These results provide support for the phytase-phytate-pectin hypothesis as the most probable explanation to the development of HTC defect (Coelho *et al.*, 2007; Galiotou-Panayotou *et al.*, 2008). The results suggest that exposure of the cowpeas to high temperature and high humidity caused activation of phytase resulting in hydrolysis of phytate. Hydrolysis of phytate caused reduction in its chelation potential thereby releasing cations that were potentially bound to pectin in the middle lamella forming pectates. The pectates in the middle lamella do not solubilise during cooking consequently restricting cell separation that is essential for softening of the cowpeas.

The differences in susceptibility to HTC defect among the cowpea types could be due to differences in the initial phytate content and the degree of phytate hydrolysis during HTHH storage. *Agripienk* was observed to be more susceptible to HTC compared to the other 2 cowpea types. *Agripienk* had the highest phytate content and the highest decrease after HTHH storage conditions. Similar observations were reported in beans (Coelho *et al.*, 2007), where longer cooking times after HTHH storage were dependent on the intial phytate content and the degree of phyate hydrolysis

3.1.6 Conclusions

HTC defect was successfully induced under the HTHH conditions used in this study as indicated by increased cooking time in all types. The susceptibility to the defect differed among the cowpea types. Storage under refrigeration (4^{o}C) conditions did not cause an increase in cooking time. The observed increase in cooking time during HTHH storage was associated with increased liberation of inorganic phosphate, decrease in phytate content and decrease in water soluble pectin. This was in support of the “phytase- phytate-pectin hypothesis”, whereas the role of lignin in HTC defect development was not established in this study.

3.2 Micronisation and hot-air roasting of cowpeas as pre-treatments to control the development of hard-to-cook phenomenon[1]

Abstract

Cowpeas, stored under conditions of high temperature and high relative humidity (HTHH), developed the hard-to-cook (HTC) defect. Cowpeas with HTC defect require long cooking times, limiting their utilisation. Heat pre-treatments aim at inactivating responsible enzymes leading to HTC development. Heat pre-treatments, i.e. micronisation and hot-air roasting were evaluated to assess their effectiveness in controlling the HTC defect development in cowpeas after storage under HTHH conditions. Micronisation and hot-air roasting as pre-treatments reduced the cooking time of cowpeas after storage under HTHH conditions compared with to the control. The differences in the effectiveness of HTC defect control between micronisation and roasting were dependent on the degree of phytase inactivation on day 0. Phytase activity was reduced by 45% and 70% by roasting and micronisation pre-treatments, respectively. Reduced phytase activity was associated with higher phytate and soluble pectin contents in micronised cowpeas than in roasted cowpeas after HTHH storage. This observation is in agreement with the phytase – phytate-pectin theory. Micronisation was more effective than hot-air roasting in controlling the development of the HTC defect. This was due to a higher degree of phytase inactivation in micronisation when compared to roasting

Key words: Heat pre-treatments, HTC-defect, phytase, phytic acid, soluble pectin

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3.2.1 Introduction

Cowpeas (*Vigna unguiculata* L. Walp) are an important source of nutrients in developing countries and are consumed extensively in Africa. Cowpeas contain about 25% proteins and 65% carbohydrates (Preet & Punia, 2000). They complement cereal-based diets by increasing the protein quality of diets especially among the low income groups in developing countries (Iqbal *et al.*, 2006). Cereal proteins are deficient in the essential amino acid, lysine. Cowpeas are comparatively rich in lysine (7 g lysine/100 g protein) (USDA, 2016) and therefore, help to alleviate protein energy malnutrition when used to fortify cereal based diets.

However, a major limitation in cowpea utilisation is the long cooking times especially during storage under tropical conditions. The high temperature and high relative humidity (HTHH) conditions of the tropical climate lead to development of the hard-to-cook (HTC) defect (Coelho *et al.*, 2007). HTC cowpeas are characterized by longer cooking times compared to normal cowpeas or even inability to soften sufficiently during cooking for consumption (Aguilera *et al.*, 2000). The longer cooking time means a high energy demand which is a challenge to developing countries due to scarcity of resources (Stanley & Aguilera, 1985).

The mechanisms leading to HTC defect are complicated and not clearly understood. Most of the proposed mechanisms leading to HTC development implicate increased enzymatic activity during storage at high temperature and high relative humidity (Hincks & Stanley, 1987). The two main hypotheses proposed to explain the HTC defect development are the ‘phytase–phytate–pectin’ (Galiotou-Panayotou *et al.*, 2008) and lignification mechanisms (Hincks & Stanley, 1987).

The ‘phytase–phytate–pectin’ hypothesis proposes degradation of phytate by increased phytase activity and subsequent interaction of the released divalent cations with pectin (Galiotou-Panayotou *et al.*, 2008). This interaction leads to crosslinking of pectin with cation bridges and insolubilisation of the pectin to form pectates, e.g. calcium pectate. During cooking, these

pectates do not dissolve easily, leading to failure of cell separation during cooking. Cell separation is necessary for softening of legumes, therefore restricted cell separation leads to long cooking time (Shomer, Paster, Lindner & Vasiliver, 1990). The lignification hypothesis proposes deposition of lignin in the cell wall from the polymerization of lignin precursors (monolignols) in the presence of peroxidase (Hohlberg & Stanley, 1987). The phytase–phytate–pectin hypothesis is the more widely accepted theory for HTC development (Hentges *et al.*, 1991; Bernal-Lugo *et al.*, 1997)

Storage of cowpeas under low temperature conditions was an effective method of preventing HTC defect (Berrios *et al.*, 1999; Coelho *et al.*, 2007). However, this might not be feasible in the scarcely-resourced regions in the tropics. Inactivation of the responsible enzymes such as phytase and peroxidase by the use of heat treatments are some of the pre-treatment strategies used to prevent the development of the HTC defect. These include sand roasting (Rivera *et al.*, 1989), microwaving (Plhak *et al.*, 1987; Cunha *et al.*, 1993), retorting (Molina *et al.*, 1976) and steaming (Molina *et al.*, 1976; Affrifah *et al.*, 2006). Affrifah *et al.* (2006) reported 64 % reduction in phytase activity of cowpeas (20% moisture content) steamed for 6 min. Steaming (121 °C, 4 min) appears to be the most effective of the heat pre-treatments reported in the literature to preventing HTC defect (Affrifah *et al.*, 2006). In all of these heat pre-treatments, total prevention of HTC development during storage was not achieved probably as a result of residual activities of the enzymes.

Infrared (IR) heating or micronisation could be a more effective heat pre-treatment strategy (Krishnamurthy *et al.*, 2008). IR heating involves use of radiant electromagnetic energy in the wavelength range of 1.8 to 3.4 µm. Hot-air roasting is a conventional heating process and heat is transferred through convection. The use of micronisation as a heat pre-treatment strategy in the control of HTC defect during storage of legumes at conditions of high temperature and high relative humidity is not reported to date.

The objective of this study was to determine the effects of micronisation and hot-air roasting on the development of HTC defect during storage under high temperature and high relative humidity.

3.2.2 Materials and methods

3.2.1.1 Raw materials

In a preliminary study, 11 cowpea cultivars from Botswana (n=4) and South African (n= 7) were screened in terms of their susceptibility to hard-to-cook defect as determined by their cooking time after storage at 40 °C and 80% RH for 40 days. *Agripienk* cultivar (Agricol, Potchefstroom, South Africa) was chosen for the study as it had a relatively quick initial cooking time (56 min) which increased 5 fold (>270 min) after storage under accelerated storage conditions.

3.2.1.2 Pre-conditioning

Cowpeas were pre-conditioned to approximately 25 % moisture content by adding deionised water. The amount of water required was calculated according to the method of Arntfield *et al.* (1997). The required amount of water was added to Zip-lock® bags containing the cowpeas and mixed by shaking and rolling the bags to ensure uniform water distribution. The bags were stored at 4 °C for 16 h for equilibration of water within the cowpea (Arntfield *et al.*, 1997)

3.2.1.3 Micronisation conditions

A small scale experimental microniser was used. The microniser components were a standard bench top 3 lamp IR module (UV+IR Engineering, Johannesburg, South Africa) mounted on a variable height stand. The heat source consisted of 3quartz-tungsten infrared lamps with a 6 kW power operating at 67% output. Pre-conditioned cowpeas (100g) were evenly spread out (single layer) on an aluminum tray and placed under the microniser. The distance between the lamps and the cowpeas was set at 25 cm. The microniser was pre-heated for 20 min before micronising the cowpeas for 5 min to a final surface temperature of 150 °C (Mwangwela *et al.*, 2006). The

micronised cowpeas were immediately spread on a tabletop and cooled at room temperature (23 ± 2 °C).

3.2.1.4 Roasting (hot-air heating) conditions

Pre-conditioned cowpeas were heated (150 °C/5 min) with a forced convection continuous tumble roaster (Roastech, Bloemfontein, South Africa). The roasted cowpeas were immediately spread on a tabletop and cooled to room temperature (23 ± 2 °C) for about 20 min.

3.2.1.5 Drying conditions

After the heat pre-treatments the cowpeas had a moisture content of 17.2 ± 0.3% and 18.8 ± 0.8% after micronisation and roasting respectively. It was desired that the treated cowpea attain a moisture content similar to that of the control (10 %). This is because higher moisture contents favour HTC development (Affrifah *et al.*, 2006) and mould growth during storage (Affrifah *et al.*, 2006). This was achieved by drying the pre-treated cowpeas at 45 °C for 5 hours in a convection air oven (Type FSOE, Labcon, Johannesburg, South Africa) at 20 ± 2% RH. Moisture contents of 10 ± 2% and 10 ± 1% were achieved for micronized and roasted cowpeas, respectively. After drying, the cowpea were spread in a tray (single layer) and sprayed with a solution of potassium sorbate in absolute methanol (1:8 w/v per kg) to control mould growth (Garruti & Bourne, 1985). They were allowed to dry overnight in a fume hood.

3.2.1.6 Accelerated storage conditions

HTC defect was induced by incubating cowpeas at 40 °C and 80% RH for 40 days according to Affrifah *et al.* (2006). Saturated potassium chloride (KCl) solution was used to give a constant RH of 80 % according to ASTM E 104-02. The temperature and % RH were monitored using humidity - temperature logger (ThermaData™, ETI ltd, West Sussex, UK). The set conditions of 40 °C and 80% RH were maintained during storage.

3.2.1.7 Flour preparation for assays

Heat pre-treated and control cowpeas were finely ground to pass through a 0.8 mm screen using a laboratory hammer mill 3100 (Falling number-3100, Huddinge, Sweden). The flours were packaged in zip lock polyethylene bags and stored at 4 °C. Analyses were conducted for all treatments (control, micronised and roasted) on day 0 and after storage for 40 days. Whole cowpea were used in the determination of cooking time.

3.2.1.8 Moisture content

Whole cowpeas and flour moisture contents were determined by AOCS Official Method Ac 2-41(AOCS, 1994) and AACC Method 44-15A (AACC, 2000) respectively.

3.2.1.9 Cooking time

Cooking time was determined by a Mattson bean cooker as described by Mwangwela *et al.* (2006). For each treatment, 25 cowpeas were positioned in the perforations of the cooker, placed in an aluminium pot with 1.5 L of deionised water and boiled. The cooking time of the cowpeas was recorded as the time when 80% of the 50 g pins had penetrated the cowpeas and plunged through the hole at the base.

3.2.1.10 Phytate

Phytate content was determined using a phytate (phytate)/total phosphorus analysis kit (K-PHYT) of Megazyme (2007). Phytate was hydrolysed by phytase and alkaline phosphatase releasing inorganic phosphate (P*i*). P*i* was then measured spectrophotometrically at 655 nm.

3.2.1.11 Phytase activity and free inorganic phosphorus (Pi)

Phytase activity was determined using the direct incubation method as described by Shen *et al.* (2005). The reaction mixture containing 1 g of flour and 100 mL of 1.5 mM Sodium Phytate was incubated at 45°C for 30 min. After 30 min the liberated phosphate was measured by the ammonium molybdate method (Eeckhout & De Paepe, 1994). Phytase activity was measured as phytase units (FTU). FTU was the amount of phytase that catalyses the release of micromoles of inorganic phosphate per minute from 1 kg of dry matter (DM) cowpea. Free inorganic phosphate (P*i*) before incubation was expressed as sample blank before incubation.

3.2.1.12 Soluble pectin extraction and determination

Soluble pectin was determined in raw and cooked (boiled for 120 min) cowpeas. Samples were prepared by mixing 5 g of ground sample with 40 mL of 95% ethanol in a 50 mL centrifuge tube to remove soluble sugars. The mixture was stirred for 10 min and centrifuged at 17300 × g for 10 min. The extraction with 40 mL of 95% ethanol was repeated twice using the residual pellet. Final extraction was done with 40 mL of absolute ethanol. The pellet (alcohol-insoluble solids) was vacuum dried at room temperature and stored in a desiccator. The alcohol insoluble solids (AIS) were extracted with cold water (cold water soluble pectin) and with hot water (hot water soluble pectin).

One gram of AIS was extracted 3 times with 10 mL of distilled water each for 10 min. The extracts were combined and considered as the cold water soluble pectin (CWSP). Determination of hot water soluble pectin (HWSP) was conducted according to the method described by Bernal-Lugo *et al.* (1997) using 1 g of the AIS. Pectin content of each fraction was expressed as glacturonic acid as determined by the metahydroxydiphenyl method of Blumenkrantz & Asboe-Hansen (1973) using galactouronic acid as a standard.

