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1. INTRODUCTION AND PROBLEM STATEMENT

In Africa, sorghum grain and cassava tuber have mostly been utilised in traditional food and beverage products (House, Osmanzai, Gomez, Monyo and Gupta, 1995; Edijala, Okoh and Anigoro, 1999). Between 2001 and 2011, per capita consumption of sorghum food products in Africa decreased slightly from 18 to 17 (kg/capita/year) compared to other cereal crops such as wheat, maize and rice (FAO, 2014). This reduction in consumption of sorghum food products is attributable to shift in consumer preference towards better tasting and ready-to-eat products such as breakfast cereals and lager and stout beer, as a result of rapid urbanisation and growth in the availability of convenience foods from cereals such as wheat and rice (reviewed by Galati, Oguntoyinbo, Moschetti, Crescimanno and Settanni, 2014). This trend is a direct effect of increasing incomes and changes in life-style of people in sub-Saharan African countries (Taylor, 2004). Therefore, diversification in the use of sorghum and cassava through commercial processing into value-added food and beverage products is necessary, as this can serve as suitable means of developing the economies of African countries.

Taylor, Schober and Bean (2006) reviewed novel food and beverage applications of sorghum such as baked goods, lager and stout beer. These authors noted that potential areas of sorghum application depend on the unique structural and chemical compositional attributes of the different sorghum types. For example, tannin-sorghum has agronomic benefits, but its application in processing of food and beverage products may be limited. This is linked to negative effects of condensed tannins on nutritional and functional properties, as a result of interactions with other grain components (Daiber, 1975; Nyachoti, Atkinson and Leeson, 1997). Problems in utilisation of tannin-sorghums have been addressed by pre-processing steps such as chemical treatment (Beta, Rooney, Marovatsanga and Taylor, 2000). However, safe and effective pre-treatment is influenced by sorghum cultivar and season (Nelles and Taylor, 2002) due to grain property differences. Therefore, there is need to investigate suitable methodology that will be effective for treating tannin-sorghum types, for application in brewing/bioethanol and beverage processing.

As reviewed by Pandey, Soccol, Nigam, Soccol, Vandenberghe and Mohan (2000), the proportion of cassava root tuber utilised in industrial processing applications is about 7% of its world production. A potential area for improving commercial utilisation of cassava is in the brewing industry. This is due to the high starch content of cassava tubers, which constitutes about 80% of the total solid material in the tuber (Charles, Sriroth and Huang, 2005). In cassava tubers, the starch granules are located within the parenchyma cells (Sriroth, Piyachchomkwan, Wanlapatit and Oates, 2000). Such cell wall materials (CWM) like cellulosic and hemicellulosic polymers are characterised by having high water holding capacity (Whistler and Daniel, 1985). This functional property of cassava CWM may constitute problem during the brewing filtration. Bioconversion techniques such as enzymatic hydrolysis and solid-state fermentation have been applied in the treatments of cassava fibrous material residues (Pandey *et al*., 2000). Therefore, development of methodology for pre-treating cassava cake in order to address the problem of poor filtration in cassava brewing is necessary.

The main aim of this study is to improve commercial utilisation of sorghum and cassava in sub-Saharan Africa through increased value addition, with specific interest in their brewing and beverage end-use, which will facilitate effective economic development of small-holder farmers.

2. LITERATURE REVIEW

This review concerns the science and technology to improve cassava and sorghum beverage processing. This involved understanding the physico-chemical properties of the cassava root tuber. Studies on improving the processing of cassava root tuber in relation to its cell wall properties are discussed in detail. It also focuses on the properties of sorghum grain types in relation to their polyphenolic compounds. Studies conducted on tannin inactivation in tannin-containing sorghums in order to reduce the negative impact of tannins on both functional and product properties in foods and beverages are reviewed.

2.1 Cassava

Cassava (*Manihot esculenta* Crantz L.) originated from South America in Brazil, a member of the *Euphorbiaceae* family, is grown mainly for its large starchy storage roots (Blagbrough, Bayoumi, Rowan and Beeching, 2010). Cassava is one of the most important food crops for millions of people in tropical and sub-tropical Africa, Asia and Latin America as a source of carbohydrate (El-Sharkawy, 2004). As reviewed by Pandey *et al*. (2000), 60% of cassava world production is used for human consumption, mostly in the form of flour, or in fermented forms such as garri (dried cassava flakes) and fufu (thick porridge made from cassava flour). Thirty three percent of cassava produced is used for animal feed and the remaining 7% in industries such as textile, paper, and some foods and fermentation. Due to the high content of starch in cassava, a potential area for improving industrial utilisation of the cassava root tuber is in beverage processing applications such as lager beer brewing (SABMiller, 2011).

Cassava roots are highly perishable with short-shelf life of one to two days after harvest, due to rapid initiation of post-harvest physiological deterioration (Ceballos, Iglesias, Perez and Dixon, 2004). Cassava root varieties are grouped into bitter and sweet varieties based on the root cyanogenic glucosides content (Oluwole, Onabolu, Mtunda and Mlingi, 2007). According to Ceballos *et al*. (2004), cyanogenic glucosides content of cassava roots ranges between 4-113 mg HCN per kg (fresh weight basis). Cyanogenic glucosides levels of the root constitute a major health concern, with exposure to cyanide been associated with health problems such as cancer (Obiri, Dodoo, Okai-Sam, Essumang and Adjorlodo-Gasokpoh, 2006), iodine

deficiency (Gbadebo and Oyesanya, 2005) and neurological syndromes (Bonmarin, Nunga and Perea, 2002). One of the objectives of cassava breeding programmes is to reduce the root cyanogenic glucosides content (Dixon, Asiedu and Bokanga, 1994) for improving cassava roots food and beverage processing applications. However, reduction of the root HCN levels below 10-20 ppm is very challenging, while cassava roots with this level of HCN are termed sweet cassava variety (Jennings and Iglesias, 2002).

The cassava root tuber is long, tapered and cylindrical, varying widely in size and shape due to influence of cultivar and environmental conditions (Wheatley and Chuzel, 1993). It has a firm homogenous flesh. Varieties vary between 3-15 cm in diameter, 15-100 cm in length and 0.17-2.35 kg in weight per tuber (Alves, 2002). The cassava root cell wall structure as described by Buschmann, Potter and Beeching (2002) is shown in Figure 2.1. Cassava xylem tissue and schlerenchymous fibres contain lignin, a hard material of cross-linked phenylpropane units (Buschmann *et al*., 2002). The tuber outer layer consists of a periderm, unlike the epidermis as in other stem and root plant crops. Components of the outer cork layer of the periderm contribute to thickening of the cell walls, which provide protection for the plant due to formation of cross-linked pigments. These include suberin, a waxy substance unique to cork tissue, as well as tannins and complex aromatic compounds of glucosides (Buschmann *et al*., 2002). The starch in cassava is referred to as storage starch because it is stored in the form of osmotically, water insoluble granules in amyloplasts (Munyikwa, Langeveld, Salehuzzaman, Jacobsen and Visser, 1997). As shown in Figure 2.2, these starch granules are located within the cassava tuber parenchyma cell walls.

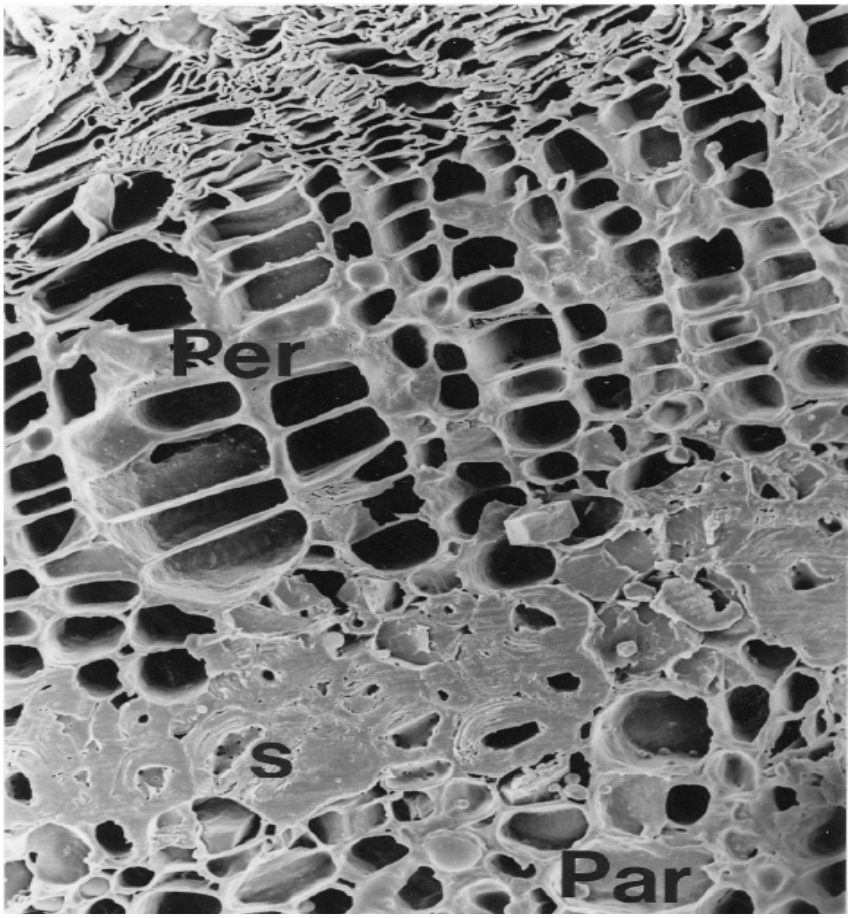


Figure 2.1: Low temperature SEM image of a cross-section through frozen-hydrated Cassava root tissue (**Par**: starch containing outer parenchyma, **S:** Sclerenchyma, **Per:** Periderm) (Buschmann *et al*., 2002).

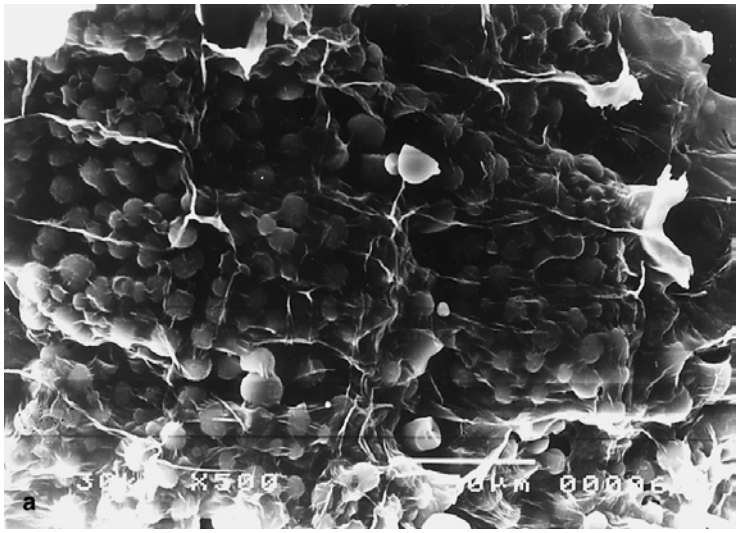


Figure 2.2: Scanning electron micrographs cassava starch granules as located within the parenchyma cell walls (Source: Sriroth, Chollakup, Chotineeranat, Piyachomkwan and Oates, 2000)

2.1.1 Physicochemical properties of the cassava root

Generally, root and tuber crops contain 70-80% water as a percentage of total weight (Hoover, 2001). Table 2.1 shows the cassava root proximate composition. It is characterised by trace levels of protein, lipids and ash (Blaghrough *et al*., 2010). The starch accounts for about 35% of the total solid materials, while the crude fibre is about 1% (Charles, Chang, Ko, Sriroth and Huang, 2004). According to Salvador, Suganuma, Kitahara, Tanoue and Ichiki (2000), cassava cell wall material is majorly composed of non-starch polysaccharides and lignin. The main classes of polysaccharides in cassava cell walls are cellulose, hemicellulose and pectin. These non-starch polysaccharides are formed by condensation of wide range of monosaccharide units into chains of glucans, xylans and arabinans. Like in most cell walls, cellulose is the principal structural component. According to findings of Salvador *et al*. (2000), cellulose constitutes about 48% of cassava cell wall material, followed by hemicellulose (22%) and pectin (18%). The remaining 12% may possibly constitute the lignified component of the cell wall materials.

Like in other plants, cassava root cell walls are composed of a matrix of polysaccharide units of glucans, xylans and arabinans (Western, Skinner and Haughn, 2000). The cassava root tuber also contains mucopolysaccharides, known as cassava tuber mucilage (CTM), which are characterised as purely hemicellulosic, mainly arabinogalactans (Charles, Huang and Chang, 2008). They are water soluble polysaccharides made up of more than 30,000 sugar units. CTM contains sugars with a (1-3)-linked β-glucan backbone, with side chain branching with non-reducing terminal residues mostly of (1-6)-linked galactose and some arabinose and glucosyl moieties (Charles *et al*., 2008). These properties of CTM will also impact on the cassava cake viscosity. As reviewed by Distelbarth and Kull (1985), CTM serve as reserve substances or provide resistance to the tuber drying out, as they contribute to moisture balance. According to Charles *et al*. (2008), biosynthesis of CTM increases with maturation of the cassava root, and this tends to reduce the tuber starch content.

Cellulose is homoglucan made up of linear chains of 1-4-β-*D*-glucopyranosyl units (Marchessault and Sundararajan, 1983). Cellulosic materials are insoluble in water and they have capacity to bind up to 0.4 g water per g of fibre (Whistler and Daniel, 1985). Hemicelluloses consist of both soluble and insoluble polymers of hexoses, glucuronic acids, pentoses and some deoxyl sugars (Saha, 2003) and also have the

capacity to bind water. The predominant hemicelluloses have a xylan backbone which consists of 1-4-β-*D*-xylopyranosyl units (Whistler and Daniel, 1985). Hemicelluloses also have branched chains made up of β-*L*-arabinofuranosyl side chains attached to the third position of some of the *D*-xylanosyl units. Lignin is made up of complex aromatic polymers based on phenylpropane units, and is not susceptible to hydrolytic enzymes (Marsden and Gray, 1986). With lignin not easily hydrolysed, high level of lignification of the cell walls may impact negatively by limiting the level of starch hydrolysis with the starch granules trapped within the parenchyma cells.

Table 2.1: Cassava tuber proximate composition

Proximate composition	**(g/100 g)**
Starch	34.2-35.1
Protein	0.8-1.1
Lipid	0.06-0.12
Crude fibre	0.6-0.8
Ash	0.7-0.9
Moisture	57.6-59.7

Source: Charles *et al*. (2004).

2.1.2 Developments in processing of cassava to improve starch yield

As stated, the major industrial application of cassava root tuber is for its starch (Teixeira, Pasquini, Curvelo, Corradini, Belgacem and Dufresne, 2009). However, this processing application for cassava has a major drawback, as a result of the significant quantity of starch which is not recovered (Dzogbefia, Ofosu and Oldman, 2008). This is because of inadequate destruction of the cell wall structure to release the starch granules, which are trapped within the cells (Kordylas, 1990). This problem results in a huge quantity of solid waste (Pandey *et al*., 2000), which accounts for between 10-15% by weight of the tuber (Sriroth *et al*., 2000). This solid waste, also known as cassava bagasse, consists of water, about 70-80% by weight, residual starch and cellulose fibres (Teixeira *et al*., 2009). The reason for the high water content of the waste is due to the high water-holding capacity of cellulosic component of the bagasse. The residual starch content of the solid waste ranges between 50 and 60% on a dry weight basis (Sriroth *et al*., 2000) and the cellulose fibre content of the bagasse ranges between 15 and 50%.

There is need to develop physical and biotechnological methods to improve starch extraction to reduce the amount of solid waste produced (Sriroth *et al*., 2000). Biotechnological approaches have been investigated involving the use of multi-

enzyme preparations to treat the cassava mash and the waste materials (Sriroth *et al*., 2000; Dzogbefia *et al*., 2008; Teixeira *et al*., 2009). Commercial hydrolytic enzymes with cellulolytic and hemicellulolytic activities are mostly used (Rahman and Rakshit, 2003). As reported by Sriroth *et al*. (2000), treatment of the cassava pulp involve multi-enzyme mixture of cellulases and pectinase. Dzogbefia *et al*. (2008) investigated a pectinase enzyme producing *Saccharomyces cerevisiae* culture to improve starch extractability from cassava. Enzymatic treatment of the cassava mash results in both fragmentation of the insoluble cell wall components and solubilisation of the soluble cell wall components with improvement in starch extraction (Demir, Acar, Sario and Mutlu, 2001; Rai, Maumdar, DasGupta and De, 2004). It was noted that there is need for studies to investigate right combination of suitable enzymes and reaction time because they are fundamental factors, which need to be considered in the pretreatment of the cassava mash in order to achieve an increase in the rate of starch extraction for maximum yield without any negative effect on the starch properties (Dzogbefia *et al*., 2008).

Another approach in the treatment of cassava cell wall materials is the application of steam explosion (Carta, Soccol, Ramos and Fontana, 1999). This treatment results in structural modification of the cell wall materials due to partial hydrolysis of the water soluble fraction, as well as chemical modification of the lignin (Ramos, 2003). These changes result from autohydrolysis and acid-catalysed breakdown of the glycosidic linkages of the cell wall materials (Figure 2.3). Nidetzky, Steiner, Hayn and Esterbauer (1993) reported that the steaming process led to the release of uronic acids and acetyl groups in the form of acetic acid from the acetylated hemicellulose. These acids play an important role in the efficiency of the treatment by removing the shielding effect of hemicellulose-lignin matrix (Ramos, 2003). It was proposed that pretreatment of the material by steam explosion treatment will effectively enhance susceptibility of the cellulosic component to enzymatic attack (Nidetzky *et al*., 1993). However, this steaming process may result in the release high concentration of these acids enabling more hydrolysis of the cell wall polymers yielding high amount of unassimilated sugars such as xylose, as well as the release of other materials such as furfural (Agu, Amadife, Ude, Onyia, Ogu, Okafor and Ezejiofor, 1997). These authors noted that high levels of these materials negatively affect metabolic activity of yeast during fermentation.

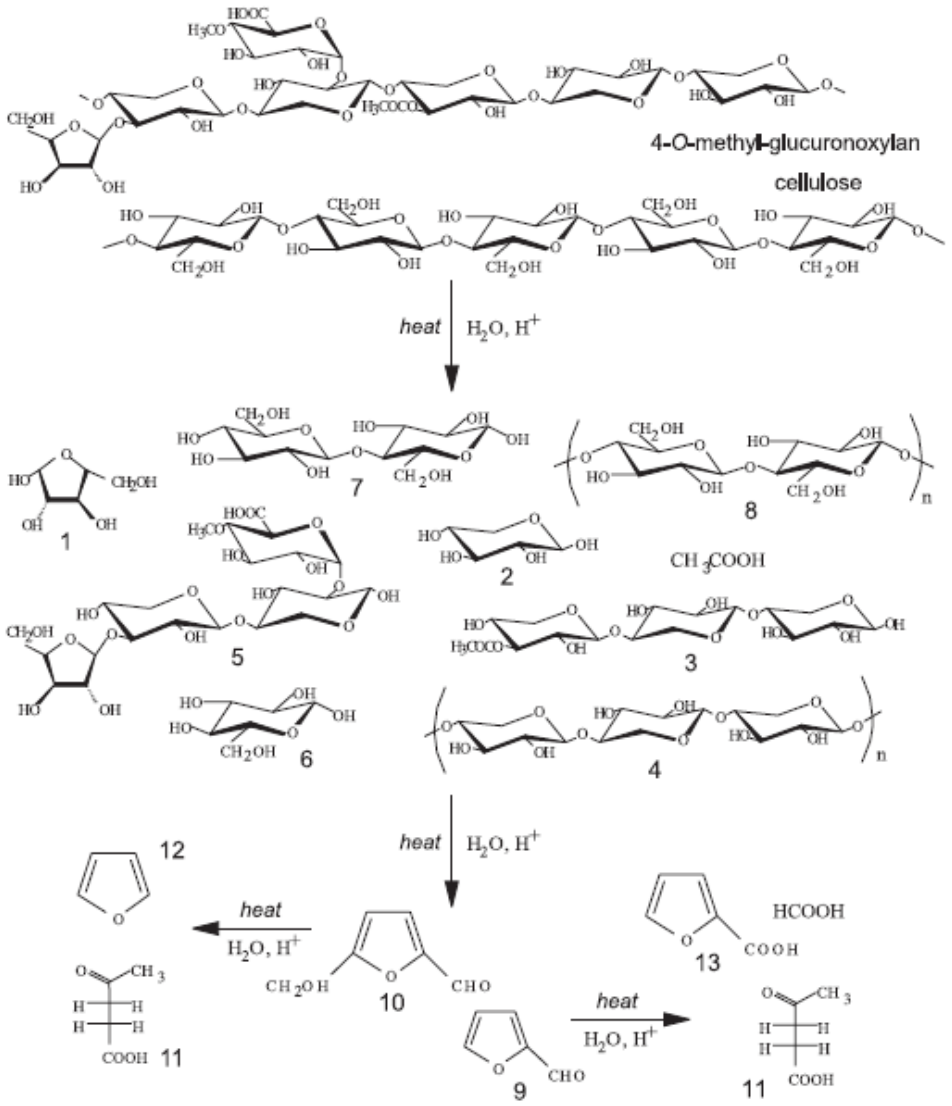


Figure 2.3: Hydrolysis of 4-O-methylglucuronoxylan and cellulose as a result of steam explosion on cell wall materials. **1.** Arabinose; **2.** Xylose; **3.** Acetylated xylo-oligomers; **4.** Xylo-oligomers of higher molecular mass; **5.** Acidic branched oligosaccharides; **6.** Glucose; **7.** Cellobiose; **8.** Cellooligomers; **9.** Furfural; **10.** Hydroxymethylfurfural; **11.** Levulini acid; **12.** Furan; **13.** 2-furoic acid (pyromucic acid). (Source: Ramos, 2003).