3.2.1.13 Determination of acid detergent lignin (ADL)

Acid detergent lignin (ADL) content was determined by the method described by Goering & Van Soest (1970) using a Foss Analytical FibreCap™ 2021/2023 system (FOSS Analytical ABB, Höganäs, Sweden). ADL was determined gravimetrically by first obtaining the acid detergent fibre, then extracting it with 72% H_2SO_4 (w/w) and ashing to determine percent ADL.

3.2.1.14 Statistical analyses

The experiment was repeated three times. The effects between heat pre-treatments before and after storage at HTHH were determined using analysis of variance (ANOVA) and Fisher's least significant difference test (LSD, $P < 0.05$). Correlation coefficients (r) were determined to establish relationships between physico-chemical characteristics. Principal component analysis (PCA) of the treatments and physico-chemical characteristics was conducted using a correlation matrix.

3.2.2 Results

The initial cooking time (day 0) of cowpeas was not significantly ($P > 0.05$) affected by heat pre-treatments (Table 3.6). Cooking time of the control and heat pre-treated cowpeas was less than 60 min. After 40 days of storage at HTHH, there was a significant increase ($P < 0.05$) in cooking time of all cowpea samples.

However the extent of the increase in cooking time of heat pre-treated cowpeas was considerably smaller than that of the control. The increase in cooking time of the cowpeas after 40 days of storage relative to day 0 was > 430%, 243% and 92% for the control, roasted and micronised cowpeas, respectively. Micronisation was more effective than hot-air roasting in controlling the HTC defect development as it minimised the development of the defect more.

Heat pre-treatments reduced the initial phytase activity (day 0) by 70% and 45% for micronisation and roasting, respectively (Table 3.7). After 40 days of storage, phytase activity was the lowest in the micronised cowpeas and highest in the control cowpeas.

Table 3.6 Changes in the cooking time of heat pre-treated cowpeas after storage at 40 °C and 80 % RH[a]

Heat pre-treatment	Storage (days)	Cooking time (min)
Control	**0**	51[a] ± 9
	40	> 270[d]
Micronised	**0**	44[a] ±3
	40	98[b] ± 9
Roasted	**0**	50[a] ± 7
	40	175[c] ± 11

[a]Means followed by the different letters in column are significantly different (n=2) (*P<0.05)*

Table 3.7 Effect of heat pre-treatments of pre-conditioned cowpea seeds followed by storage at 40 °C and 80 % RH on phytase, released Pi and phytate [a]

Heat pre-treatment	Storage (days)	Phytase activity (FTU/kg)	Released P*i* (g/kg)	Phytate (g/kg)
Control	**0**	375[e]±14	0.93[a]±0.10	10.08[e]±0.10
	40	267[d]±24	4.09[d]±0.34	6.86[a]±0.11
Micronised	**0**	102[ab]±9	0.90[a]±0.04	9.62[d]±0.10
	40	74[a]±10	2.47[b]±0.05	8.81[c]±0.22
Roasted	**0**	204[c]±17	0.89[a]±0.06	9.66[d]±0.23
	40	130[b]±23	2.80[c]±0.06	7.82[b]±0.20

[a]Means followed by the different letters in column are significantly different (n=2) (*P<0.05*)

Heat pre-treatments reduced the initial phytase activity (day 0) by 70% and 45% for micronisation and roasting, respectively (Table 3.7). After 40 days of storage, phytase activity

was the lowest in the micronised cowpeas and highest in the control cowpeas. Phytase activity of micronized and roasted cowpeas after 40 days was 72% and 51% lower in comparison with the control. These percentages of residual activities were similar to those at day 0 indicating that the partial inactivation of phytase achieved initially was maintained during storage. This observation was supported by lower liberated phosphate (P*i*) content in heat pre-treated cowpeas when compared to the control. Phytase hydrolysis of phytate is accompanied by an increase in inorganic phosphate in legume grains (Mafuleka *et al.*, 1993). The control had the greatest increase in P*i* while the micronised cowpeas had the smallest increase on day 40 (Table 3.7). After 40 days of storage at HTHH, phytate was reduced by 31%, 19% and 8% in the control, roasted and micronised cowpeas, respectively. There was a slight reduction in the initial phytate content (day 0) after heat pre-treatments.

Both the CWSP and the HWSP significantly increased after heat pre-treatments on day 0 (Table 3.8). During storage using HTHH conditions there was a decrease in the CWSP and HWSP for both cooked and raw cowpeas. Despite the decrease in the CWSP and HWSP for both cooked and raw cowpeas in all the treatments after HTHH storage (Table 3.8), heat pre-treated cowpeas had higher cold and hot water soluble pectin contents than the control.

Heat pre-treatments did not have a significant ($P > 0.05$) effect on the lignin content of cowpeas either on day 0 or after 40 d storage (Table 3.9).

Table 3.8 Effect of heat pre-treatments of pre-conditioned cowpea seeds (raw and cooked) followed by storage at 40^{o}C and 80 % RH on cold and hot water soluble pectin content[a]

Heat pre-treatment	Storage (days)	Raw samples		Cooked samples	
		CWSP[b] (g/kg)	HWSP[c] (g/kg)	CWSP (g/kg)	HWSP (g/kg)
Control	**Day 0**	1.44^{c}±0.03	2.27^{d}±0.02	1.62^{c}±0.01	2.30^{d}±0.06
	Day 40	1.22^{a}±0.03	1.38$_{a}$±0.04	1.30^{a}±0.03	1.39^{a}±0.03
Micronised	**Day 0**	1.68^{e}±0.06	2.56^{f}±0.03	2.24^{f}±0.01	2.58^{f}±0.05
	Day 40	1.63^{e}±0.04	2.09^{c}±0.07	1.90^{d}±0.02	2.21^{c}±0.06
Roasted	**Day 0**	1.54^{d}±0.05	2.37^{e}±0.02	2.09^{e}± 0.04	2.41^{e}±0.05
	Day 40	1.36^{b}±0.03	1.63^{b}±0.01	1.52^{b}±0.01	1.63^{b}±0.02

[a]Means followed by the different letters in a column are significantly different (n=2) ($P<0.05$)

[b]Cold water soluble pectin

[c]Hot water soluble pectin

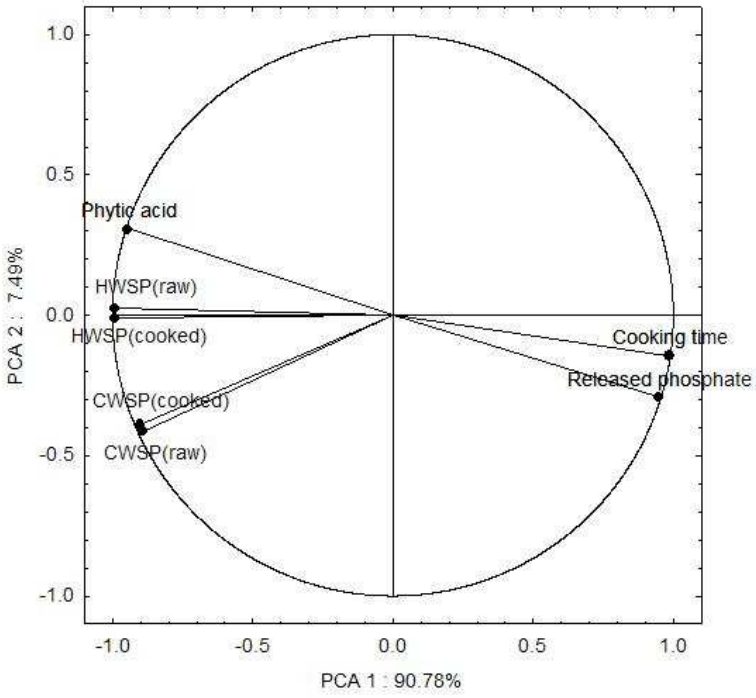
Table 3.9 Changes in the lignin content of heat pre-treated cowpeas after storage at 40 °C and 80 % RH[a]

Heat pre-treatment	Storage (days)	Lignin (g/kg)
Control	**Day 0**	32.0[a]±0.6
	Day 40	31.0[a]±1.0
Micronised	**Day 0**	31.1[a]±0.7
	Day 40	30.8[a]±0.3
Roasted	**Day 0**	31.4[a]±0.8
	Day 40	30.7[a]±0.6

[a]Means followed by the different letters in a column are significantly different (n=2) ($P < 0.05$)

PCA was used to better understand the potential relationships between the independent variables (heat pre-treated cowpeas stored under HTHH conditions) and the dependent variables (physico-chemical characteristics) that differed significantly (Figure 3.3). The first two principal components accounting for 98% of the variation within the data were used to explain relationships between the variables. PC 1 explained 90 % of the variance in the data. PC 1 separated cowpeas (micronised day 40 and roasted day 40) with long cooking times, higher released phosphate to the right of the loading plot from those on the left (Control day 0, micronised day 0, roasted day 0 and micronised day 40) that exhibited relatively shorter cooking times. The cowpeas on the left of the map were characterised by higher phytate and soluble pectin contents. These were generally treatments which had not undergone storage. It was also observed that cowpea samples with higher phytate and higher soluble pectin contents exhibited shorter cooking times. Correlation analysis showed similar observations (Table 3.10). Cooking time was highly positively correlated with released phosphate (r = 0.96) and highly negatively correlated with phytate (r = -0.97), HWSP (raw) (r = -0.97), HWSP (cooked) (r = 0.97), CWSP (raw) (r =-0.82) and CWSP (cooked) (r = -0.82).

a)



b)

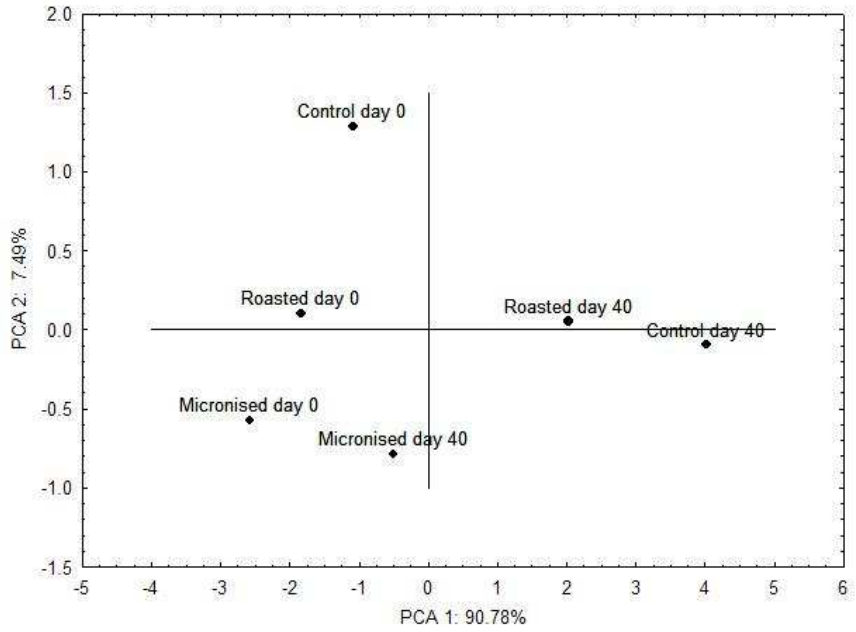


Figure 3.3 Principal component analysis (PCA) of heat pre-treated cowpea seeds under HTHH storage. (a) Plot of first two principal component loading vectors of physico-chemical characteristics. (b) Plot of the first two principal component scores of the cowpea seeds

Table 3.10 Correlations coefficients between physico-chemical characteristics of cowpeas at level P < 0.05[a]

	Cooking time	**Released P*i***	**Phytase activity**	**Phytate**	**HWSP[c] (raw)**	**CWSP[b] (raw)**	**HWSP[c] (cooked)**	**CWSP (cooked)**
Cooking time	1.00	**0.96**	0.07	**-0.97**	**-0.97**	**-0.82**	**-0.97**	**-0.82**
Released P*i*		1.00	-0.08	**-0.96**	**-0.94**	**-0.69**	**-0.92**	**-0.76**
Phytase activity			1.00	0.12	-0.12	-0.53	-0.14	-0.49
Phytate				1.00	**0.94**	**0.71**	**0.93**	**0.72**
HWSP[b](raw)					1.00	**0.85**	**0.99**	**0.89**
CWSP[c](raw)						1.00	**0.87**	**0.91**
HWSP (cooked)							1.00	**0.89**
CWSP(cooked)								1.00

[a] Significant correlations are indicated in bold

[b]Cold water soluble pectin

[c]Hot water soluble pectin

3.2.3 Discussion

The heat pre-treatments used in this study did not significantly decrease the initial cooking time of the cowpeas (Table 3.6). However, Mwangwela *et al.* (2006) reported a 36% reduction in cooking time of pre-conditioned (41% moisture) cowpeas micronised at 153 ^{o}C for 5 min. In the current study, it is probable that the lack of reduction in cooking time after the heat pre-treatments was due to the low pre-conditioning moisture content (25%). Micronisation of cowpeas with high moisture (> 40%) content improves water absorption during cooking due to fissuring of the cowpea by the escaping water vapour (Mwangwela *et al.*, 2006). High pre-conditioning moisture also increases the degree of starch gelatinization in micronised legumes (Arntfield *et al.*, 1997). Increased water absorption and increased degree of starch gelatinisation are associated with shorter cooking times. Therefore, the pre-conditioning moisture content of 25% was not sufficient to result in a decrease in cooking time. Moisture content was set at 25% since higher moisture contents (about 40%) was reported to lead to fissuring (Mwangwela *et al.*, 2006) and cracking of lentils (Cenkowski & Sosulski, 1997) when micronized at higher temperatures. Cracked seeds are undesirable since the cracks would allow possible more moisture penetration in the seed thereby promoting HTC defect development. Cracked cowpeas are perceived as of a low quality when compared to whole cowpeas.