2.2 Sorghum

Sorghum (*Sorghum bicolor* L. Moench) originates from Africa and is widely grown in tropical, subtropical and arid regions of the world (Waniska and Rooney, 2000). The sorghum kernel consists of three main parts: the pericarp (bran), the endosperm (starch-rich storage tissue) and the germ (embryo) (Figure 2.4). The relative proportion of these parts varies based on the influence of genetic and environmental factors (Rooney and Miller, 1982). Sorghum varieties differ from another in terms of

pericarp colour and thickness, presence or absence of pigmented testa, endosperm texture and colour (Beta, Rooney, Marovatsanga and Taylor, 1999). Through extensive breeding programmes, a large number of improved grain quality sorghum types have been released (Obilana, 1998; Chisi, 2003). These improvements focused on the development of grain properties suitable for processing and end-use product quality for effective diversification of sorghum utilisation (Rohrbach and Obilana, 2003). The following sorghum grain types have been identified based on their phenolic grain quality attributes: white tan-plant and red non-tannin (Type I), white tannin (Type II) and red tannin (Type III) (Awika and Rooney, 2004). In the Type I, no significant amounts of tannins can be extracted with 1% acidified methanol, while the Type II has tannins that are extractable in 1% acidified methanol and not in methanol alone. The Type III tannins can be extracted in both acidified methanol and methanol alone. Grain quality characterisation of these sorghum types revealed significant differences in their physical and chemical properties (Adetunji, 2011).

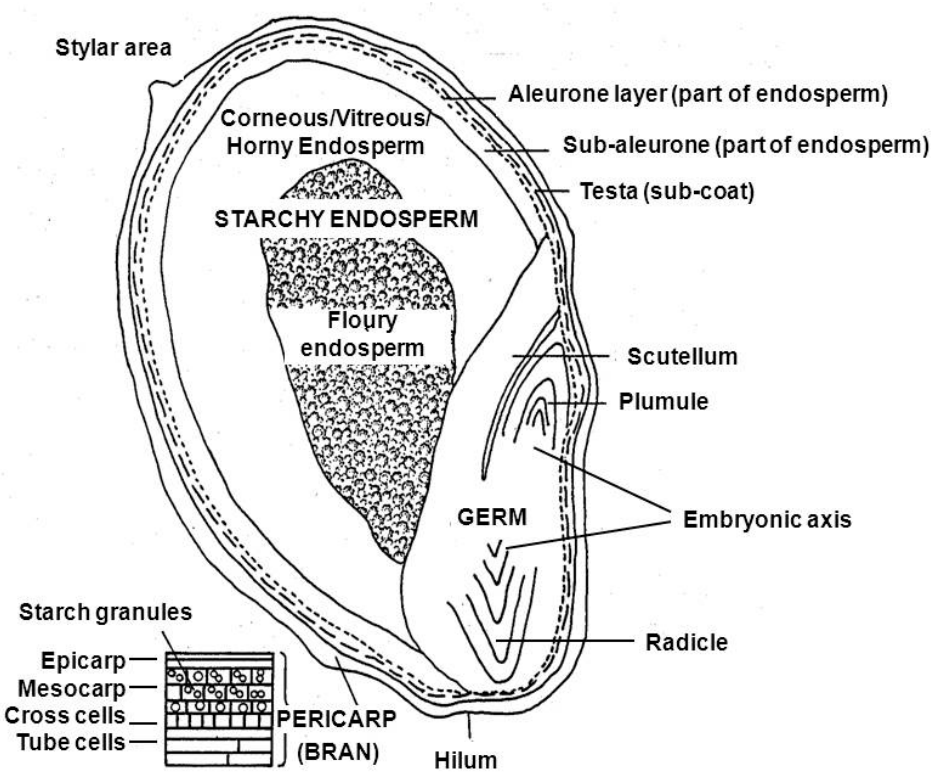


Figure 2.4: Schematic longitudinal section through a sorghum grain showing cereal kernel component parts. Source: Taylor and Emmambux (2008).

2.2.1 Physicochemical properties of sorghum grain

Importantly, unlike other major cereal grains, sorghum can be differentiated based on the presence of unique polyphenolic compounds, like condensed tannins. Tannin-containing sorghum grain types possess a prominent pigmented testa beneath the pericarp (Rooney and Miller, 1982), whereas non-tannin varieties do not (Figure 2.5). This unique kernel structure makes tannin sorghum grain types distinctive, as it contributes to tannin sorghum grain functionality (Elkin, Freed, Hamaker, Zhang and Parsons, 1996). The presence of tannins in sorghum grain has agronomic benefits by serving as a defence mechanism against bird predation, mould and possible insect attack (Waniska, Poe and Bandyopadhyay, 1989). However, the tannin property is an important factor to consider in the selection of sorghum types for food and industrial utilisation because of interaction between condensed tannins and other grain components such as starch and protein (reviewed by Taylor and Duodu, 2009). This interaction of tannins with other grain components leads to negative effects on the processing, functionality and nutritional properties of food and beverage products from tannin sorghums. For example, in the brewing mashing process, condensed tannins inactivate amylase enzymes required for starch hydrolysis in the production of fermentable sugars (Daiber, 1975).

The presence of tannins in the pigmented testa is controlled by the B_1 and B_2 genes that indicate tannin content but not pericarp colour and its intensity (Rooney, 2000). Sorghums with a pigmented testa have both dominant genes ($B_1\_B_2\_$), while the presence of tannins in the epicarp and endocarp is controlled by spreader genes *S*. The Type III tannin sorghums contain dominant $B_1\_B_2\_SS$ genes, while the Type II contains dominant ($B_1\_B_2\_$) and recessive *ss* genes. This may explain the difference in how tannins are deposited in the Type II and III tannin sorghums. Type II tannin sorghum grain has tannins deposited in the vesicles within the testa layer, while tannins are deposited along the cell walls of the testa and some in the pericarp in the Type III tannin sorghums (Earp, McDonough, Awika and Rooney, 2004). These differences in tannin deposition in the testa layer in these tannin sorghum types have been noted to influence their level of extractable tannins (Earp *et al*., 2004). As explained by Asquith, Izuno and Butler (1983), structural and chemical properties of condensed tannins are factors that influence tannin extraction. The Type II tannins contain acid-labile bonds such as a glycoside or an ester linking them to methanol-

insoluble components of the grain. In terms of structural properties, Type II condensed tannins were reported to have relatively higher degree of polymerisation than the Type III tannins (Bullard, York and Kilburn 1981). These factors coupled with the form of tannin deposition in the Type II sorghums explain the difficulty of their tannin extraction in comparison to Type III sorghums.

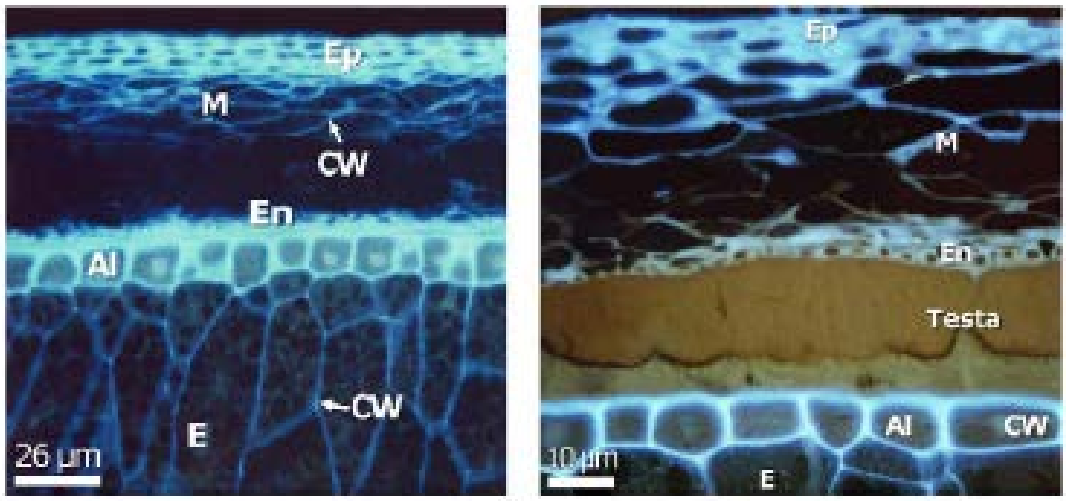


Figure 2.5: Fluorescence photomicrographs of cross-sections of a non-tannin (left) and a tannin sorghum kernel (right). Al- aleurone; CW- cell wall; E- endosperm; En- endocarp; Ep- epicarp; M- mesocarp; T- pigmented testa (Awika and Rooney (2004)).

2.2.2 Sorghum phenolic compounds and their chemical properties

Sorghum grain is characterised as containing several wide groups of phenolic compounds (Dykes and Rooney, 2006), which are mostly located in the pericarp. These phenolic compounds can be categorised into phenolic acids, flavonoids and condensed tannins. Table 2.2 shows the different types of flavonoids and condensed tannins that have been found in sorghum grains. However, their compositions and content in different sorghums differ widely due to influence of genetic and environmental factors (Dykes, Seitz, Rooney and Rooney, 2009). This review focuses on flavonoids and condensed tannins because they are likely to influence sorghum product functional and nutritional quality based on the complexity of their structural and chemical properties as compared to phenolic acids.

Table 2.2: Types of flavonoid and tannin phenolic compounds reported in sorghum (modified from Dykes & Rooney, 2006)

Phenolic group		**Phenolic compounds**
Flavonoids	***Anthocyanidins***	Apigeninidin
		Apegeninidin 5-glucoside
		Luteolinidin
		5-Methoxyluteolinidin
		7-Methoxyapigeninidin
		Luteolinidin 5-glucoside
	Flavan-4-ols	Apiforol
	Flavones	Apigenin
		Luteolin
	Flavanones	Eriodictyol
		Naringenin
	Flavonols	Kaempferol 3-rutinoside-7-glucuronide
	Dihydroflavonols	Taxifolin
Condensed tannins	***Proanthocyanidins (Polymers)***	Procyanidins
		Prodelphinidin
		Proapigeninidin
		Proluteolinidin

2.2.2.1 Flavonoids

Flavonoids are characterised by a benzo-*y*-pyrone structure (Figure 2.6a) (Yao, Jiang Shi, Tomas-Barberan, Datta, Singanusong and Chen, 2004). They are low molecular weight polyphenolics based on a flavan nucleus (Coultate, 1990), which consist of 15 carbon atoms having a C6-C3-C6 configuration. Flavonoids are derivatives of the 2-phenylchromone parent compound which comprise of A, B (phenolics) and C (pyran) rings. According to Cook and Samman (1996), flavonoid classification is based on their chemical structure and the major groups include flavanols (catechins), flavones, flavonones, anthocyanidins, isoflavones, dihydroflavonols and chalcones (Figure 2.6b). Variation in the substitution patterns of their C ring is the main factor of grouping these major flavonoids, based on the level of their hydroxylation and methylation (Hollman and Katan, 1999). Flavonoids are also present as glycosides (Cook and Samman, 1996). According to Yao *et al*. (2004), the chemical properties of these flavonoids in terms degree of hydroxylation, other substitutions and conjugations, as well as the degree of polymerisation are the major factors that determine their biochemical activities. As shown in Table 2.2, many different flavonoids have been identified and characterised in sorghum (Dykes and Rooney, 2006).