The heat pre-treatments had some degree of control over the development of HTC defect during storage at HTHH as shown by the resulting cooking times (Table 3.6). Micronisation was more effective in reducing HTC defect development since micronised cowpeas exhibited the shortest cooking time after storage. The proposition that HTC defect is due to increased enzyme activity suggests that inactivation of the responsible enzymes should prevent HTC defect development. Heat pre-treatment strategies aim to prevent HTC defect mainly through inactivation of enzymes by heat (Rivera *et al.*, 1989; Cunha *et al.*, 1993; Affrifah *et al.*, 2006). The effectiveness of heat pre-treatments appears to be dependent on temperature, time and moisture conditions of the cowpeas (Affrifah *et al.*, 2006). The inability of heat pre-treatments to totally prevent HTC defect development might be linked to residual enzyme activities in the legume grains.

The decreased phytase activity (Table 3.7) was presumably due to denaturation of the enzyme by heat. Partial inactivation of phytase was achieved by both heat pre-treatments. However there was residual phytase activity indicating incomplete inactivation of phytase by the heating conditions used. In literature there is little information on the inactivation kinetics of legume phytases. Thermal inactivation studies of phytase in cowpeas (Affrifah *et al.*, 2006) and flour (Affrifah & Chinnan, 2005) are indicative of a high thermal stability. Steaming (121^{o}C, 6 min) cowpeas at 20% moisture content reduced the activity of the enzyme to 64% of original activity(Affrifah *et al.*, 2006). The thermal stability of phytase was also demonstrated in heating cowpea flour. Heating the flour with 35% moisture at 95 ^{o}C for 32 minutes reduced phytase activity by 64% (Affrifah *et al.*, 2005).

Severe heat treatments such as increasing the temperature and time could result in increased inactivation of phytase. However, these severe conditions could as well lead to structural damage, loss of nutritional and sensory properties (Plhak *et al.*, 1987). Mwangwela *et al.* (2007) reported reduction in pasting properties, loss of foaming capacity and reduction in gelation properties of flour from cowpeas (41 % moisture) micronised at 170 ^{o}C for 5 minutes.

The observed reduction in phytate content in all treatments during storage is suggested to be as a result of phytase activity which enzymatically hydrolyses phytate to lower inositol phosphates (Reyes-Moreno *et al.*, 2001; Yousif *et al.*, 2007). However, with heat pre-treatments, there was less hydrolysis during storage when compared to the control possibly as a result of partial inactivation of phytase by heat.

Despite the decrease in the CWSP and HWSP for both cooked and raw cowpeas in all the treatments after HTHH storage (Table 3.8), heat pre-treated cowpeas had higher contents of these soluble pectin fractions than the control. This observation indicated less insolubilisation of pectin in heat pre-treated cowpeas. The decrease in water soluble pectin during storage has been mainly attributed to formation of water insoluble pectates (Galiotou-Panayotou *et al.*, 2008).

Formation of pectates is reported to be due to release of divalent cations e.g. calcium from phytate during hydrolysis by phytase. These divalent cations e.g. calcium binds to pectin forming pectates which are insoluble in water (Galiotou-Panayotou *et al.*, 2008). However the extent of reduction in solubility of pectin was significantly ($P > 0.05$) smaller for the heat pre-treated cowpeas than the control. The observation that HWSP was always slightly higher than CWSP, could be attributed to increased pectin solubility. A Similar increase in soluble pectin in cooked cowpeas when compared to the respective raw cowpeas was observed. These increases were probably due to degradation of pectin via the β-elimination reaction due to heat (Bernal-Lugo *et al.*, 1997). The β-eliminative degradation of pectin leads to dissociation of hydrogen bonds in the pectin polymer (Bernal-Lugo *et al.*, 1997). Therefore, pectin was depolymerised and made more soluble (Liu *et al.*, 1993b) leading to an increase in water soluble pectin when cowpeas were either cooked or extracted with hot water.

According to the lignification theory, lignin precursors could be polymerized into lignins due to increased peroxidase activity in the cell wall (Hincks & Stanley, 1987). Heat pre-treatments did not have a significant ($P > 0.05$) effect on lignin content of cowpeas either on day 0 or after 40 d storage (Table 3.9). The suggested increase in peroxidase activity during storage at HTHH conditions was not established in an earlier experiment. Peroxidase activity of cowpeas (*Agripienk*) was lower (0.5ΔA/min/g) after storage for 40 days under accelerated HTHH conditions when compared to day 0 (0.76ΔA/min/g). Rivera *et al.* (1989) reported a decrease in peroxidase activity during HTHH storage of black beans. According Rivera *et al.* (1989), peroxidase activity was not related to an increase or rate of bean hardening. Some authors suggest that HTC defect occurs in sequence; the phytase-phytate-pectin mechanism takes place initially followed by the lignification mechanism that occurs as storage time increases (Hincks & Stanley, 1986; Aguilera & Rivera, 1992a; Del Valle & Stanley, 1995). However, there is lack of quantitative data critically evaluating this proposition. Nasar-Abbas *et al.* (2008) observed a 3 fold increase in lignin content during storage of faba beans at 50^{o}C for 12 months compared to those stored at 5^{o}C. Studies reporting increased lignin content during storage at HTHH are done over a relatively longer time as compared to accelerated storage in the current study (Jones &

Boulter, 1983; Mafuleka *et al.*, 1993; Nasar-Abbas *et al.*, 2008). It may be that the accelerated HTHH conditions used in this study did not allow for lignin synthesis

It can be inferred from the PCA and correlation analysis that the differences in cooking time of the cowpeas were in support of the proposed phytase-phytate-pectin hypothesis. The increase in cooking time during storage was linked to increased inorganic phosphate (increased phytase activity), decreased phytate content and decreased pectin solubility. The observed differences in cooking time between the control and heat pre-treated cowpeas after storage appeared to be due to partial inactivation of phytase. The partially inactivated phytase resulted in less hydrolysis of phytate in heat treated cowpeas. This was possibly accompanied with less liberation of cations available to bind with pectin. Therefore, heat pre-treated cowpeas exhibited more soluble pectin than the control after HTHH storage. During cooking after HTHH storage, more soluble pectin in heat pre-treated cowpeas would indicate better hydration and ease in separation of cells than in control cowpeas. Therefore, shorter cooking time of stored heat pre-treated cowpeas was achieved.

Micronisation was more effective than hot-air roasting as a pre-treatment in preventing the HTC defect development as demonstrated by the shortest cooking time. The observed differences are suggested to be due to the superior heating properties of micronisation. Micronisation is reported to have better heating properties when compared to hot air heating (Sakai & Hanzawa, 1994). During micronisation the IR waves absorbed during penetration, cause molecular vibrations that generate heat (Sakai & Hanzawa, 1994). Therefore IR heating takes place at the surface and inside the inner layers of a material simultaneously (Sakai & Hanzawa, 1994; Krishnamurthy *et al.*, 2008). In contrast, hot air roasting involves conduction of heat from the cowpea surface to the inner layers. IR radiation also has high heat intensity, about 20-100% higher than that of convective heat intensity (Ginzburg, 1969) such as hot-air roasting. In the literature, the advantages of IR heating over hot air heating include reduction in processing time and energy saving (Hebbar, Vishwanathan & Ramesh, 2004). Energy saving of 54.5 % was achieved with infrared heating when compared to conventional ovens for baking rice crackers

(Sakai & Hanzawa, 1994). The baking time of the rice crackers was reduced by 33% when infrared heating was compared with conventional oven (Sakai & Hanzawa, 1994). The results in this study indicate that, the penetration power of IR radiation coupled with the high heat intensity lead to a faster and more uniform increase in temperature within the cowpeas than during hot air. Therefore, the effectiveness of micronisation over roasting was possibly due to the greater degree of heat inactivation of phytase.

3.2.4 Conclusions

Micronisation was a better heat pre-treatment than hot-air roasting in controlling the development of HTC defect of cowpeas during HTHH storage. The extent of phytase inactivation by heat influences the phytate and soluble pectin content during storage, which eventually influences the cooking time.

3.3 Micronisation and hot-air roasting in controlling hard-to-cook phenomenon in cowpeas: structural and physico-chemical changes

Abstract

Micronised and hot-air roasted cowpeas had lower chelator soluble pectin compared to the control after HTHH storage. This indicated lower content of pectates in the heat pre-treated cowpeas. During cooking of cowpeas stored under HTHH conditions, adjacent parenchyma cells of the heat pre-treated cowpeas were observed to separate as cooking progressed. While the parenchyma cells of the non-heat pre-treated cowpeas remained closely adhered together even after 2 h of cooking. The 2 heat pre-treatments increased the transition onset (T_o), peak (T_p) and endset (T_c) temperature of isolated cowpea starch. However, the pre-treatments did not have an effect on the gelatinisation enthalpy. HTHH storage did not influence the transition temperatures or gelatinisation enthalpy of starch within a treatment significantly ($P > 0.05$). Paste of flours from micronised and hot-air roasted cowpeas had lower viscosity when compared to the contol. However, cowpea flours from each treatment after HTHH storage had similar viscosities to storage did not have a significant effect on the pasting properties of the flour. HTHH storage did not have a significant effect on the pasting properties of flours.

3.3.1 Introduction

Deterioration of cowpeas and other legumes cooking quality due to development of hard-to-cook (HTC) defect during high temperature and high humidity (HTHH) storage is a major factor limiting their utilization (Reyes-Moreno & Paredes-Lopez, 1993). Legumes with the HTC defect require long cooking times to attain the soft texture characterised with cooked legumes (Hincks & Stanley, 1987). In contrast, easy to cook legumes are characterised with short cooking times in attaining the desired soft texture (Moscoso, Bourne & Hood, 1984).

In the previous section (3.2), heat pre-treatments were shown to reduce the incidence of HTC during HTHH storage as demonstrated by shorter cooking times of heat pre-treated cowpeas when compared to the control. This indicated that after HTHH storage, heat pre-treated cowpeas attained a softer texture at a faster rate than the control during cooking. Thermal softening of dry

legume seeds during cooking is mainly been attributed to parenchyma cell separation (Sefa-Dedeh & Stanley, 1979; Bernal-Lugo *et al.*, 1997; Aguilera, 2000) and starch gelatinization (Sefa-Dedeh & Stanley, 1979; Arntfield *et al.*, 2001; Yousif *et al.*, 2007).

The loss of intercellular adhesive strength due to dissolution of pectin results in cell separation that promotes tissue softening of legumes during cooking. The observed reduced rate or lack of cell separation during cooking of legumes with HTC defect is reportedly due to presence of water insoluble pectin in the middle lamella (Galiotou-Panayotou *et al.*, 2008). According to the phytase–phytate–pectin theory, insolubilisation of the middle lamella pectin is due to pectate formation which limits parenchyma cell separation in the cotyledon. Therefore, longer cooking time is required to soften the legumes with HTC defect (Hentges *et al.*, 1991; Galiotou-Panayotou *et al.*, 2008).

Starch is the major macromolecular constituents of cowpeas (Kerr *et al.*, 2001). Starch gelatinisation during cooking contributes to the soft texture of legumes (Arntfield *et al.*, 2001). Changes in the properties of starch such as increase in starch granule crystallinity during HTHH storage have been reported to influence starch gelatinisation (Hohlberg & Stanley, 1987; Garcia & Lajolo, 1994). Increased starch gelatinisation enthalpy and temperature reported in HTHH stored legume is suggested to be due to increase in starch granule crystalinity (Garcia & Lajolo, 1994; Reyes-Moreno *et al.*, 2001). The increase in gelatinisation temperatures and enthalpy would thus result in more energy required to gelatinize the starch (Liu *et al.*, 1992a; Garcia & Lajolo, 1994; Kaur & Singh, 2007). Therefore, it can be inferred that high gelatinization temperature would result in higher cooking times as observed in rice (Fitzgerald, McCouch & Hall, 2009; Cuevas, Daygon, Corpuz, Nora, Reinke, Waters & Fitzgerald, 2010).

Hydrothermal pre-treatments such as micronisation have been reported to result in reduced cooking time of legumes due to partial starch gelatinisation and increased pectin solubility (Bellido *et al.*, 2006; Mwangwela *et al.*, 2006). Micronisation has been shown to alter the thermal and pasting properties of starch (Cenkowski & Sosulski, 1997; Mwangwela *et al.*, 2006).

In order to understand further the mechanisms whereby micronisation and hot-air roasting of pre-conditioned cowpeas improved the cooking time after HTHH storage, this study examined the (1) chelator soluble pectin content which indicates pectate content (2) adhesion/separation of the parenchyma cells in the cotyledon of cowpeas during cooking and (3) thermal and pasting properties of starch.

3.3.2 Materials and methods

3.3.2.1 Raw materials

Agripienk cowpea seeds and flour samples were prepared as reported in sections 3.2.2.1 and 3.2.2.7.

3.3.2.2 Chelator soluble pectin (CSP)

CSP was determined according to a modified procedure of Hentges *et al.* (1991) after extraction of CWSP as reported in section 3.2.2.12. The water-insoluble pellet was extracted 3 times with 10 mL of 0.5% Ethylenediaminetetraacetic Acid (EDTA) each for 10 minutes. The extracts were combined and considered as the chelator soluble pectin (CSP). Pectin content was expressed as glacturonic acid as determined by the metahydroxydiphenyl method of Blumenkrantz & Asboe-Hansen (1973), using galactouronic acid as a standard.

3.3.2.3 Confocal Laser Scanning microscopy

Tissue blocks (1 mm^3) were cut from the cooked cotyledon. These were fixed in 2.5% (w/v) formaldehyde overnight. The fixed cotyledon tissues were rinsed three times for 10 min in 0.075 M phosphate buffer. The tissues were dehydrated at room temperature at 10 min intervals in a graded series of aqueous ethanol (50%, 70%, 90%, 100%, 100%, 100%). Dehydrated tissues were infiltrated with 50% (v/v) LR White resin in ethanol for 1 h and 100% LR White resin overnight at room temperature. The tissues were then embedded in gelatin capsules containing LR White and polymerised for 24 hours. Sections with thickness of 3.0 μm were cut and placed on a glass slide. Cell wall material was located by staining the sections with Calcofluor White

MR2 (Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min. The stained sections were washed in running tap water and dried. Samples were then viewed under a CLSM (ZEISS LSM 510, Zeiss SMT, Jena Germany). The microscope system was equipped with a Plan-Apochromat 20×/0.75 objective lens. Excitation was at 405 nm using a 420 long pass filter, with a pinhole set at 48 µm. The digital images obtained were processed using Zeiss LSM image browser.

3.3.2.4 Starch extraction

Starch was isolated from the cowpeas based on the method of Taylor, Novellie & Liebenberg (1984). About 100 g of defatted cowpea flour (from treated and untreated cowpea seeds) was mixed with 500 ml deionised water and stirred at 22 °C for 2 h. The slurry was wet milled in a Retsch Mill ZM 200 (Haan, Germany) with a 250 µm opening screen. The slurry was filtered through sieves (Labotech, Johannesburg, South Africa) with aperture sizes 212, 108, 75, 45 and 38 µm on to a collecting pan. The sediments on the sieves were suspended in distilled water, wet milled and filtered through the sieves. The wet milling process was repeated until the filtrate was clear. The filtrate was centrifuged at 3880 *g* for 5 min (15 °C). The supernatant was carefully decanted and the brown layer on top of the residue was scrapped off. The residue was re-suspended in deionised water and vigorously mixed, centrifuged and decanted. This procedure was repeated for 7 times until a white pellet (starch) was obtained. The starch samples were then freeze-dried.

3.3.2.5 Thermal analyses

Thermal properties of cowpea flours and extracted starch were determined using a Metler Toledo HPDSC-827 DSC (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Each sample (10 mg) was weighed into a 40 µL aluminium sample pan and 30 mg of water was added. The pans were hermetically sealed and the samples equilibrated overnight at room temperature. The instrument was calibrated using indium, and an empty aluminium pan was used as reference. The measurements were done using a heating rate of 10 °C min^{-1} between 30 and 120 °C. The

following thermal parameters were measured: the melting enthalpy (ΔH Jg-1) peak onset (T_o), peak (T_p) and peak end (T_c) temperatures.

3.3.2.6 Pasting properties

Pasting properties were measured using a Rapid Visco Analyser (RVA Model 3D) (Newport Scientific, Warriewood, Australia). Cowpea flour (3 g corrected to 14% moisture) was suspended in distilled water and the weight adjusted to 28 g. Samples were heated from an initial temperature of 50–90 °C in 7.3 min, held at this temperature for 5 min and cooled to 50 °C in 7.3 min at a speed of 160 rpm for the first 10 s and 960 rpm for the remainder of the cycle.

3.3.2.7 Statistical analyses

The effects of heat pre-treatments before and after storage at HTHH on CDTA soluble pectin, thermal properties and pasting properties were determined using analysis of variance (ANOVA) and Fisher's least significant difference test (LSD, $P \leq 0.05$).

3.3.2.8 Results and discussions

Heat pre-treatments did not influence the EDTA soluble pectin content of the cowpeas before storage (Table 3.11).

Table 3.11 Effect of heat pre-treatments followed by storage on chelator (EDTA) soluble pectin*

Treatment	Storage (days)	EDTA soluble pectin (mg/g)
Control	**0**	$0.08^{a} \pm 0.01$
	40	$0.14^{c} \pm 0.01$
Micronised	**0**	$0.08^{a} \pm 0.02$
	40	$0.09^{a} \pm 0.01$
Hot-air roasted	**0**	$0.08^{a} \pm 0.01$
	40	$0.11^{b} \pm 0.01$

*Means followed by the different letters in column are significantly different (n=2) ($P < 0.05$)

During storage the control and roasted cowpeas had a significant increase in EDTA soluble pectin ($P < 0.05$) while there was no change in the micronised cowpeas. The control day 40 cowpeas had the highest content of EDTA soluble pectin. Increase in chelator soluble pectin during storage of legumes under HTHH conditions was reported (Hentges *et al.*, 1991). EDTA extracts divalent cation bound pectins from the middle lamella (Yashoda, Prabha & Tharanathan, 2006). Increased EDTA soluble pectin during storage indicated increased presence of pectates (divalent cations bound to pectin).

This further strengthens the results of the previous work (section 3.2) that showed the HTC defect was partially dependent on the extent of phytate hydrolysis as suggested in the "the phytase-phyate–pectin" theory. Heat pre-treatments reduced the extent of phytate hydrolysis due to partial inactivation of phytase (section 3.2). Therefore, during storage fewer cations were released in the micronised cowpeas when compared to the control. The higher degree of phytase inactivation in the micronised cowpeas when compared to the roasted cowpeas resulted in fewer cations being available to bind with pectin and thus lower amount of EDTA soluble pectin

Microstructures of the parenchyma cells were observed after boiling (cooking) the cowpeas for 30, 60, 90 and 120 minutes (Figure 3.4 to Figure 3.7). The focus of this microstructural study was to observe the separation or adhesion of parenchyma cells in the cotyledons. The cell walls (CW) were stained specifically by Calcofluor white as shown in figures. No clear differences were observed in the microstructure of all treatments cooked for 30 minutes (Figure 3.4). The cells were closely packed, isodiametric and of a regular shape. The cell walls were intact, with very small intercellular spaces (IS) and the tri-cellular junctions (TCJ) were clearly visible. After 60 minutes of cooking, there was an increase in the size of intercellular spaces while the cells separated from one another in all day 0 samples (Figure 3.5). The compact structure of the cells was lost as the cells started to "round off" and separate from one another. On day 40 cowpea seeds that were untreated had the cells still adhered to one another, the intercellular spaces (IS) were still very small and the tri-cellular junctions were still intact. The micronised and roasted day 40 cowpeas showed slight increase in intercellular spaces but the compact structure was still present.

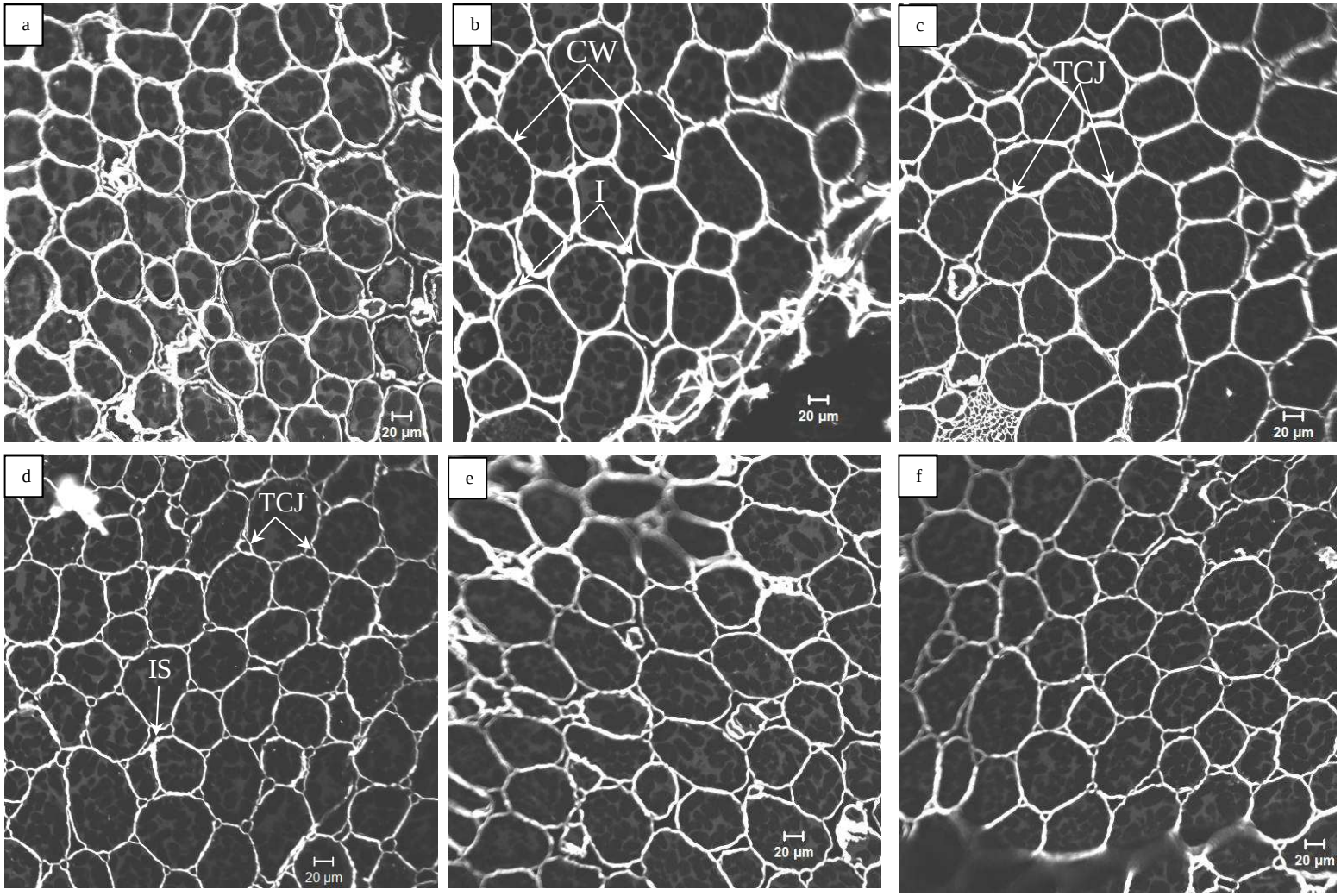


Figure 3.4 CLSM micrographs of cowpea cooked for 30 min. (a) control day 0, (b) micronised day 0, (c) roasted day 0, (d) control day 40, (e) micronised day 40, (f) roasted day 40. CW: cell wall; TCJ: tri-cellular junction; IS: intercellular space

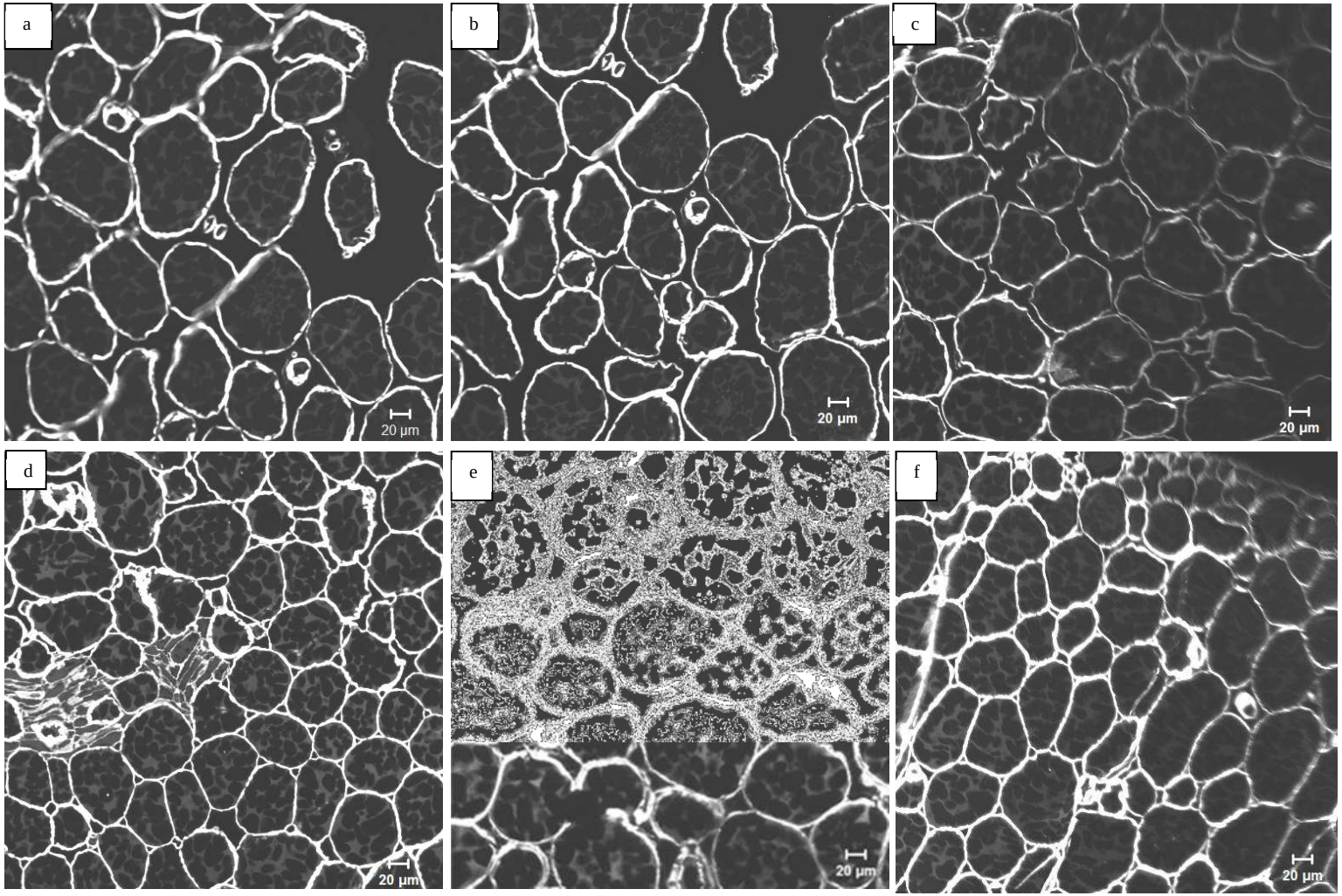


Figure 3.5 CLSM micrographs of cowpea cooked for 60 min. (a) control day 0, (b) micronised day 0, (c) roasted day 0, (d) control day 40, (e) micronised day 40, (f) roasted day 40.