Sorghum anthocyanidins are called 3-deoxyanthocyanidins. This is because sorghum anthocyanidins lack a hydroxyl group in the carbon 3-position of their C-ring (Figure 2.7). This unique feature of sorghum anthocyanidins confers on them more stability at high acidic pH than the common anthocyanidins from other sources (Awika, Rooney and Waniska, 2004). Several classes of 3-deoxyanthocyanidin compounds have been identified in different varieties of sorghums (Wu and Prior, 2005). Studies by Dykes *et al*. (2009) and Dykes, Peterson, Rooney and Rooney (2011) identified four major 3-deoxyanthocyanidins in sorghum. These include luteolinidin (LUT), apigeninidin (AP), 5-methoxyluteolinidin (5-MeO-LUT) and 7-methoxyapigeninidin (7-MeO-AP). Sorghums with a red/purple plant colour (especially the black sorghum varieties) had much higher level of 3-deoxyanthocyanidins (32-680 µg/g) compared to the tan-plant sorghums (0-1 µg/g) (Dykes *et al*., 2009). Their findings further show that the 3-deoxyanthocyanidin profile in terms of composition varied widely in the sorghums studied. The red plant sorghums had a combination of apigeninidin (AP) and 7-MeO-AP as the predominant 3-deoxyanthocyanidin. The purple plant sorghums had higher levels of luteolinidin (LUT) and 5-MeO-LUT combined. It was suggested that secondary plant colour may possibly be used to determine sorghum 3-deoxyanthocyanidin compositions.

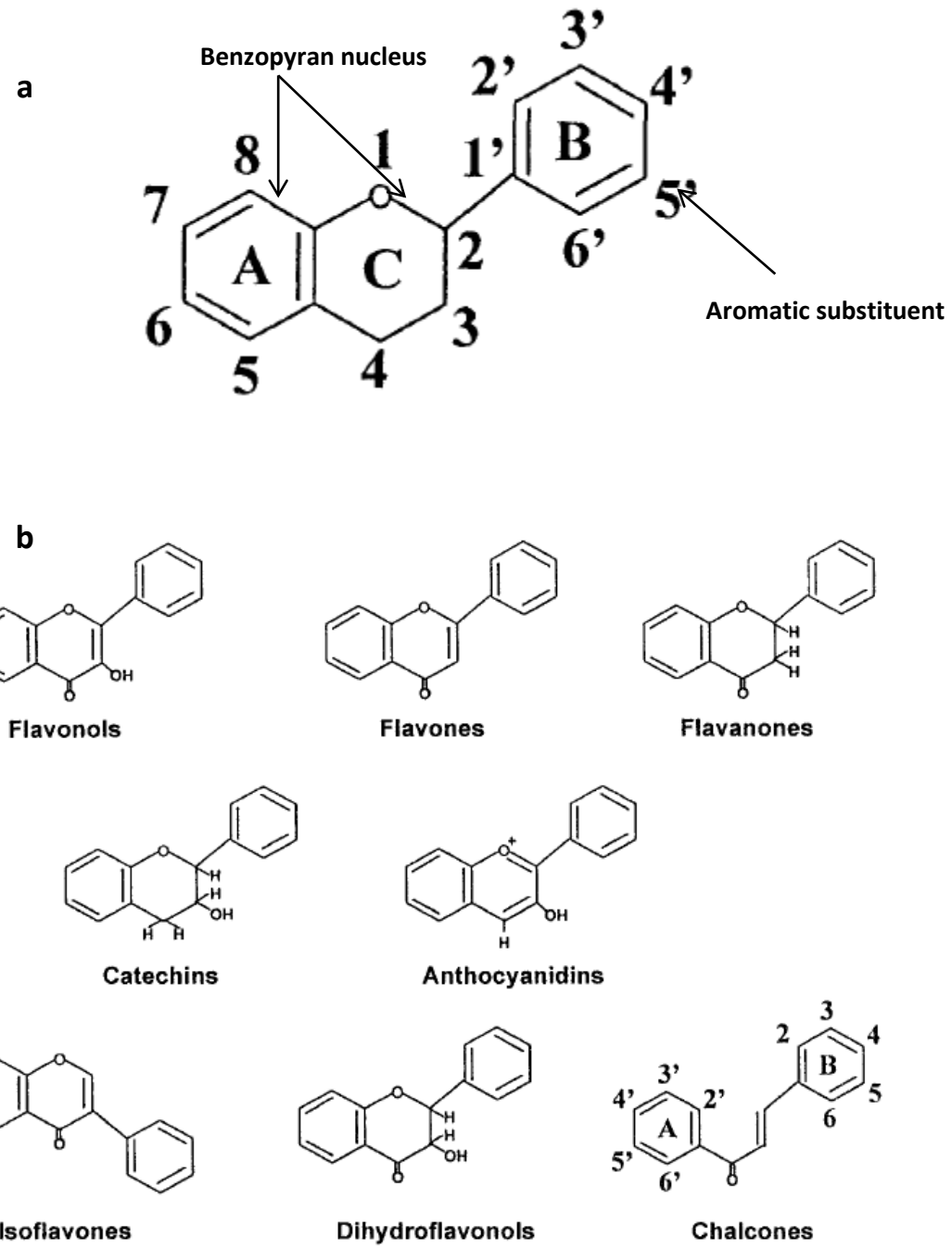
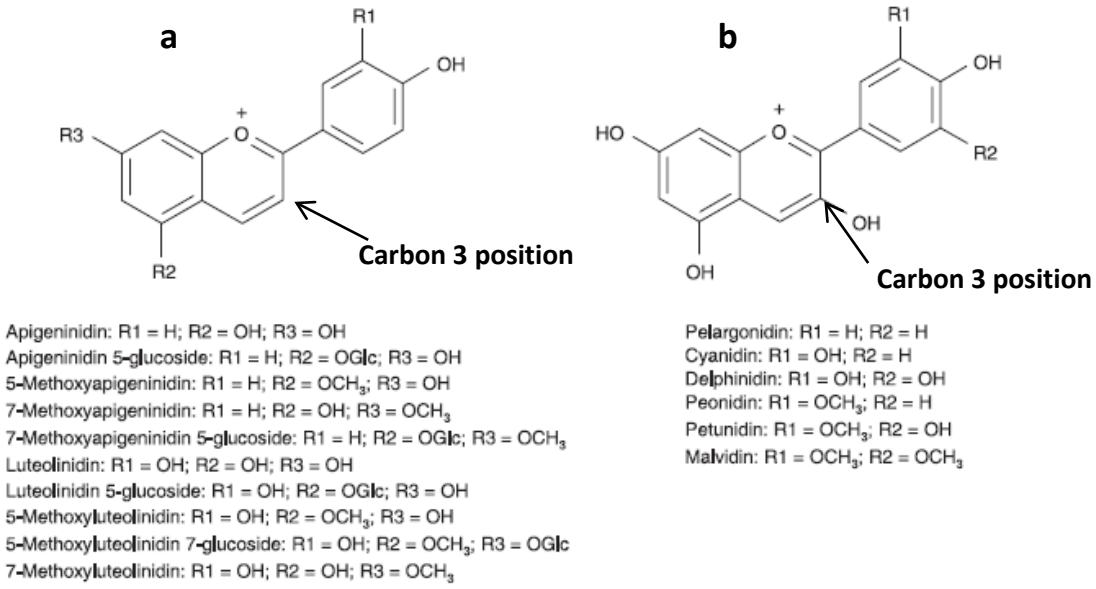


Figure 2.6: Structure of flavonoids based on benzo-γ-pyrone pattern; **a:** Generic structure of flavonoids; **b:** Structures of the major classes of flavonoids; A: Benzopyran nucleus; B: Aromatic substituents; Numbers represent the carbon positions (Cook and Samman, 1996).



Apigeninidin: R1 = H; R2 = OH; R3 = OH
Apigeninidin 5-glucoside: R1 = H; R2 = OGlc; R3 = OH
5-Methoxyapigeninidin: R1 = H; R2 = OCH_3; R3 = OH
7-Methoxyapigeninidin: R1 = H; R2 = OH; R3 = OCH_3
7-Methoxyapigeninidin 5-glucoside: R1 = H; R2 = OGlc; R3 = OCH_3
Luteolinidin: R1 = OH; R2 = OH; R3 = OH
Luteolinidin 5-glucoside: R1 = OH; R2 = OGlc; R3 = OH
5-Methoxyluteolinidin: R1 = OH; R2 = OCH_3; R3 = OH
5-Methoxyluteolinidin 7-glucoside: R1 = OH; R2 = OCH_3; R3 = OGlc
7-Methoxyluteolinidin: R1 = OH; R2 = OH; R3 = OCH_3

Pelargonidin: R1 = H; R2 = H
Cyanidin: R1 = OH; R2 = H
Delphinidin: R1 = OH; R2 = OH
Peonidin: R1 = OCH_3; R2 = H
Petunidin: R1 = OCH_3; R2 = OH
Malvidin: R1 = OCH_3; R2 = OCH_3

Figure 2.7: Structures of the (a) 3-deoxyanthocyanidins and their derivatives found in sorghum compared to six common (b) anthocyanidins found in other sources including other cereals (Dykes and Rooney, 2006).

2.2.2.2 Condensed Tannins

Condensed tannins consist of a group of polyhydroxy-flavan-3-ol oligomers and polymers with C-C linked bonds subunits (Schofield, Mbugua and Pell, 2001). They are also known as proanthocyanidins due to the ability of the flavan-3-ol oligomers to depolymerise, yielding monomeric anthocyanidin pigments (cyanidin) under acidic condition (Butler, 1982). Variations in condensed tannins are mainly due to the following: the nature of their extension and terminal units, oxygenation pattern of their flavan-3-ol units, as well as their potential to add substituents to the ring hydroxyls especially the C-3 hydroxyl (Waterman and Mole, 1994). Proanthocyanidin molecular size is expressed as the degree of polymerisation (DP) (Porter, 1988). Based on their DP, proanthocyanidins are classified as follows: DP between 2 and 10 as oligomers, while those with DP greater than 10 are polymers (Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi and Prior, 2002). Polymeric proanthocyanidin structural complexity is based on the variations in hydroxylation patterns of their flavan chain extender units, stereochemistry of the three chiral centers of their heterocyclic ring, location and the type of interflavan bonds, as well as the structure of their terminal units (Hemingway, 1989).