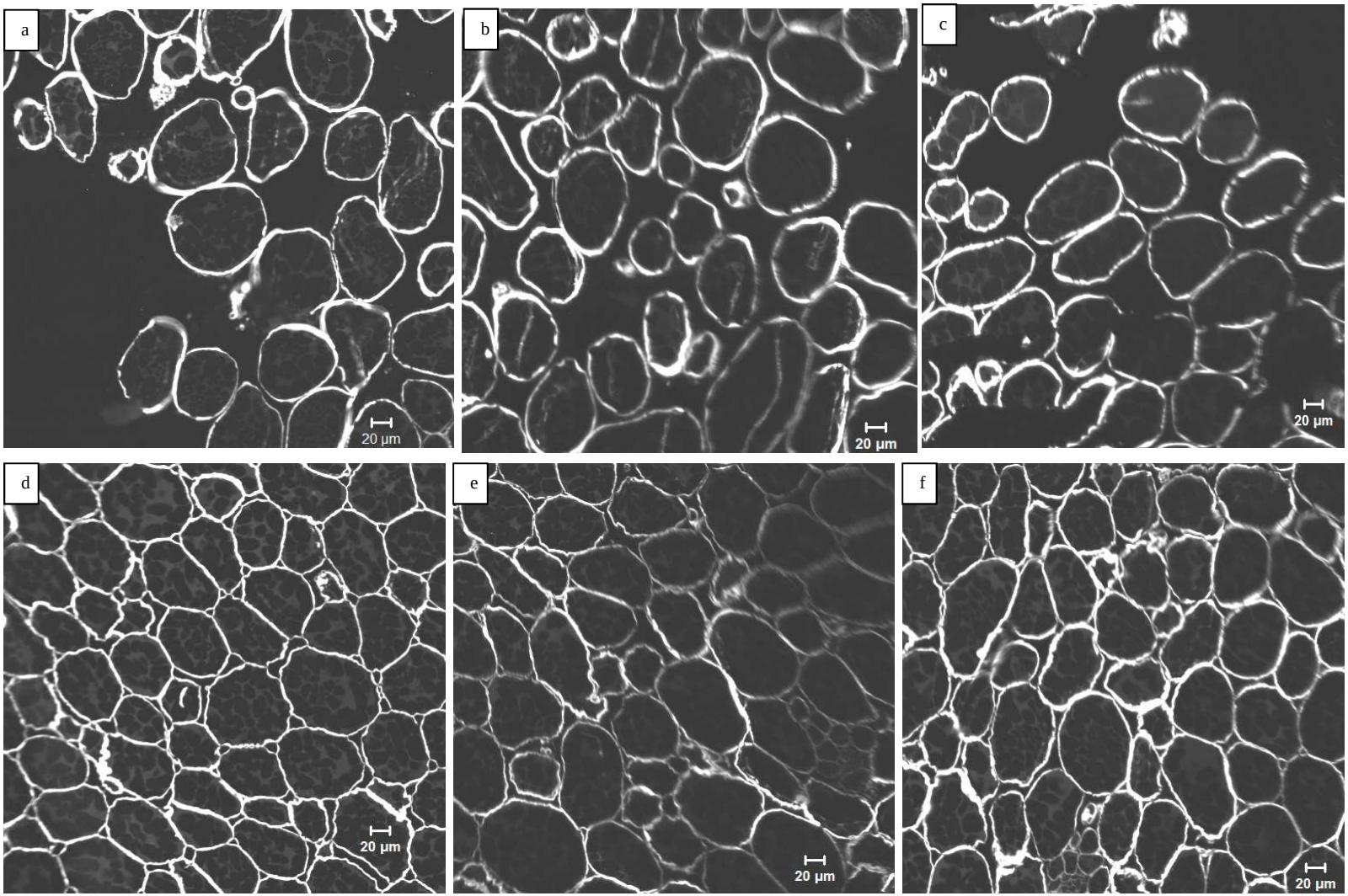


Figure 3.6 CLSM micrographs of cowpea cooked for 90 min. (a) control day 0, (b) micronised day 0, (c) roasted day 0, (d) control day 40, (e) micronised day 40 (f) roasted day 40.

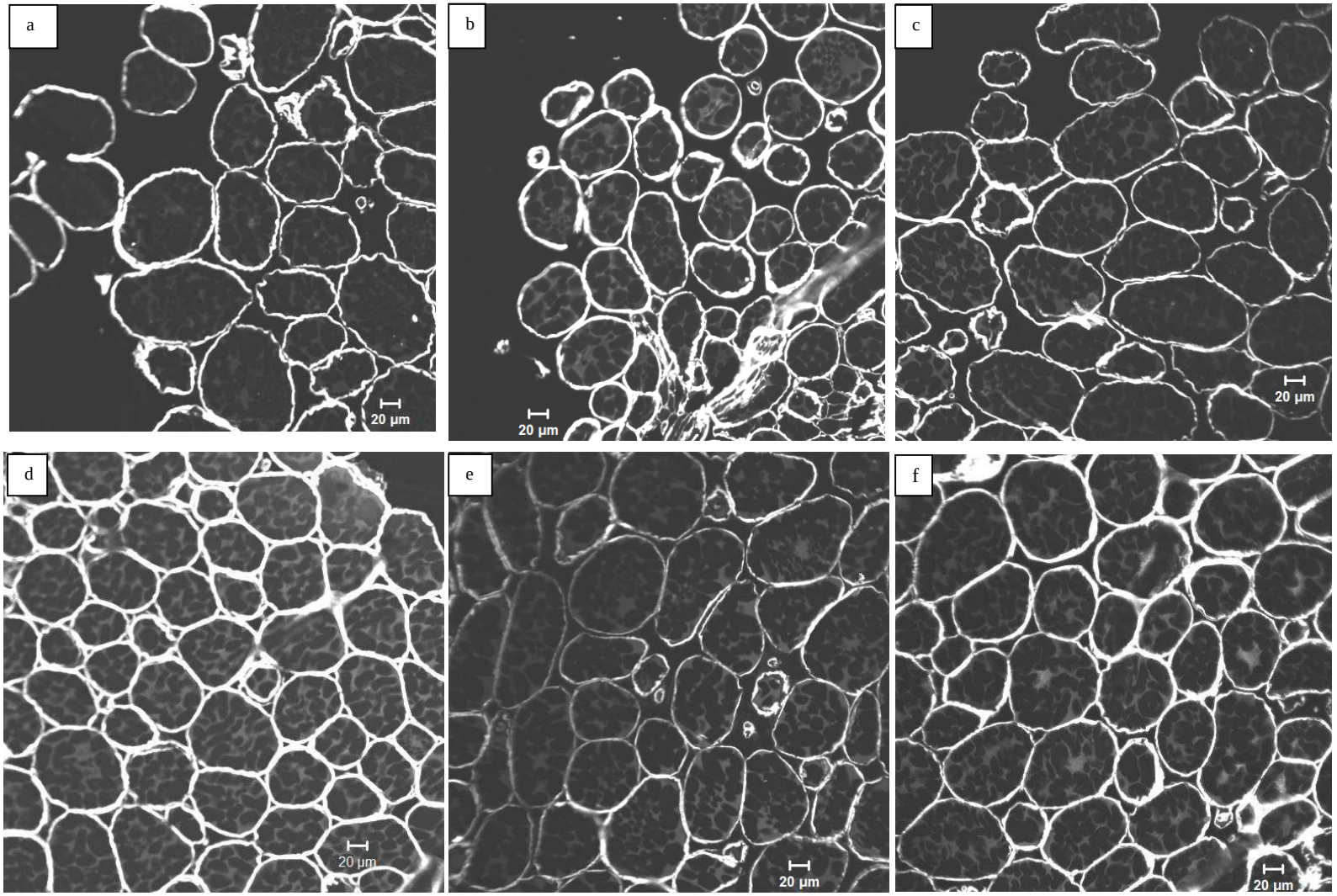


Figure 3.7 CLSM micrographs of cowpea cooked for 120 min. (a) control day 0, (b) micronised day 0, (c) roasted day 0, (d) control day 40, (e) micronised day 40 (f), roasted day 40.

All day 0 cowpeas were characterised by complete cell separation and complete "rounding off" of cells after 90 minutes of cooking (Figure 3.6). However, the structure of the control day 40 cells still remained unchanged. The micronised day 40 cowpeas were characterised by "rounding off" of the cells and increase in the intercellular spaces. Although, the disappearance of the tri-cellular junctions was observed in the roasted day 40 cowpeas, the cells were still close together. Further, cooking for 120 minutes (Figure 3.7) resulted in a tissue structure similar to that observed after 90 min for the respective day 0 samples. It was interesting to note that even after 2 hours of cooking the structure of control day 40 remained compact as observed in the previous cooking times. This was contrasted with increased cell separation and "rounding off" of cells in the stored heat pre-treated cowpeas. The extent of separation and "rounding off" of cells was more pronounced in the micronised day 40 samples when compared to the roasted day 40 samples.

The changes in microstructure of cowpeas during cooking could possibly explain the observed differences in cooking times as reported in the chapter 3.1 and 3.2. Cooking was achieved under an hour for all day 0 cowpeas whereas after storage there was increased cooking time with the control day 40 cowpeas having the longest cooking time. The microstructural study indicated that samples with the shortest cooking time (day 0 cowpeas) had their cells completely separated after 60 minutes of cooking. The increase in intercellular space and cell separation indicated dissolution of the middle lamella pectins. On the other hand control day 40 cowpeas which were characterised with the longest cooking time showed minimal cell separation even after 2 hours of cooking. This observation was in agreement with studies that reported ease in separation of parenchyma cells along the middle lamella for easy to cook legumes, whereas, the HTC legumes are characterised by limited cell separation (Bhatty & Slinkard, 1989). The attainment of the soft texture of cooked legume is associated with cell separation which is facilitated by heat induced dissolution/degradation of the middle lamella pectin. Therefore, failure of pectin dissolution/degradation during cooking restricts cell separation and thus longer cooking times (Aguilera & Stanley, 1985). In earlier part of this study (chapter 3.1), a decrease in water soluble pectin was shown to contribute to HTC defect development. The role of pectin in the middle lamella is to cement cells together and therefore during cooking easier solubilisation of the pectin

facilitates separation of adjacent cells and subsequent softening of the legume grain during cooking (Galiotou-Panayotou *et al.*, 2008). Therefore it can be inferred that the ease in solubilisation of pectin and subsequent cell separation contributed to shorter cooking times in the day 0 cowpeas. The lack of separation of cells in control day 40 and the longer time for separation of cells to occur in the heat pre-treated stored sample was probably due to insolubilisation of pectin. The insolubilisation was partly due to formation of pectates as observed with the increased EDTA pectin. The day 40 cowpeas were found to have decreased hot and cold water soluble (chapter 3.2) with day 40 control having the lowest amount. These results suggest that the reduced cooking time observed in heat pre-treated cowpeas was related to the ease in middle lamella pectin dissolution that caused cell wall separation and therefore softening.

A single endothermic peak in the DSC curves of flours and starches isolated from cowpea samples was observed under the test conditions. The transition onset (T_o) and peak (T_p) temperatures (Table 3.12 and Table 3.13) were indicative of endothermic transitions associated with starch gelatinisation in cowpeas. These values were in agreement with those in literature which show the values for cowpea starch to be between 67 to 78 °C (Agunbiade & Longe, 1999; Abu, Duodu & Minnaar, 2006; Mwangwela *et al.*, 2006). Flours from cowpea seeds had higher thermal transitional temperatures values when compared to their respective starch samples. Similar findings were reported by Mwangwela *et al.* (2007) in fresh cowpeas. Heat pre-treatment increased the transition onset (T_o), peak (T_p) and endset (T_c) of isolated cowpea starch (Table 3.13). Starch from micronised seeds had the highest T_o , T_p and T_c.

Similar increases in thermal transition temperatures of starch isolated from micronised (130 and 170^{o}C) pre-conditioned cowpeas (41% moisture) were reported (Mwangwela *et al.*, 2007). Storage did not influence the transition temperatures of starch within a treatment significantly ($P > 0.05$). Zamponi, Giner, Lupano & Anon (1990), reported higher thermal transition temperatures in the starch of hot-air (80^{o}C) dried wheat grains with 25.5% moisture when compared to the control.