Condensed tannins are grouped into A- and B-type proanthocyanidins based on the location and the type of their interflavan bonds (reviewed by Dykes and Rooney, 2006). The B type condensed tannins are single linked proanthocyanidins mostly linked either by C4–C6 or C4–C8 interflavan bonds (Figure 2.8a) (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz and Prior, 2003). The A type condensed tannins in addition to the C4-C6 or C4-C8 interflavan bond have a double linked ether bond between C2-O7 due to C–O oxidative coupling (Figure 2.8b) (Gu *et al*., 2003). Proanthocyanidins are further grouped into three major subdivisions, which include procyanidins, propelargonidins and prodelphinidins. Proanthocyanidins with flavan-3-ol monomer units of (+)-catechin and/or (-)-epicatechin are designated as procyanidins (Gu *et* al., 2003; Xu and Chang, 2007). Both propelargonidins and prodelphinidins are not common in nature but rather coexist with procyanidins containing (epi)afzelechin or (epi)gallocatechin subunits, respectively (Gu *et al*., 2003).

In sorghum, the proanthocyanidins are of the B-type with (-)-epicatechin extension and catechin as terminal units (Gupta and Haslam, 1978). They consist of polymerised flavan-3-ol or flavan-3, 4-diol units linked mainly by C4-C8 interflavan bonds (Figure 2.9). A number of variations in the condensed tannins in sorghum have been reported based on the type of flavan-3-ol linkages at the extension and terminal units (Gupta and Haslam, 1978; Krueger, Vestling and Reed, 2003). As identified by Gupta and Haslam (1978), sorghum proanthocyanidins consist of 2, 3, *cis* procyanidin units and a catechin terminal unit (Figure 2.9). According to studies by Brandon, Foo, Porter and Meredith (1982), a similar condensed tannins structural property was identified. Their findings further show that 20% of the extension units consist of 2, 3-*trans* procyanidins, with small proportion of the units (8%) having the prodelphinidin oxidation pattern. This clearly revealed that the proanthocyanidins present in sorghum are structurally heterogeneous in terms of their stereochemistry and composition.

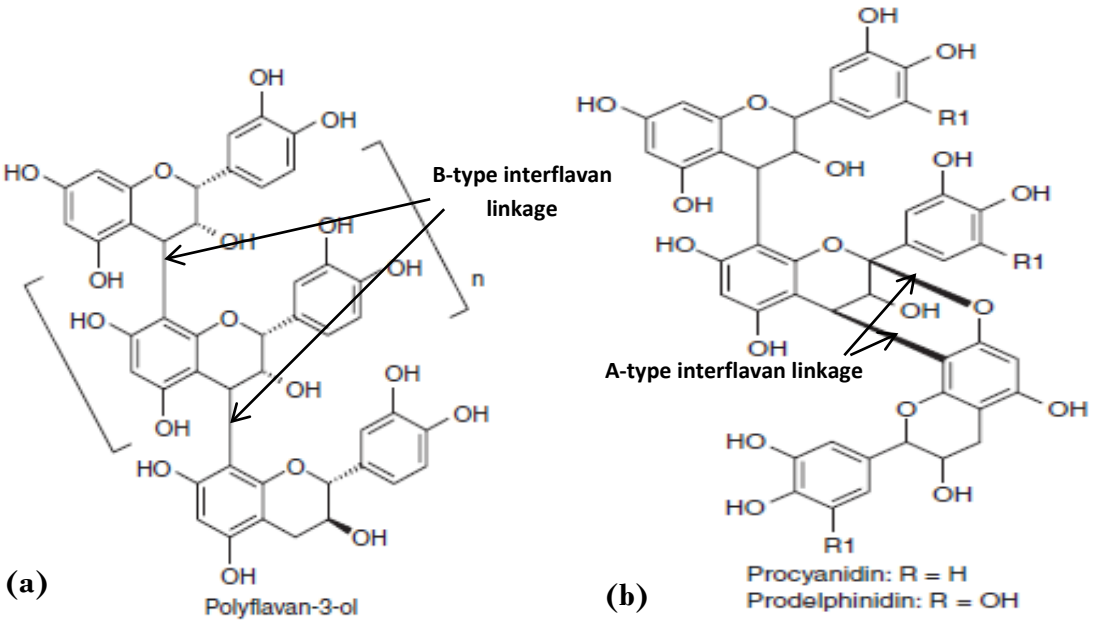


Figure 2.8: Structure of proanthocyanidins: (a) Polyflavan-3-ol with B-type interflavan linkages (Gu *et al*., 2002); (b) heteropolyflavan-3-ols with A- and B-type interflavan linkages (Krueger *et al*., 2003); n = 1 to > 10.

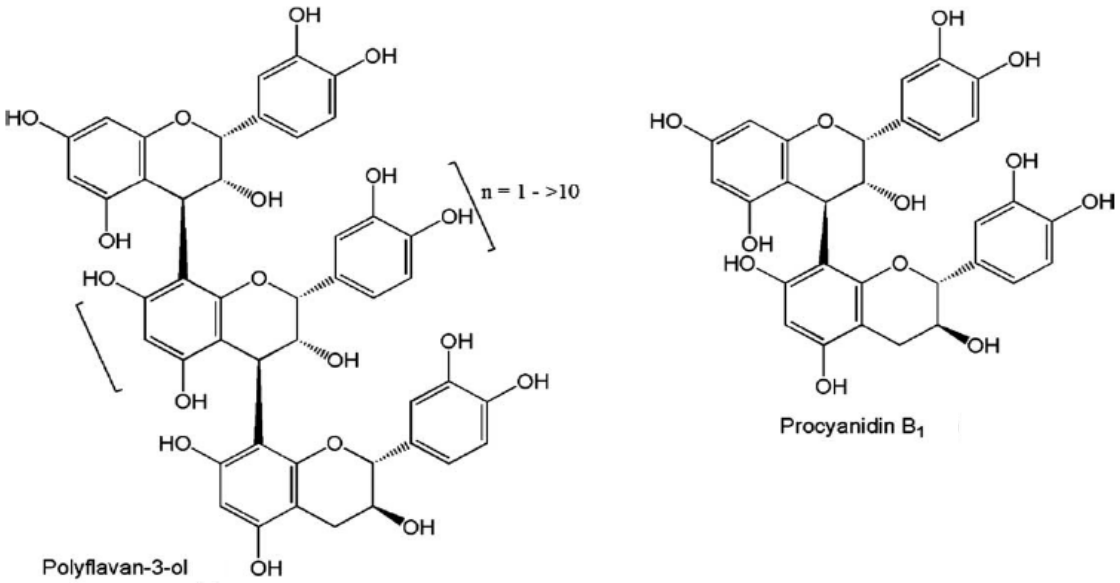


Figure 2.9: The types of proanthocyanidins most commonly reported in sorghum (Awika and Rooney, 2004).

2.2.3 Developments in condensed tannin inactivation and their limitations

A number of approaches such as decortication, malting and chemical pretreatments have been investigated to improve food and industrial utilisation of tannin-containing

sorghums, involving removing or inactivating tannins in the grain. Removal of tannins by decortication based on abrasive removal of the pericarp and seed coat layer from the grain has been applied in reducing tannin content in tannin sorghum (Chibber, Mertz and Axtell, 1978). However, the efficiency of decortication in removing tannins is counterbalanced by the soft grain characteristic of tannin sorghums as this result in kernel breakage during decortication. The application of malting (germination) in reducing the negative effect of tannins has been reported (Yan, Wu, MacRitchie and Wang, 2009). Malting reduces the level of extractable tannins in the grain (Beta *et al*., 2000; Omary, Fong, Rothschild and Finney, 2012). The mechanism of reduction in grain tannin content has been linked to both hydrogen bonding and non-polar hydrophobic interactions between tannins and proteins (Butler, Riedl, Lebryk and Blytt, 1984). However, with the germination method, the tannins were found to be still active (Beta *et al*., 2000).

Chemical detoxification techniques are based on steeping in dilute chemical solutions such as HCl (Reichert, Fleming and Schwab, 1980; Beta *et al*., 2000), formaldehyde (Daiber and Taylor, 1982; Beta *et al*., 2000) and NaOH (Reichert *et al*., 1980; Dewar, Orovan and Taylor, 1997; Beta *et al*., 2000; Nelles and Taylor, 2002), as a preprocessing step in sorghum malt production. Formaldehyde inactivation of tannins is based on its preferential reaction with tannins due to the location of tannins in the testa layer of the grain (Reichert *et al*., 1980). Interaction between tannins and formaldehyde results in a high degree of cross-linking of tannin monomer units (Roux, Ferreira and Botha, 1980), as shown in Figure 2.10. However, reaction between formaldehyde and proteins also occurs (Fraenkel-Conrat and Olcott, 1948). Further, the use of formaldehyde in food is limited due to safety reasons (Cheftel, Cuq and Lorient, 1985).

Pretreatment in dilute alkaline solution using NaOH has been widely investigated in tannin sorghum malting to improve the malt quality properties (Okolo and Ezeogu, 1996; Beta *et al*., 2000; Nelles and Taylor, 2002) and is applied commercially in sorghum malting in Nigeria (Taylor, 2003). However, it has been noted that effectiveness of alkaline treatment in deactivating tannins in sorghums is influenced by cultivar differences and season (Nelles and Taylor, 2002). As proposed by Porter (1992), the mechanism of inactivation using dilute NaOH solution may be due to the prevailing alkaline condition promoting oxidative polymerisation of condensed

tannins resulting in oxidation of the phenolic groups under moist condition. Kennedy, Munro, Powell and Porter (1984) explained that condensed tannins undergo C-ring opening coupled with rearrangement via radical reactions involving traces of oxygen.

A study on condensed tannin inactivation in sorghum grain investigated the use of ozonation (Yan, Wu, Faubion, Bean, Cai, Shi, Sun and Wang, 2012). These authors noted that ozone has ability to degrade macromolecules such as cellulose, lignin and tannins due to its oxidising property. According to the study, ozone treatment significantly reduced the grain tannin content, as reflected by significant improvement in fermentation efficiency and ethanol yield using tannin sorghum. These authors linked the mechanism of tannin inactivation to depolymerisation of condensed tannin molecules, resulting from the formation of carboxyl and carbonyl groups. Depolymerised tannin molecules have been reported not to bind or interact with protein (Butler, 1982). However, there is a safety concern with regard to the use of ozone in tannin inactivation because it is a strong oxidising agent (Yan *et al*., 2012).

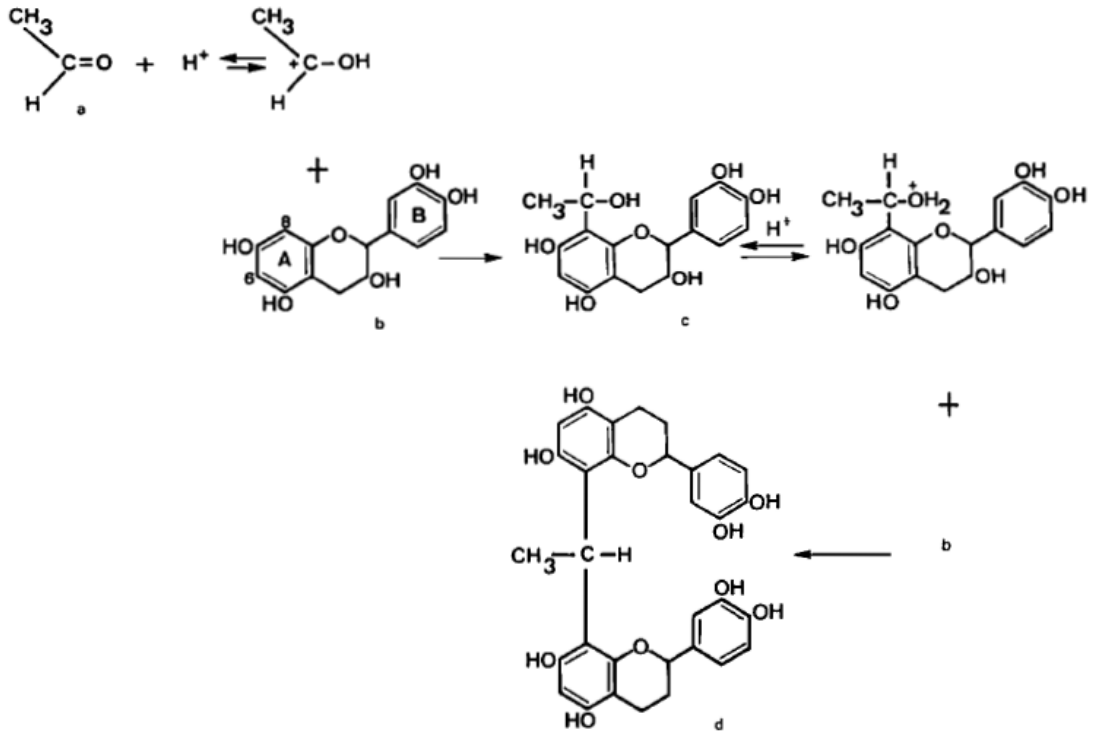


Figure 2.10: Condensation reactions between acetaldehyde (a) and catechin (b) enabling cross-linking of catechin; (c): benzylic alcohol; (d): condensation product (Delcour, Dondeyne, Trousdale and Singleton, 1982).

2.3 Analytical techniques for cell wall and condensed tannin determination

2.3.1 Cassava cell wall materials

Few studies on the characterisation of cell wall materials in cassava root tuber have been carried out. However, detailed information regarding the cell wall materials is vital in order to effectively control and improve cassava processing applications. This is because information on the cell wall composition and organisation enable better understanding of their molecular mechanism in relation to their biotechnological properties (Andersson, Westerlund and Aman, 2006). General analytical methods noted in the determination of cell wall polysaccharides include gravimetric and enzymatic gravimetric methods based on proximate analysis, while component analysis system techniques involved methods based on colorimetry and enzymatic chemical methods based on gas liquid chromatography (GLC) (Andersson *et al*., 2006). They also noted that cell wall polysaccharides component analysis method systems are best applied when studying physiological, nutritional and technological attributes of cell wall materials. As reviewed by Andersson *et al*. (2006), HPLC is a significant improvement in analytical characterisation of cell wall components and their sugar compositions. This technique enables determination of both hydrolysable and non-hydrolysable cell wall polysaccharides. Application of mass spectrometry is a more preferred technique for analysing the structural components of complex carbohydrates (Ciucanu, 2006).

Extraction of cell wall polysaccharides

As reviewed by Andersson *et al*. (2006), extraction of cell wall polysaccharides is an important step as this affects the effectiveness of the cell wall characterization. However, there is no universal extraction method. This was attributed to the structural complexity and natural variation of these polysaccharides as influenced by species, organ, stage of development and growth conditions. It was also noted that sample preparation in relation to extraction conditions is critical to determination of the yield and composition of the cell wall polysaccharides. High temperature pretreatment of cell wall materials following starch degradation is able to yield a much higher soluble cell wall polysaccharides component than with acidic buffer extraction (Graham, Gron Rydberg and Aman, 1988).

Different reagents are required for the extraction of the various cell wall polysaccharides due to variations in their chemical and physical properties. Extraction

of pectin is generally carried out with an aqueous solution of chelating agents such as ammonium oxalate or ethyldiamine tetraacetic acid (EDTA) (Selvendran and Ryden, 1990). Extraction using 1, 2-cyclohexane diaminetetracetate (CDTA) may be more preferable, as reported by Jarvis (1982). This is due to less pectin degradation as a result of β-elimination (Selvendran, 1985). Extraction of other non-cellulosic polysaccharides such as arabinoxylans, xyloglucans and glucomannans is preferentially carried out using an aqueous alkali solution containing sodium borohydride (Andersson *et al*., 2006). According to Selvendran and Ryden (1990), addition of borohydride prevents alkaline degradation by converting the reducing end groups of these non-cellulosic polysaccharides to a hydroxymethyl group. With plant tissues having lignin, delignification is generally necessary before extraction of the polysaccharides by using sodium chlorite-acetic acid (Andersson *et al*., 2006). Extraction of the cellulosic component of cell wall polysaccharides is by using N-methylmorpholine N-oxide because cellulose is resistant to alkali (Chanzy, Chumpitazi and Peguy, 1982).

HPLC and MS methods

These techniques are used for quantifying the monosaccharides constituent of the cell wall hydrolysates (Salvador *et al*., 2000). HPLC is mostly with pre-column derivatisation in the quantification of sugars (Anumula, 1994). As reviewed by Ciucanu (2006), derivatisation of cell wall material is generally necessary because it is difficult to analyse because the monosaccharides can adopt different structures in terms of ring sizes and conformation, as well as lack of chromophores or fluorophores making detection difficult. Permethylation of the cell wall material by using dimethylsulphoxide (DMSO) is a commonly used derivatisation technique (Ciucanu, 2006). This converts the hydroxyl groups to methyl ethers in order to reduce polarity and to increase thermal stability. With derivatisation-based HPLC methods, the possibility of excess reagent and reaction media, and production of multiple derivatives from one solute have been noted to cause interference (Heftmann, 1992).

As reviewed by Lee (1996), the development of high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has led to an improvement in monosaccharide analysis. This is because HPAEC-PAD does not require pre- or post-column derivatisation and gives high resolution and sensitivity.

The principle is based on high pH that is alkaline mode. The separation of the sugar components is then based on anomers, positional isomers and degree of polymerisation. Salvador *et al*. (2000) applied HPAEC-PAD in the determination of cell wall polysaccharide fractions from cassava and other root tubers by comparing different CarboPac column types. These CarboPac columns are made of non-porous pellicular resins design to meet different analytical requirements, which are suitable for separation of underivatized carbohydrates at high pH. Their findings show that a CarboPac PA 10 column gave a complete separation of the arabinose, rhamnose, xylose and mannose sugar components of the cell wall polysaccharides. CarboPac PA 1 and CarboPac PA 100 columns resulted in incomplete separation due to co-elution of these sugars. According to the manufacturer, CarboPac 10 analytical columns are specifically recommended for monosaccharides composition analysis, as against CarboPac1 which is a general-purpose column for glycoprotein monosaccharides analysis and CarboPac 100 develop specifically for oligosaccharides (Dionex, 2010).

Furthermore, pretreatment of the cell wall materials with 12 M H_2SO_4 before hydrolysis with 1 M H_2SO_4 is necessary for complete cell wall hydrolysis in combination with HPAEC-PAD to give better estimation of the sugar compositions (Salvador *et al*., 2000). A further development in carbohydrate analysis is the use of MS detection of the eluent for identification and peak confirmation based on their mass to charge ratio (Bruggink, Maurer, Herrmann, Cavalli and Hoefler, 2005). It was noted that the use of MS detection in carbohydrate analysis enables determination of coelutions of compounds.

2.3.2 Sorghum phenolics

Analytical techniques used in characterising polyphenolics in sorghum normally start with determination of total phenolic content or quantification of individual phenolics or specific groups of phenolic compounds. According to Naczk and Shahidi (2004), quantification of polyphenolics is mostly carried out by spectrophotometric techniques. The assays are based on different principles as they depend on determination of particular phenolics or various groups of phenolic compounds. In the determination of total phenolics, the most common methods include Folin-Ciocalteu (Singleton and Rossi, 1965) and Prussian Blue assay (Graham, 1992). With specific phenolic compounds such as condensed tannins, the principles used in quantifying are

based on the following: oxidative depolymerisation of proanthocyanidins, reactions of the A ring with an aromatic aldehyde, and oxidation-reduction reactions (Waterman and Mole, 1994). Other methods as indicated by Schofield *et al*. (2001) involved acid cleavage reactions, precipitation reactions, enzyme and microbial inhibition. According to Kelm, Hammerstone and Schmitz (2005), HPLC and MS are appropriate analytical techniques for quantitative and qualitative analysis of polyphenols such as flavanol and proanthocyanidins. The most important of these analytical techniques will be discussed briefly.

Extraction of polyphenolics

Importantly, polyphenolic compounds must first be extracted from the plant material prior to analysis. This step is very critical for their effective identification and quantification (Ignat, Volf and Popa, 2011). These authors noted that there is no single and standard extraction method. The most common technique for polyphenolics extraction is by solvent extraction (Castanedo-Ovando, Pacheco-Hernandez, Paez-Hernandez, Rodriguez and Galan-Vidal, 2009), which is based on solid-liquid phase extraction. This result in co-extraction of non-phenolic substances such as sugar, fats and proteins, which makes it necessary for subsequent purification processes such as Solid-Phase Extraction (SPE), as reviewed by Castanedo-Ovando *et al*. (2009). Solvents commonly used are aqueous mixtures of ethanol or methanol or acetone (Kähkönen, Hopia and Heinonen, 2001). The use of acidified methanol has been reported to be more efficient (Kapasakalidis, Rastall and Gordon, 2006). A number of factors were stated by Naczk and Shahidi (2004) to affect the solubility of phenolic compounds during extraction, including the type of solvent (polarity), degree of polymerisation of the phenolics, interaction of the phenolics with other constituents and formation of insoluble complexes. It was also noted that a universal or completely satisfactory procedure suitable for extraction of all phenolics or specific group of phenolic compounds in plant material is not available.

Condensed Tannin Analysis

The structural complexity of condensed tannins is the main factor that poses a challenge in terms of their separation, identification and quantification (Schofield *et al*., 2001). With most spectrophotometry assays for condensed tannins, detection is based on their specific chemical attributes (Waterman and Mole, 1994). The assays are based on soluble condensed tannins extracted with aqueous acetone or methanol,

without taking into consideration the insoluble tannins (Schofield *et al*., 2001). The insoluble fractions are higher molecular weight tannins bound to other materials such as non-starch polysaccharides, starch and protein. Techniques used in the analysis of these insoluble tannins fractions may involve ^{13}C-NMR (Makkar, Gamble and Becker, 1999).

Vanillin HCl assay

The most commonly used assay for tannin content is the vanillin HCl assay. This assay is based on the reaction between vanillin and condensed tannins with the formation of colour complexes (Schofield *et al*., 2001). This assay is critically affected by the type of solvent, nature and concentration of acid, reaction time, temperature, vanillin concentration and reference standard used (Scalbert, 1992; Makkar and Becker, 1993). Temperature is an important factor in vanillin assay because an increase of 1.4ºC can lead to 11% increase in absorbance (Waterman and Mole, 1994). According to Schofield *et al*. (2001), a major drawback of this assay is the variation in reactivity of the tannin polymer subunits. As they explained, not all the internal flavan-3-ol units in the tannins polymer react with vanillin. This is indicative of structural variations of proanthocyanidins.