Table 3.12 Effects of heat pre-treatments of pre-conditioned cowpea seeds followed by storage at 40 °C and 80% RH on the thermal properties of cowpea flour*

Treatment	Storage (days)	Onset T_o (^oC)	Peak T_p (^oC)	Endset T_c (^oC)	ΔH(J/g)
Control	**0**	$72.43^{a} \pm 2.16$	$77.32^{a} \pm 1.53$	$85.13^{a} \pm 2.58$	$2.9^{a} \pm 0.9$
	40	$72.24^{ab} \pm 1.53$	$77.66^{a} \pm 1.47$	$84.69^{a} \pm 1.95$	$3.1^{a} \pm 0.3$
Micronised	**0**	$74.44^{abc} \pm 1.96$	$80.12^{b} \pm 1.85$	$89.03^{ab} \pm 3.51$	$3.5^{a} \pm 1.6$
	40	$76.13^{c} \pm 0.28$	$81.51^{b} \pm 0.18$	$92.40^{b} \pm 1.17$	$3.4^{a} \pm 2.0$
Roasted	**0**	$75.36^{bc} \pm 0.43$	$80.30^{b} \pm 1.10$	$90.64^{b} \pm 3.40$	$3.4^{a} \pm 1.6$
	40	$74.77^{bc} \pm 0.73$	$79.78^{b} \pm 0.61$	$90.24^{b} \pm 3.92$	$3.2^{a} \pm 0.7$

*Means followed by the different letters in column are significantly different (n=2) ($P<0.05$)

The observed increases in gelatinisation temperatures of starch and flours from heat treated cowpeas were possibly due to modification of the crystalline structure and nature of the starch granules due to the hydrothermal treatments. Heat moisture treatments (HMT) are known to increase thermal transitional temperatures of starches (da Rosa Zavareze & Dias, 2011). These temperatures increase as the heat and moisture intensity increase (da Rosa Zavareze & Dias, 2011). The increases have been noted in other heat-moisture treated legume starches such as peas and lentils (Chung, Liu & Hoover, 2010). The increases are attributed to structural changes within the starch molecule, which involve amylose-amylose (AM-AM], amylose-amylopectin [AM-AMP] and amylose-lipid [AM-L] interactions (Hoover & Vasanthan, 1994; Jacobs & Delcour, 1998; Gunaratne & Hoover, 2002). It is suggested that the AM-AM and AM–L interactions reduce the mobility of the amorphous region. Therefore, higher temperatures are required for swelling and disruption of the crystalline regions, leading to the observed increases in T_o, T_p, and T_c (da Rosa Zavareze & Dias, 2011).

Table 3.13 Effects of heat pre-treatments of pre-conditioned cowpea seeds followed by storage at 40 °C and 80% RH on the thermal properties of cowpea starch*

Treatment	Storage (days)	Onset T_o (^oC)	Peak T_p (^oC)	Endset T_c (^oC)	ΔH(J/g)
Control	**0**	66.93^{ab} ± 0.52	72.69^{a} ± 0.01	79.45^{a} ± 0.88	7.8^{a} ± 0.7
	40	66.67^{a} ± 0.11	73.00^{a} ± 0.01	79.01^{a} ± 1.27	8.9^{a} ± 0.5
Micronised	**0**	69.97^{de} ± 0.73	75.77^{c} ± 0.28	83.48^{c} ± 0.47	9.7^{a} ± 0.1
	40	71.17^{e} ± 0.17	76.27^{c} ± 0.25	83.68^{c} ± 0.87	9.2^{a} ± 1.6
Roasted	**0**	68.13^{bc} ± 0.93	74.22^{b} ± 0.22	81.33^{b} ± 0.71	9.8^{a} ± 0.4
	40	68.53^{cd} ± 0.76	74.20^{b} ± 0.06	81.49^{b} ± 0.36	9.9^{a} ± 0.2

*Means followed by the different letters in column are significantly different (n=2) ($P < 0.05$)

Storage did not affect the thermal properties of both flour and starch samples within a treatment. It is suggested by some researchers that HTHH storage of legume grains may lead to an increase in starch granule crystallinity (Hohlberg & Stanley, 1987; Hentges *et al.*, 1991) which could increase gelatinisation temperatures (T_o and T_p) and gelatinisation enthalpy(ΔH). However, in this study such increases were not observed. The results in this study were in agreement with some researchers who reported no significant changes in gelatinisation temperatures of legume flours (Paredes-Lopez, Maza-Calvino & González-Castañeda, 1989a; Liu *et al.*, 1992a).

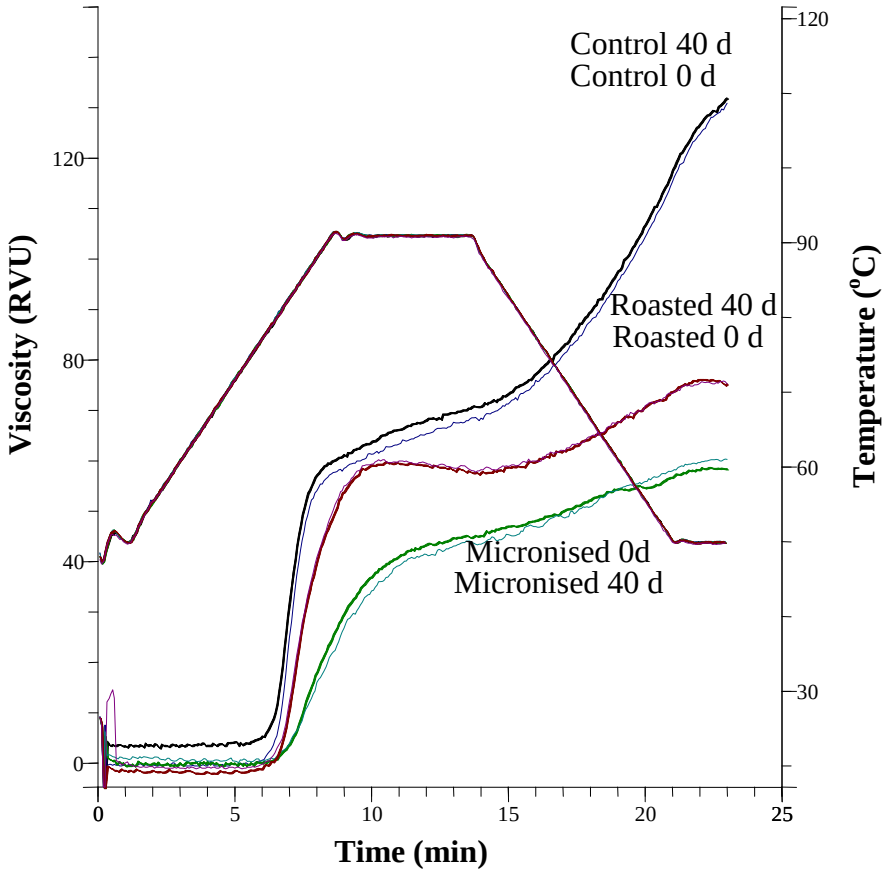


Figure 3.8 Effect of heat treatments followed by storage on pasting properties of cowpea flour

The pasting curves of the cowpea flours showed differences in viscosity as influenced by the heat pre-treatments but not as a result of storage (Figure 3.8). The heat treatments however decreased the viscosity of flours when compared to the control. The micronised flours had the lowest viscosity. Roasting (Plahar, Annan & Nti, 1997) and micronisation (Mwangwela *et al.*, 2007) have been shown to reduce pasting viscosities of cowpea flours. Starch granulea are embeded in the protein matrix, therefore the reduced viscocity could be due to denaturation of protein due to heating that could prevent starch granule hydration and dispersion during pasting. The unfolding of protein molecule due to heat increases the exposed hydrophobic sites leading to aggregration and possible gel formation (Clark *et al.*, 2001). The reduction in viscosity could also be due to heat damage of starch which could reduce the swelling and hydration of starch granules (Hoover, Swamidas & Vasanthan, 1993; Hoover & Manuel, 1996; Yue, Rayas-Duarte

& Elias, 1999). In kidney beans, (Kaur & Singh, 2007) observed lower paste viscosity of starch from HTC beans when compare to the control. However, Paredes-Lopez *et al.* (1989a) reported a higher paste viscosity of starches from HTC beans when compared to fresh beans. According to Rupollo, Vanier, Zavareze, de Oliveira, Pereira, Paraginski, Dias & Elias (2011) changes in pasting properties during storage of grains under different conditions are more likely due to changes in crystallinity of the starches. The storage conditions in this study did not impact on the starch pasting properties of the cowpeas similar to what was observed in the thermal properties of starch.

3.3.3 Conclusions

The lower content of chelator soluble pectin (pectates) in heat-pretreated cowpeas after HTHH storage promotes faster parenchyma cell separation during cooking. The higher pectate content in the control after HTHH storage is associated with lack of parenchyma cell separation even after 2 hours of cooking. Micronised cowpeas have lower content of chelator soluble pectin and more cell separation when compared to the hot-air roasted cowpeas during cooking. Both heat pre-treatments have effects on the thermal and pasting properties of starch but HTHH storage did not significantly influence these properties.

4 GENERAL DISCUSSION

The discussion is divided into three sections. The discussion will first examine the strengths and weaknesses of the experimental design and some of the methodologies used during the research. The second section will examine the possible mechanisms by which high temperature and high relative humidity storage causes the development of HTC in cowpeas. The last section examines the possible mechanisms involved in the reduction of the HTC defect development in cowpeas by the use of micronisation and hot air roasting. Possible scientific mechanisms on how micronisation and hot air roasting were effective in reducing the HTC defect will be put forward.

4.1 Critical review of experimental design and methodologies

4.1.1 Raw material selection

It was essential to evaluate different cowpea types' susceptibility to HTC before selecting the type that was used in the study. 11 cowpea types from Botswana (n=4) and South Africa (n= 7) were screened in terms of their initial cooking time. 8 types were found to have excessively long cooking times (> 270 minutes) which indicated they already had the HTC defect. Therefore only 3 types were selected for the inducement of HTC defect, after which one cowpea type (*Agripienk*), that was most susceptible to HTC ,was selected for heat pre-treatment studies.

4.1.2 Model system to induce HTC

Tropical conditions of high temperature (≥ 25 ^{o}C) and high relative humidity (≥ 65%) promotes the HTC defect in legumes (Reyes-Moreno, Romero-Urias, Milan-Carrillo, Valdez-Torres & Zarate-Marquez, 2000b; Reyes-Moreno *et al.*, 2000c) when stored for a long period (> 6 months). Therefore in studying HTC defect fast laboratory procedures are used (Jackson & Varriano-Marston, 1981; Vindiola *et al.*, 1986; Paredes-Lopez *et al.*, 1989b) in inducing HTC defect in legumes. Accelerated storage involves storage of legumes under conditions that mimic tropical conditions for shorter durations (Reyes-Moreno *et al.*, 2000c). Martin-Cabrejas, Jaime, Karanja, Downie, Parker, Lopez-Andreu, Maina, Esteban, Smith & Waldron (1999) demonstrated that HTC development in beans stored under accelerated conditions (42^{o} C, 80% RH) for 6 weeks were comparable to beans stored at tropical conditions for 1 year. Therefore quality defects can be observed in a shorter period when using accelerated storage conditions. In this study, accelerated HTHH storage conditions were achieved by storing the cowpeas above a

saturated potassium chloride (KCl) solution in an airtight plastic container. The KCl solution maintained a constant humidity condition of 80%. The container was then placed in a growth chamber at 40 °C. This procedure was effective in maintaining the desired HTHH (40 °C, 80% RH) conditions throughout the 40 days of storage as shown by humidity-temperature logger that was placed inside the container. The storage conditions were effective in inducing HTC defect as observed in the > 3 fold increase cooking time.

4.1.3 Pre-conditioning

Dry legume seeds are usually of low moisture content of about 10%. Therefore, before heat pre-treatments are applied to the seeds, the moisture level is increased. Moisture content is critical for generation of heat as well as heat transfer during the heat pre-treatments since water has a high specific heat in comparison to other food components (Adams, 1991). Pre-conditioning or tempering involves increasing the moisture content of the seeds to target moisture content. The cowpeas had a moisture content of 8%, therefore dry heating of the cowpeas at low moisture content would have been ineffective in inactivating the enzymes and might have caused burning of the seeds. The pre-conditioning procedure as described by (Arntfield *et al.*, 1997) was successful in adjusting the moisture content of the cowpeas to the desired level of 25%.

4.1.4 Micronisation

Micronisation or Infrared (IR) heat processing involves heating with radiant electromagnetic energy in the wavelenth range of 1.8 to 3.4 µm (Fasina *et al.*, 1999). A table top experimental microniser used in this study comprised of 3 quartz-tungsten infrared lamps with a 6 kW power. Two out of 3 the three lamps were used. The distance between the lamps in the micronizing unit and the sample being irradiated is adjusted depending on the desired heating temperature (Bellido *et al.*, 2006). In this study a distance of 25 cm was used to attain a heating temperature of 150 ^{o}C. Rotation of individual seeds by using vibrating troughs or belts as demonstrated by (Bellido *et al.*, 2006) would have been a better procedure in ensuring uniform exposure of IR energy on all seed surfaces. However, small seeded legumes such as the cowpea variety used in this study can be micronised effectively while on stationery surface (Fasina *et al.*, 2001; Mwangwela *et al.*, 2007). Although, the surface temperature of the cowpeas was monitored

using a temperature probe, an indication of the temperature inside the cowpea seeds could also have been monitored by inserting thermocouples into the centre of some seeds.

4.1.5 Phytase

Phytase activity was determined by direct incubation of the cowpea flour in a buffered sodium phytate solution. This colorimetric assay in phytase activity determination was based on the quantification of inorganic phosphate (P*i*) liberated from added sodium phytate after 30 min incubation period (Eeckhout & De Paepe, 1994; Shen *et al.*, 2005). The principle of this enzyme assay relied on measuring the generation of a reaction product (phosphate) over one period of time (one point method). Phytase catalyses the hydrolysis of phytate yielding inorganic phosphate (P*i*). The phytase activity was determined by color formation between the released P*i* and molybdate ions(Eeckhout & De Paepe, 1994). The total amount of P*i* liberated after 30 min was calibrated for background (free) P*i* already present before incubation. The one point method assumes a linear rate of P*i* liberation under the assay conditions used (Eeckhout & De Paepe, 1994). It was observed that after HTHH storage of samples the determined phytase activity decreased rather than increase as expected. This was contrasted by the observation that phytate content decreased, while P*i* increased during HTHH storage which indicated increased phytase activity. Higher levels of Pi are reported to cause interference during phytase activity determination in two ways (Ullah, 1988; Kim & Lei, 2005). According to Ullah (1988), high content of P*i* inhibits the completion of phytate hydrolysis by phytase during determination. Therefore, high contents of P*i* due to endogenous phytase activity in the cowpeas stored under HTHH conditions could have inhibited phytase hydrolysis of sodium phytate in the assay. Secondly, high background P*i* results in strong colour formation in blanks that decreases the color contributions by the enzymatic hydrolysis (Kim & Lei, 2005). The high background P*i* after HTHH storage was quite high and could have resulted in the low values observed. To overcome this challenge, a measure of Pi present in the cowpeas seeds before and after storage was adopted as a measure of phytase activity during storage. This gave a relative value of changes in phytase activity. The measure of inorganic phosphate in beans before and after storage had previously been used by Mafuleka *et al.* (1993) as an indirect measure of phytase activity.

4.1.6 Phytate

The amount of phytate present in cowpeas was determined using Megazyme test kit K-PHYT (Megazyme, 2007). The method involved extraction of phytate from the cowpea flour with hydrochloric acid. This was followed by enzyme treatments with phytase and alkaline phosphatase. Phytase hydrolyses phytate and lower *myo*-inositol phosphate forms (i.e. inositol pentakisphosphate to inositol bisphosphate releasing inorganic phosphate. Alkaline phosphatase released phosphate from inositol monophosphate that is relatively resistant to the action of phytase. Phytate was measured as the total phosphorus liberated by phytase and alkaline phosphatase using a colourimetric method. The K-PHYT quantitative method for determination of phytate is rapid and simple when compared to precipitation and ion exchange methods that are widely used for phytate assays. Both of these methods are time consuming and could overestimate phytate content since they include partially dephosphorylated isomers of phytic acid (Wu, Tian, Walker & Wang, 2009). The generally accepted AOAC Method 986.11 is laborious due to the step gradient anion-exchange purification step (Megazyme, 2007).

4.1.7 Cooking time

The classification of legumes as “easy to cook” or “hard to cook” is determined by the time required for the legumes to attain acceptable softness for consumption during cooking. The presence of HTC defect or its absence in the cowpeas was therefore dependent on the cooking time. Cooking time was determined using a commonly used Mattson bean cooker (MBC) (Proctor & Watts, 1987). The cooking time as determined by the MBC involved placing 25 cowpeas seeds each in a perforation at the cylindrical base of the MBC. Steel rods of equal weight (50 g) with tapering ends are then placed on top of the seeds and the MBC is immersed in boiling water. Cooking time was recorded as the time when 80% (20/25) of the rods had fully penetrated the cowpea seeds. The main challenge with this method was that it was time consuming and required keen observation by the operator to record each time a rod went through the cooked seed. Some of the cowpeas with HTC defect required observation for over 4 hours. Penicela (2011) observed that it was quite a challenge for the cowpeas to remain in position once the rods were placed or during immersion of the MBC in boiling water. This is because the perforations were larger and meant for beans which have a larger surface area than cowpeas. A

Similar problem was experienced by the experimenter, to overcome this challenge, a cylindrical base with perforations that matched the cowpea size was used. The use of automatic recording system connected to the MBC device (reference) would eliminate the manual aspect of recording penetration times during the whole duration of determination. Although the MBC is widely used in determination of cooking time, it is always a challenge to compare results from different studies due to differences in the determination procedure (Mwangwela *et al.*, 2006). These differences include; weight of the rods (e.g. 49.8 g (Penicela, 2011) and 90 g (Mwangwela *et al.*, 2006; Salvador, 2007), cooking time as determined by % of rods penetrating the seeds(e.g. 50%, 80 %,100% (Akinyele *et al.*, 1986; Berrios *et al.*, 1999; Mwangwela *et al.*, 2006) and pre-determination procedures such as soaking.

4.1.8 Pectin

Pectin determination was based on quantifying the amount of galacturonic acid present. This is because, pectins are polysaccharides composed mainly of polymers of D-galacturonic acid (80-90%) whose monomers are joined by α-1, 4-glycosidic bonds (Vazquez-Blanco, Vazquez-Oderiz, Lopez-Hernandez, Simal-Lozano & Romero-Rodriguez, 1993). The initial step in the extraction of pectin involved preparation of an alcohol insoluble residue (AIR). This procedure separated polymeric materials from alcohol soluble solids such as any monomeric galcturonic acid or other soluble small molecules that would later interfere with the galacturonic acid analysis after pectin hydrolysis (Qi, Moore & Orchard, 2000). The AIR was then extracted with cold water, hot water and EDTA to yield cold water soluble pectin, hot water soluble pectin and the EDTA soluble pectin fractions respectively. The pectin fractions were hydrolysed in hot concentrated H_2SO_4 yielding galacturonic acid residues. The strong acidic medium ensured that the glycosidic linkages were cleaved. The resulting galacturonic acid residues were quantified colorimetrically with m-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973)

4.1.9 Microscopy

Softening during cooking of legumes is associated with separation of parenchyma cells at the middle lamella. Microstructure analysis was done to show whether there was separation of parenchyma cells at the middle lamella during cooking. In identifying the cell wall of individual cells, Calcofluor-white was used. Calcofluor-white is a fluorescent stain that selectively binds to

cellulose and fluoresces when exposed to long-wavelength ultraviolet (Knox, 2008). Therefore, in using this stain the cell wall could be distinguished for the cell contents. In this study, staining with Calcoflour was successful as the cell wall was virtually the only visible fluorescent component when viewed using Confocal Laser Scanning Microscopy (CLSM) at an excitation wavelength of 405 nm. Different levels of cell wall separation in adjacent parenchyma cells were observed.

4.2 The effect of high temperature and high relative humidity storage on HTC defect development in cowpeas

Storage of three cowpea types under accelerated conditions (42^{o} C, 80% RH) of HTHH conditions led to development of HTC defect in the cowpeas. The susceptibility of cowpeas to HTC defect was demonstrated by increased cooking time after HTHH storage. Although the 3 cowpea types were stored under similar conditions the degree of HTC development differed among the types. *Agripienk* cowpea type had more than 5-fold increase in cooking time while *Bechuana white* had a 1.5 -fold increase. This is in agreement with several legume studies which report differences in proneness to HTC defect development among different types of legumes of the same species (Reyes-Moreno & Paredes-Lopez, 1993; Bernal-Lugo *et al.*, 1997; Coelho *et al.*, 2007). However, storage at refrigerated conditions (4 ^{o}C) did not result in HTC defect development therefore demonstrating that changes during the HTHH conditions promote HTC defect development. (Knox, 2008)

The two main theories to explain the HTC phenomenon, “phytase-mineral and mineral-pectin interaction” and lignification, implicate activation/increased enzyme activity due to HTHH conditions. In studying the effect of accelerated HTHH storage conditions on the cowpeas, investigation of the physicochemical properties of the cowpeas associated with these two main theories was carried out.

Lignin content did not change significantly in both refrigeration and HTHH storage in all the cowpea types. Peroxidase, the main enzyme reported to promote lignification (Rivera *et al.*, 1989) also did not change significantly during HTHH storage. Therefore, the role of lignification of the cell wall was not established in this study. There are studies that suggest over short periods

of HTHH storage such as used in this study (40 days), HTC development is more likely to be due to phytase activation than lignification. The studies suggest that the first stage of HTC is due to "phytase-mineral and mineral-pectin interaction" and the later one due to lignification (Hincks & Stanley, 1987; Mafuleka *et al.*, 1993; Martín-Cabrejas *et al.*, 1997). Martín-Cabrejas *et al.* (1997) observed that with extended (5 years) HTHH storage of beans, changes in phytate did not correlate with the extent of increased hardening. Mafuleka *et al.* (1993) observed a significant correlation (r = 0.821) between lignin content and hardness after 8 m storage at HTHH. However lignin was not significantly correlated with hardness at 4 months (r = 0.232) at the same storage conditions. Lignification was observed to take place after long duration of storage while the changes in phytate in the early stages of hardening suggested "phytase-mineral and mineral-pectin interaction". In support of the view that HTC defect occurs in different stages. Del Valle & Stanley (1995) and Aguilera & Rivera (1992b) observed that a degree of hardness in beans stored under HTHH conditions was reversed by soaking the hardened beans in the chelating agent, EDTA. They termed the hardness reduced by EDTA as "reversible hardening" and the one that remained as "irreversible hardening". The reversal by EDTA was due to the removal of divalent cations from HTC beans therefore counteracting the hardening effect due to the "phytase-mineral and mineral-pectin interaction" (Del Valle & Stanley, 1995). They reported that "reversible hardness" was associated with the intial or earlier days of storage and was due to the "phytase-mineral and mineral-pectin interaction" (Aguilera & Rivera, 1992a; Del Valle & Stanley, 1995). The irreversible hardening which occurred later during HTHH storage was associated with the lignification mechanism (Del Valle & Stanley, 1995).

In all three cowpea types, HTC defect development (long cooking time) was associated with changes in physico-chemical characteristics that are related to the "phytase-mineral and mineral-pectin interaction". Storage at HTHH conditions led to increased phosphate liberation (indicating increased phytase activity), decreased phytate content and decreased soluble pectin in all three cowpea types (Table 4.1). These observations were in agreement with the mechanisms proposed in putting forward the "phytase-mineral and mineral-pectin interaction" theory. In contrast, storage under refrigeration conditions did not result in any significant changes in phytase activity, phytate content and soluble pectin content (Table 4.1).

Table 4.1 Summary of changes in physicochemical properties of cowpea samples following storage at different conditions in comparison to control

Physicochemical property		Percentage change (%) in comparison to cowpeas at day 0 (Control)		
		Bechuana White	Agripienk	Mae–e–tsiliwane
Cooking time	Day 40 at 4° C	↔	↔	↔
	Day 40 at HTHH	51 ↑	>382 ↑	>131↑
Released phosphate	Day 40 at 4° C	↔	14	↔
	Day 40 at HTHH	86 ↑	139↑	124↑
Phytate	Day 40 at 4° C	↔	↔	↔
	Day 40 at HTHH	13↓	23 ↓	29↓
Water soluble pectin	Day 40 at 4° C	↔	↔	↔
	Day 40 at HTHH	9↓	18↓	17 ↓
Peroxidase	Day 40 at 4° C		34↓	
	Day 40 at HTHH	↔	↔	↔
Lignin	Day 40 at 4° C	↔	↔	↔
	Day 40 at HTHH	↔	↔	↔
Moisture	Day 40 at 4° C	↔	11	↔
	Day 40 at HTHH	29 ↑	13↑	23 ↑
Water activity	Day 40 at 4° C	↔	↔	8↓
	Day 40 at HTHH	33 ↑	17↑	↔
pH	Day 40 at 4° C	1↑	↔	↔
	Day 40 at HTHH	3↓	6 ↓	6↓

↔ = No significant change, ↓ = significant decrease and ↑ = significant increase

It is proposed that during HTHH storage, phytase was activated leading to enzymatic hydrolysis of phytate that resulted in liberation of divalent cations. The released cations were subsequently crosslinked with pectin in the middle lamella. This led to formation of insoluble pectates that hindered cell separation during cooking. Increased cooking time was therefore due to reduced rate of cell separation. The soft texture associated with cooked legumes is dependent on the ease or rate of cell separation. The proposed mechanisms in HTC defect development were based on the "phytase-mineral and mineral-pectin interaction" as illustrated in Figure 4.1.

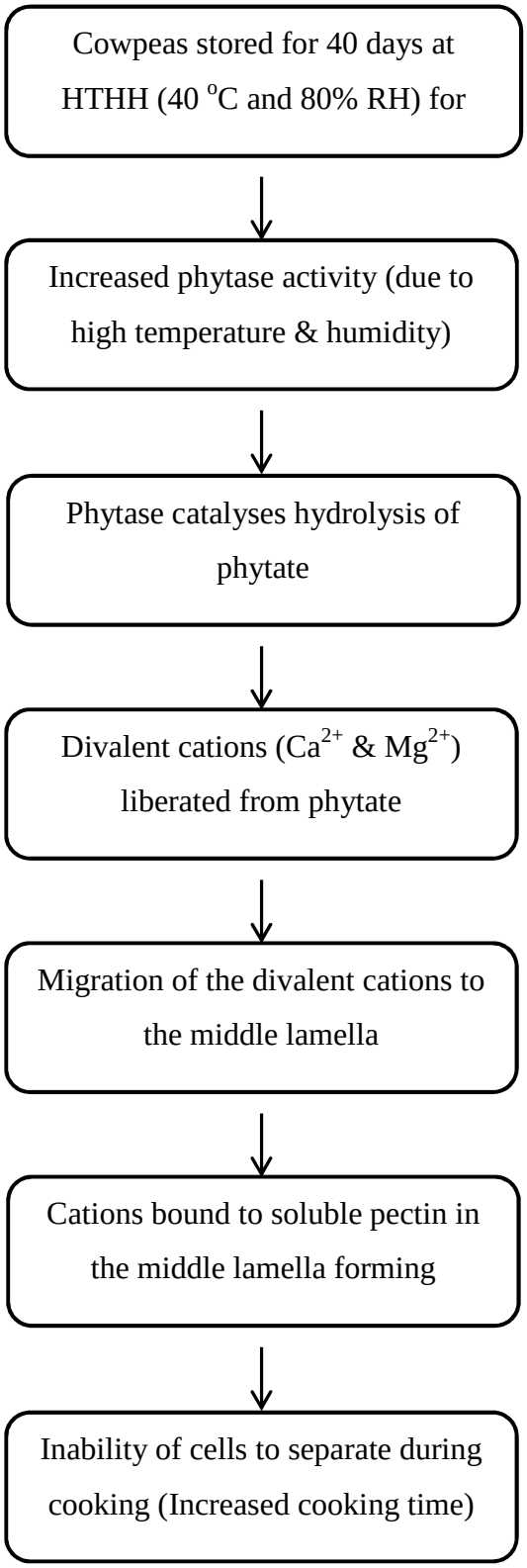


Figure 4.1 The proposed mechanism in HTC defect development in the cowpeas

4.3 The effects of micronisation and hot-air roasting of cowpeas as pre-treatments in the control of hard-to-cook phenomenon during HTHH storage

HTC defect development in the HTHH storage conditions used in this study was shown to be contributed by increased phytase activity. The heat pre-treatments were expected to prevent HTC defect development by inactivating phytase. The partial inactivation of phytase achieved in this study was due to the hydrothermal energy generated when pre-conditioned cowpeas were exposed to micronisation and roasting.