HPLC and MS methods

Reversed-phase (RP) HPLC has been the primary method in the quantification of proanthocyanidins (Lazarus, Adamson, Hammerstone and Schmitz, 1999). RP-HPLC is limited to analysis of lower molecular weight proanthocyanidins because higher polymers (DP>3) co-elute, as the number of isomers having similar polarity increases with increase in their degree of polymerisation DP (Lazarus, Hammerstone, Adamson and Schmitz, 2001). An improvement in proanthocyanidin analysis is the application of normal-phase (NP) HPLC for the separation and quantification of procyanidin oligomers with DP up to decamers, as reviewed by Lazarus *et al*. (2001). Separation by NP-HPLC is based on degree of polymerisation and the order of elution increase with increase in degree of polymerisation. NP-HPLC is able to analyse higher proanthocyanidins up to decamers, while higher polymers (DP>10) co-elute as distinct single peak by using a sharp gradient elution at the end of the HPLC run (Gu *et al*., 2002). The principle of NP-HPLC separation is based on hydrogen bonding interactions between silica hydroxyls with the higher oligomeric procyanidins due to

more extensive interactions and this result in longer retention times of the higher molecular weight procyanidins (Waterhouse, Ignelzi and Shirley, 2000).

HPLC proanthocyanidin (PA) detection is most commonly by ultraviolet (UV) detection (Waterman and Mole, 1994). In the presence of other polyphenols, UV detection is not specific for PA. Detection using fluorescence (excitation wavelength 276 nm and emission wavelength 316 nm) was noted to offer better sensitivity and selectivity for proanthocyanidins (Lazarus *et al*., 1999). Separation of proanthocyanidins is achieved using gradient elution of dichloromethane-aqueous methanol-formic acid, which is from non-polar to polar (Kelm *et al*., 2005). The elution of the procyanidin constituents is based on retention times corresponding with increase in degree of polymerisation.

However, NP-HPLC determination of the proportion of individual proanthocyanidins is not possible and it is also limited to extractable proanthocyanidins, leading to underestimation of total proanthocyanidin content (Hellström and Mattila, 2008). This is because of the tendency of proanthocyanidins to form complexes with insoluble polymeric plant materials (Rohr, Meier and Sticher, 2000). In analysing unextractable proanthocyanidins, thioacidolysis which is an acid-catalysed depolymerisation of the proanthocyanidins in the presence of suitable nucleophilic reagent such as benzyl mercaptan can be applied (Hellström and Mattila, 2008). This process converts the flavan-3-ol extender units into benzyl-thioethers, while the terminal units are released as monomeric flavan-3-ols (Kelm *et al*., 2005). The nature of these thiolysis products are then determined by RP-HPLC using UV detection. Determination of average DP is by calculating the ratio between total units (terminal units plus thioethers) and the terminal units. However, this HPLC acid-catalysis approach in proanthocyanidin analysis is a destructive method (Kelm *et al*., 2005).

HPLC coupled with MS is able to provide qualitative information in relation to structural and molecular properties of higher oligomeric procyanidins (Hammerstone, Lazarus, Mitchell, Rucker and Schmitz, 1999). Studies by Lazarus *et al*. (1999) demonstrated that HPLC/MS has the ability to separate singly and doubly linked procyanidins and prodelphinidins, as well as copolymer oligomers and their galloylated derivatives in food materials.

A major limitation in applying these HPLC techniques in quantitative analysis, especially of higher oligomers and polymers is the requirement for pure standards. However, oligomers with DP>3 are not commercially available and this necessitates the need for isolation of these higher oligomeric standards, which may not be feasible due to lack of time and technicality (Kelm *et al.*, 2005). It is therefore assumed, perhaps wrongly, that oligomers are identical in their molar ionisation efficiencies.

^{13}C-NMR

^{13}C-NMR spectroscopy is non-destructive technique that can resolve separate cell wall components such as tannins and is also capable of measuring relative changes in the components (Gamble, Akin, Makkar and Becker, 1996). As explained by Waterman and Mole (1994), the principle ^{13}C-NMR is based on the use of magnetic resonance, where by atomic nuclei take up electromagnetic energy at a characteristic wavelength to attain higher state of resonance. Application of ^{13}C-NMR in tannin analysis requires ^{1}H and ^{13}C isotopes with the ability to exhibit nuclei magnetic resonance (NMR). Samples are dissolved in an appropriate solvent that does not resonate in overlapping parts of the spectrum to ^{1}H such as deuteriochloroform ($CDCl_3$) and deuterium oxide (D_2O). The spectra are measured in solution state. With ^{13}C-NMR, the range of resonance is far larger than ^{1}H-NMR. Carbon spectra occur unmodified as singlets, doublets, triplets or quartets, and these give an indication of the number of hydrogen atoms attached to the carbon directly.

2.4 Conclusions

This review clearly shows that the structural and compositional properties of cell wall materials in cassava root tuber are important to its processing. Their unique physicochemical properties in terms of water-holding capacity and structural organisation of the cell wall matrix are major factors that could limit processing applications of cassava such as in lager beer brewing. Modification of these cell wall materials based on the highlighted techniques, in particular cellulolytic and hemicellulolytic enzyme treatment could lead to improved cassava starch extraction and cassava bagasse processing.

In relation to sorghum, its polyphenolic, especially the condensed tannins which are present in some sorghum types is a major factor limiting tannin sorghum food and beverage utilisation. This is because of their negative effects on both processing and food product quality characteristics. This review shows that the flavonoids and

condensed tannins in sorghum are complex because they are structurally and compositionally heterogeneous. These attributes are responsible for difficulties in their extraction and determination. The need to develop technology that can effectively inactivate condensed tannins in tannin sorghums is critical to tannin sorghum food and beverage processing utilisation. Application of an alkaline treatment for tannin inactivation to milled sorghum grain is desirable since sorghum is mostly used milled in food, beverage and bioethanol processing. Further, better understanding of the chemistry involved in inactivating condensed tannins is needed to enable the development of more effective and efficient inactivation processes.

3. HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

a. Cellulolytic and hemicellulolytic treatment of cassava cake (wet milled cassava) will result in modification in the structure and composition of cassava cell walls, thereby reducing their water-holding capacity. Bioconversion techniques involving the use of hydrolytic enzymes with cellulolytic and hemicellulolytic activities have been applied to cassava mash in improving starch extraction (Rahman and Rakshit, 2003). By using a multi-enzyme mixture of cellulases, pectinases and hemicellulases (Sriroth *et al*., 2000), this type of enzymatic treatment of cell wall materials result in the hydrolysis of the soluble cell wall components and fragmentation of the insoluble cell wall components (Demir *et al*., 2001; Rai *et al*., 2004).

b. Steeping of milled tannin sorghum grain in dilute sodium hydroxide solution will effectively reduce the negative effects of the tannins in tannin sorghums on the brewing and bioethanol mashing processes. With dilute NaOH steeping of whole tannin sorghum grain which was then milled and used in brewing, tannin were found to still negatively affect wort quality attributes (Adetunji, 2011). According to Gaffet, Bernard, Niepce, Charlot, Gras, Le Caër, Guichard, Delcroix, Mocellin and Tillement (1999), milling process is a means of modifying conditions in which chemical reaction occur by changing reactivity, which enables increase in reaction rates. The effect of modification in grain structure due to germination during malting explains inactivation of tannins in tannin sorghum (Yan *et al*., 2009).

c. The mechanism of tannin inactivation by NaOH will be due to chemical modification of tannin molecules, resulting from the direct chemical reaction between tannins and NaOH. According to Kiatgrajai, Wellons, Gollob and White (1982), alkaline extraction of condensed tannins is known to result in tannins that are less reactive with aldehydes compared to neutral-solvent extraction. Reaction between tannins and alkali involves C-ring opening and rearrangement via radical reactions in the presence of traces of oxygen (Kennedy *et al*., 1984). This structural modification of tannins due to the reaction between tannins and alkali can explain reduction in tannin reactivity (Laks, Hemingway and Conner, 1987; Hashida and Ohara, 2002).

3.2 Objectives

a. To evaluate the effects of cellulolytic and hemicellulolytic enzyme activities on the structural and compositional properties of cassava cell walls.

b. To evaluate the effects of steeping milled tannin sorghum grains in different concentrations of dilute NaOH solution on tannin content and tannin inhibitory activity.

c. To understand the chemistry of reaction between tannins and NaOH, resulting in tannin inactivation.

4.1 RESEARCH CHAPTER

Hydrolysis of the fibre material in cassava cake with cellulolytic and hemicellulolytic enzymes to improve wort filtrability

ABSTRACT

The levels of high fibrous materials in cassava root tubers and their unique functional properties constitute a major problem after starch hydrolysis in brewing processing application of cassava tuber. This is due to the cellulolsic and hemicellulosic cell wall polymers having high water holding capacity (85-90%), resulting in poor wort filtration during brewing. This problem was addressed by applying cellulolytic and hemicellulolytic enzymes in the treatment of cassava cake (wet milled cassava tuber). This enzymatic treatment of cassava cake resulted in a reduction in total solids volume and in the amount of insoluble solids. The amount of free starch granules also increased with increase in enzyme concentration. A combination of multiple enzyme activities gave a synergistic effect by significantly reducing the viscosity of cassava cake. These effects can be attributed to hydrolysis of the starch-containing parenchyma cell wall polymers. As determined by GC, cassava CWM monosaccharide units consisted of arabinose, rhamnose, xylose, galactose and glucose, with glucose being the most sugar units followed by xylose. This suggests that cellulosic type material is the main components of cassava CWM. Enzymatic treatment of cassava CWM can potentially improve processing of cassava in brewing.

4.1.1 INTRODUCTION

Cassava (*Manihot esculenta* Crantz L.) is ranked sixth in the world food production for 2013 after sugarcane, maize, rice, wheat and potatoes (FAO, 2015), and serves as a major source of food and dietary calories for many people in the tropical countries of Africa, Asia and Latin America (Pandey, Soccol, Nigam, Soccol, Vandenberghe and Mohan, 2000). Cassava can to grow on impoverished and marginal soils with little technological input (Buschmann, Potter and Beeching, 2002). Cassava production in Africa represents about 57% of the world production in 2013 (FAO, 2014). As reviewed by Pandey *et al*. (2000), only a low proportion of cassava world production, about 7%, is used commercially in industries such as paper, textile, food and fermentation. Therefore, there is need for increased value addition to cassava in food and beverage product processing, especially in lager beer brewing, due to its high starch content.