During enzyme inactivation, the heating parameters (temperature and time) and the intrinsic characteristics of the material such as moisture content are the critical factors in inactivating enzymes. The temperature, time and moisture conditions used in this study were 150 ^{o}C, 5 min and 25% respectively. These conditions were more slightly severe than those used by Affrifah *et al.* (2006) who reduced phytase activity to 64% by steaming cowpeas (20% moisture) at 121°C for 4-6 minutes. In the current study, phytase activity was reduced to 70% and 45% by micronisation and roasting, respectively. Initial moisture content and heating temperature were shown to be the significant factors in phytase inactivation. At high temperature (> 90^{o}C), heating time was observed to be significant up to a point after which increased exposure time did not seem to make a difference on the degree of inactivation. This "cut-off" or leveling off occurred after about 4 min of exposure. Therefore, in selecting a slightly higher temperature of 150 ^{o}C a higher degree of inactivation was achieved in the micronized cowpeas. However, complete inactivation of phytase was not achieved suggesting a high thermal stability as shown by Affrifah & Chinnan (2005). Although increasing the "temperature-time–moisture" conditons might increase the level of enzyme inactivation`, undesirable changes in the physico-chemical characteristic of the seeds may occur. Temperatures of above 160 ^{o}C are associated with burning (Mwangwela *et al.*, 2006) and increased seed hardness (Arntfield *et al.*, 2001). Micronisation of cowpeas at higher moisture contents (40%) was shown to result in fissuring (Mwangwela *et al.*, 2006) due to intense vapour pressure build up within the seed. In the current study only one "temperature- time – moisture" combination was used (150 ^{o}C - 5 min - 25%). It would be useful in future to study the impact of varying the heating parameters on phytase inactivation and eventual impact on HTC defect development.

Heat pre-treatments reduced the incidence of HTC defect development during HTHH storage as demonstrated by the shorter cooking time when compared to the control. Cooking time was least increased in heat pre-treated cowpeas during HTHH storage. Therefore, heat pre-treatment controlled HTC defect to a degree but did not completely prevent the defect from developing. Heating reduced phytase activity partially as seen in the significant reduction in released P*i* in heat pre-treated seeds.

Heat pre-treatment resulted in decreased phytase activity, decreased phytate hydrolysis, higher HWSP content and lower EDTA soluble pectin in the cowpeas when compared to the control samples after HTHH storage. The activation of phytase during HTHH initiates the HTC defect development as suggested in the "phytase-mineral and mineral-pectin interaction" theory. Therefore, controlling the defect might be achieved by inactivating phytase as demonstrated in this study. The observed lack of cell separation during the cooking of the cowpea further strengthens the hypothesis that HTC results from changes in the middle lamella due to insolubilisation of pectin that prevents cell separation during cooking.

The effect of heat pre-treatment in preventing HTC was dependent on the extent of phytase inactivation, which was in agreement with the observation that the "phytase-mineral and mineral-pectin interaction" led to HTC defect development during HTHH storage. When compared to roasting, micronisation was more effective in controlling the development of the HTC defect. This was observed to be due to a higher degree of phytase inactivation in micronised cowpeas when compared to hot-air roasted cowpeas.

The mechanistic model proposed in explaining the reduced cooking time in heat pre-treated cowpeas during HTHH storage is shown in (Figure 4.2). This study demonstrated that by partially inactivating phytase, phytate is less hydrolysed therefore achieving some degree of control on HTC defect defect. Micronisation, a technique not previously used in the prevention of HTC was more effective when compared to hot-air roasting.

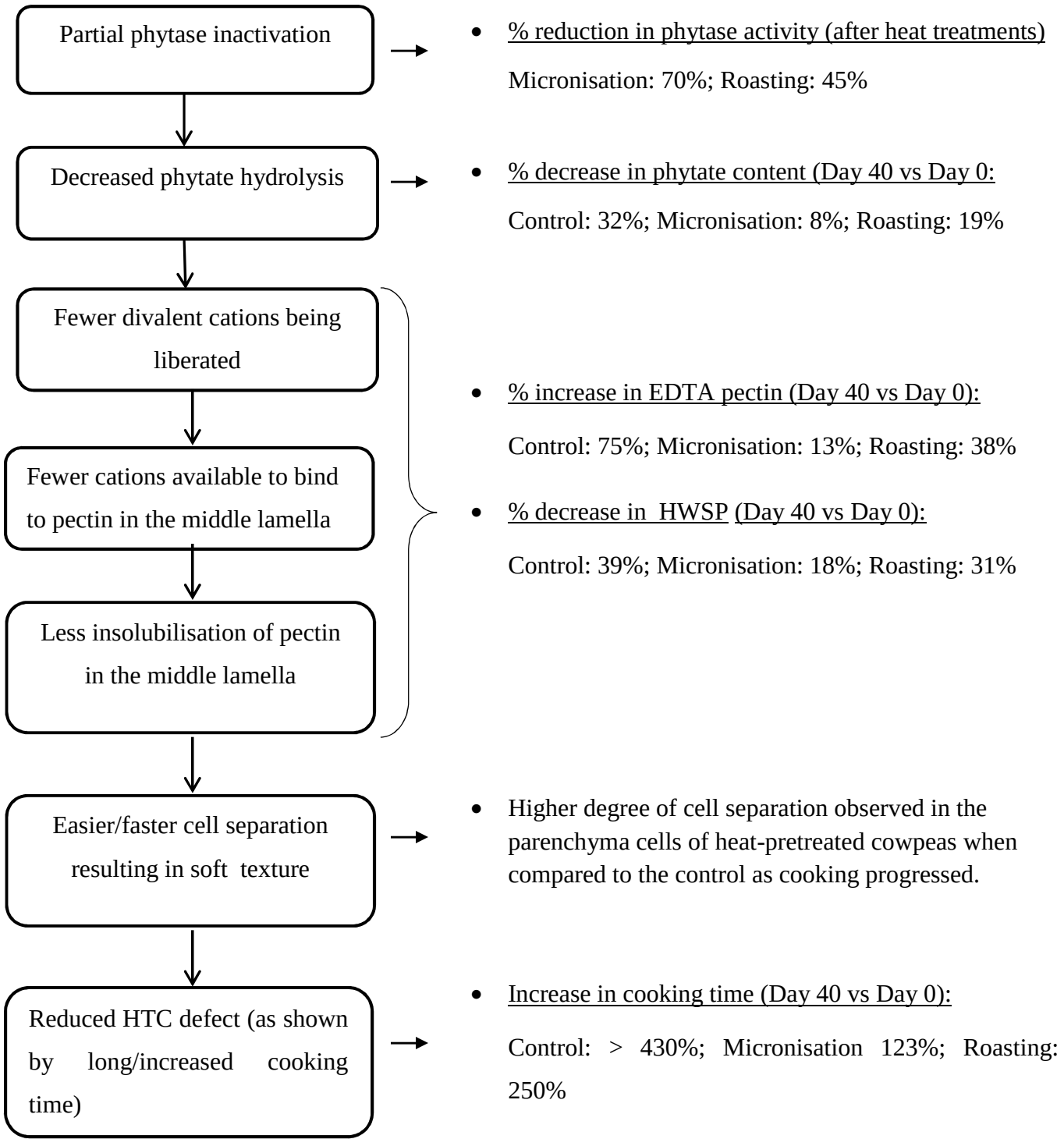


Figure 4.2 Mechanistic model proposed to explain the prevention of HTC defect development by micronisation and hot-air roasting

Micronisation was more effective in controlling HTC defect than hot-air roasting after HTHH storage. The effectiveness of the heat pre-treatments was shown to be dependent on the level of phytase inactivation. Micronisation achieved more inactivation of phytase when compared to hot-air roasting under similar heating conditions (150 ^{o}C, 5 min, cowpeas at 25% moisture). The effectiveness of micronisation over hot-air roasting was due to superior heating properties. During micronisation, the radiated IR waves are absorbed during penetration, causing molecular vibrations that generate intensive heat inside the seed (Sakai & Hanzawa, 1994). IR heating takes place at the surface and inside the inner layers of the cowpea simultaneously (Sakai & Hanzawa, 1994; Krishnamurthy *et al.*, 2008) thereby reducing the temperature gradient within a short period. In contrast, hot air roasting involves convective heating of the cowpea surface by hot air and subsequent conduction of heat from the surface to the inner layers. The high heating intensity, faster and more uniform heating within the cowpea during micronisation resulted in more phytase inactivation and subsequent reduction in phytate hydrolysis when compared to hot-air roasting during HTHH storage. Therefore, micronised cowpeas had higher content of soluble pectin that ensured easier cell separation at the middle lamella during cooking.

5. CONCLUSIONS AND RECOMMENDATIONS

Storage of cowpeas at high temperature and high humidity (HTHH) conditions lead to development of hard-to cook-defect (HTC) as shown by the increased cooking time. Accelerated storage conditions (40 ^{o}C and 80% RH for 40 days) that simulate tropical conditions of HTHH result in increased cooking time of legumes by 51 - 382% after storage. Based on the results, the increased cooking time is attributed to increased phytase activity at the HTHH conditions. Increased phytase activity is highly correlated with increased cooking time, decrease in phytate content and decrease in pectin solubility. These corroborate the proposition that the "phytase-phytate-pectin" pathway is the main mechanism controlling the HTC defect development. Lignin content and peroxidase activity did not increase during HTHH storage. According to the lignification theory, increased peroxidase activity during HTHH storage leads to increase in lignin content in the cell wall. Increased lignin content limits water penetration and restricts cell separation thereby promoting HTC. Therefore, the probable cause of HTC defect in the cowpeas is as a result of increase phytase activity and not due to lignification. Cowpeas stored under refrigeration conditions (4 ^{o}C) do not develop the defect as they had similar cooking time with their respective day 0 samples. Phytase activity did not increase and phytate content was similar to that of day 0. Therefore, refrigeration conditions do not lead to HTC defect as the low temperature conditions fail to promote increased enzyme activity.

HTC defect development can be prevented by storage of cowpeas at low temperature. However, this is not an affordable option to developing countries where legumes are a major dietary source. Therefore one of the key strategies in prevention of HTC defect lies in employing strategies that stop the deteriorative reactions before storage at HTHH conditions. Micronisation and hot-air roasting were used as heat pre-treatments with the aim of inactivating phytase before HTHH storage of cowpeas. The heating temperature, time (150 ^{o}C, 5 min) and cowpea pre-conditioning moisture content (25%) was similar during both heat pre-treatments. Phytase inactivation was only achieved partially in both treatments. Micronisation reduced phytase activity by 70% while hot-air roasting reduced the activity by 45%. Phytase has been described as a quite heat stable enzyme and currently no study has shown its complete

inactivation in legumes. This study shows that the effectiveness of the heat pretreatments in preventing HTC is dependent on the degree of phytase inactivation. Micronised cowpeas had the lowest phytase activity and the lowest increase in cooking time after HTHH storage. Cowpeas that were not heat-pretreated had the highest increase in cooking time indicating that phytase inactivation is critical in prevention of HTC. According to this study the mechanism of HTC prevention by heat pre-treatments is based on partial phytase inactivation that leads to less hydrolysis of phytate. Therefore, there are less divalent cations released from phytate to crosslink with pectin in the middle lamella. Therefore, there is less formation of water insoluble pectates that allows for easier cell separation to take place at a faster rate during cooking as shown microstructurally. Micronisation is more effective in reducing the incidence of HTC defect development since it achieves more phytase inactivation when compared to hot-air roasting. The effectiveness of micronisation when compared to hot-air roasting is due to its superior heating properties such as high heating intensity and penetration of the waves causing heating inside the cowpeas.

Microstructural study focused on the parenchyma cell separation at the middle lamella during cooking based on the "phytase-phytate-pectin" theory. However, there is need to also investigate intracellular changes such as the swelling and gelatinisation of starch granules that simultaneously take place with separation of the parenchyma cells in the cotyledon.

This study shows that HTC defect can be controlled by inactivation of responsible enzymes such as phytase. The heating parameters (150 ^{o}C, 5 min) of moisture-conditioned (25%) cowpeas do not lead to complete inactivation of phytase. It is recommended that a study on the optimisation of the parameters would need to be conducted to establish the most effective combination in achieving maximum phytase inactivation or complete inactivation. The success of the heat pre-treatments is finally determined by consumer acceptance of the cowpeas after HTHH storage. Therefore, the determination of the sensory quality of heat pre-treated HTHH stored cowpeas is recommended.

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