Proximate composition of cassava root shows that moisture is about 64% and carbohydrates about 34%, which are the main chemical components (Rickard and Behn, 1987). According to a study by Charles, Sriroth and Huang (2005) on five different genotypes of cassava, carbohydrate and crude fibre composition ranges between 80-86% and 1.5-3.5% dry weight basis, respectively. Among different cultivars, the protein content of cassava root tuber ranges from 0.5 to 1.9 g/100 g dry matter (El-Sharkawy, 2004). In terms of the root cyanogenic quality, cassava roots with levels of hydrocyanic acid below 10 mg/100 g are generally considered sweet cassava (Jennings and Iglesias, 2002). Salvador, Suganuwa, Kitahara, Tanoue, and Ichiki (2000) investigated the cell wall components in cassava root tuber. Fractionation of the cell wall material components identified cellulose (about 48%), hemicellulose (about 22%) and pectin (about 17%) as the major non-starch polysaccharides components of the cell wall materials.

During extraction of starch from cassava, fibre materials are the major solid residue constituting about 15-20% by weight of the cassava tuber processed (Swain and Ray, 2007). As a result of this high residual fibrous material content in cassava root, its application in brewing may constitute considerable problems after starch hydrolysis. This is due to the high water retention capacity of the fibre material (85-90%) (Pandey

et al., 2000). In particular, this may constitute a considerable drawback in the filtration of the wort. As reviewed by Pandey *et al*. (2000), bioconversion techniques such as enzymatic hydrolysis and solid-state fermentation have been applied in the treatment of cassava fibrous material residues. Therefore, enzymatic hydrolysis could be applied in the treatment of cassava fibre material in order to reduce its negative effect. This study focused on improving the cassava wort filtration processing step through treatment of the cassava with cellulolytic and hemicellulolytic enzymes in order to reduce the amount of fibre material residue left after the mashing (starch hydrolysis) step.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Materials

Cassava cake (wet milled cassava tuber) was obtained from SABMiller (ex. Nampula, Mozambique) and stored at 6-8ºC until analysis. Fresh cassava root tubers from Mozambique were obtained from a retailed outlet in Pretoria. The commercial enzyme preparations used were Viscozyme L and Ultraflo Max (cellulolytic and hemicellulolytic enzymes), and Termamyl SC (Thermal-stable α-amylase enzyme) kindly provided by Novozymes (Benmore, South Africa).

4.1.2.2 Methods

Pre-weighed fresh cassava root tubers were washed, peeled, chopped and weighed again. The cassava chips were milled using a waring blender, with small quantity of water added to aid the milling process. After milling, the cassava cake obtained was weighed to determine the yield. The cassava cake (wet milled cassava tuber) obtained was stored at 6 °C until required.

4.1.2.2.1 Enzymatic treatments

Effect of different levels of Viscozyme treatment

Cassava cake (450 g) was diluted with 450 ml distilled water (50:50) to give a cassava slurry of about 900 g. Viscozyme enzyme was diluted and added to give the following concentrations of the enzyme in the slurry: 0, 250, 500, 700 ppm and overdose of the enzyme (relative to cassava cake solids). The control sample was diluted with distilled water without the addition of enzyme. The cassava slurries were incubated at ambient temperature (about 24ºC) for two weeks. The treatments were performed in duplicate.

Effect of different types of enzyme preparations

Cassava cake (450 g) was weighed into beakers. Nine ml of diluted Viscozyme and Ultraflo enzymes were added to give 250 ppm of the enzyme (relative to cassava cake solids) and mix thoroughly. Combined Viscozyme and Ultraflo enzymes to give 250 ppm of the enzyme was obtained by adding 4.5 ml of diluted Viscozyme and Ultraflo enzymes each and mix thoroughly with the cassava cake. After mixing thoroughly, the cassava cake samples were covered with parafilm to ensure anaerobic condition in order to prevent mould growth. The control sample was mixed with 9 ml distilled water without added enzyme. The cassava cake samples were incubated at ambient temperature (about 24ºC) for two weeks. The treatments were performed in duplicate. The activity of the enzymes in the cassava cake samples for one week incubation period was stopped by storing at -20 ºC and at the end of two weeks incubation the cassava cake samples were also stored frozen to stop the activity of the enzymes until required for analysis. The frozen cassava cake samples after incubation periods were thawed at 24ºC for analysis.

4.1.2.2.2 Starch hydrolysis

This was carried out using a BRF mashing bath (Brewing Research Foundation, Nutfield, UK). During mashing, the slurry was stirred manually due to difficulty in stirring the mash by magnetic stirring and the beakers covered with watch glasses. Mashing was performed by quantitatively weighing the treated and the control cassava cake samples into the mashing beaker. The pH was adjusted with 0.1 M NaOH solution to pH 5.0, which is the optimum pH for the α-amylase enzyme used. This was followed by addition of 6 ml diluted Termamyl SC to give 100 ppm of the enzyme in the slurry and cooked for 1 hr. After cooking for 1 hr, one ml of full strength Termamyl SC was added to the mash. Mashing was carried out at 100ºC until the starch was negative by iodine. After mashing, the samples were centrifuge at 470 *g* for 2 min and the clear supernatant carefully removed. The insoluble solids residue stored by freezing for further analyses. Mashing was performed in duplicate.

4.1.2.2.3 Purification of enzyme treated cassava cake and mashed solid materials

After incubation, 25 g of the samples were weighed and diluted to 50 g with distilled water in 100 ml glass centrifuge tubes. This was followed by centrifugation at 470 *g* for 2 min and the clear supernatant carefully removed. The samples were then re-suspended in distilled water and re-centrifuged in order to completely wash out the

soluble solids. The purified residual solid materials of both the control and enzyme treated cassava cake samples before and after starch hydrolysis by α-amylase enzyme were analysed for the following: total solids, starch, soluble and insoluble fibre contents, particle size (sieving) and light microscopy. Part of the purified residual insoluble cell walls materials were freeze dried and analysed for compositional and structural properties by gas chromatography.

4.1.2.3 Analyses

4.1.2.3.1 Titratable acidity and pH determination

Titratable acidity of the cassava cake was determined according to GEA Niro analytical method A 19 a (GEA Niro, 2006), with slight modification. Samples were prepared by weighing 10 g cassava samples and diluted with 20 ml distilled water. The pH of the cassava cake was determined using pH meter.

4.1.2.3.2 Viscosity determination

Cassava cake samples viscosity in terms of flowability was determined using a Bostwick viscometer. The samples were allowed to equilibrate to ambient temperature (24ºC) before determination of viscosity. Cassava cake (100 g) was poured into the Bostwick cell with the bridge closed. The bridge was opened completely to allow the sample to flow down the trough section for 30 seconds and the length covered was recorded in mm.

4.1.2.3.3 Total solids content determination

Total solids content of the cassava cake was determined based on dry matter remaining, by drying at 103ºC for 3 hr. The total solids content of the treated samples were determined after washing and centrifuging to remove the soluble solids. The results were expressed in percentage (w/w) wet basis.

4.1.2.3.4 Total starch content determination

Total starch content was determined using the Megazyme Total Starch Assay Procedure (Amyloglucosidase/α-Amylase Method) (Megazyme International, 2011). The starch content of the total solids in the cake was expressed in percentage (w/w) dry basis.

4.1.2.3.5 Insoluble fibre content determination

After complete starch hydrolysis and washing of the solid material left to remove the soluble solids, total insoluble fibre content was estimated based on the dry matter

remaining, by drying at 103ºC for 3 hr. The insoluble fibre content of the total solids in the cake was expressed in percentage w/w dry basis.

4.1.2.3.6 Particle size determination

Particle size was determined by weighing 10 g sample of purified insoluble solids by removing soluble solids from the cassava cake samples before and after starch hydrolysis by α-amylase enzyme and then sieved through 500 and 250 µm opening sieves with small amount of distilled water. The solid materials retained by the 500 and 250 µm sieves and the materials that passed through the 250 µm sieve were weighed and dried. The proportion of the particle size fractions expressed in percentage w/w dry basis.

4.1.2.3.7 Light microscopy

Microscopic examination of the fibre materials was carried out before and after starch hydrolysis by α-amylase enzyme, as well as on the particle size fractions. The samples before starch removal by α-amylase hydrolysis were stained with iodine solution to identify the starch granules.

4.1.2.3.8 Gas chromatography characterisation of residual cell wall materials

The freeze dried residual cell wall samples were characterised for compositional and structural properties of as described by Ciucanu and Caprita (2007). Hydrolysis of the cassava residual cell wall samples into their monosaccharide components were carried out with acidified methanol. Samples for structural analysis were first per-methylated using dimethyl sulphoxide (DMSO). This was followed by hydrolysis with acidified methanol. Analysis of cell wall monosaccharide compositions and structural linkages were performed using GC- flame ionisation detection (FID).

4.1.2.4 Statistical analysis

All experiments were repeated at least once because the experiments were repeatable. The independent variables were treatment of the cassava cake with enzyme and incubation period, while the dependent variables were the total starch content and insoluble CWM properties determined. Data were analysed by one-way analysis of variance (ANOVA). Significant differences among the means were determined by Fisher's least significant difference (LSD) test at $p<0.05$.

4.1.3 RESULTS AND DISCUSSION

The cassava cake used in this study contained about 60% moisture (Table 4.1.1). The starch in the total solids was about 81% (dry weight basis). Hence, the starch content of the cassava cake was about 32%, similar to the 34.7% reported by Charles, Chang, Ko, Sriroth and Huang (2004). The remaining 19% of the total solids probably consisted mainly of non-starch polysaccharides (both soluble and insoluble cell wall materials). The derived amount of non-starch solid materials in the cake was about 7.4%, similar to 7.6% reported by Carrie and Brillouet (1986).

Table 4.1.1: Proximate composition of cassava cake

Components	**(g/100 g cake)**
Moisture	60.3±0.1
Total solids	39.7±0.2
Starch[a]	32.3±0.3 (81.4)[2]
Cell wall materials[a1]	7.4±0.2 (18.6)[2]

[a]Percentage of solids component
[1]This may include trace amounts of protein, lipids and ash
[2]Relative proportions in brackets between starch and CWM

4.1.3.1 Effects of Viscozyme on cassava cake composition

After two weeks incubation of the cassava slurry with different concentrations of Viscozyme, a reduction in insoluble solids with increase in concentration of the enzyme was observed after washing and centrifugation to remove the soluble solids (Figure 4.1.1). It was observed that the amount of freed starch in the cassava slurry increased with the enzyme concentration (Figure 4.1.1a). Quantitatively, the starch content of the cake also increased slightly with increase in enzyme concentration, except for enzyme overdose treatment sample (Table 4.1.2). All these effects can be attributed to hydrolysis of the fibre material by the Viscozyme in releasing the trapped starch granules. A possible explanation for the low starch concentration in the enzyme overdose treatment is due to starch hydrolysis by the enzyme. This may suggest that at significantly high concentration, the enzyme will hydrolyse the starch. As shown in Figure 4.1.1b, an increase in volume of the insoluble fibre material also occurred while hydrolysing the starch during mashing. This increase in volume may be due to hydration of the insoluble fibre materials as a result of wet cooking. However, there

was a reduction in volume of the insoluble solids with increase in Viscozyme concentration. As shown in Figure 4.1.1b and Table 4.1.2, the level of reduction in the volume of insoluble fibre materials corresponded with the amount of insoluble fibre remaining in the cake. This can be attributed to hydrolysis of the fibre materials, as it affects water holding capacity of the insoluble fibre in the cake.

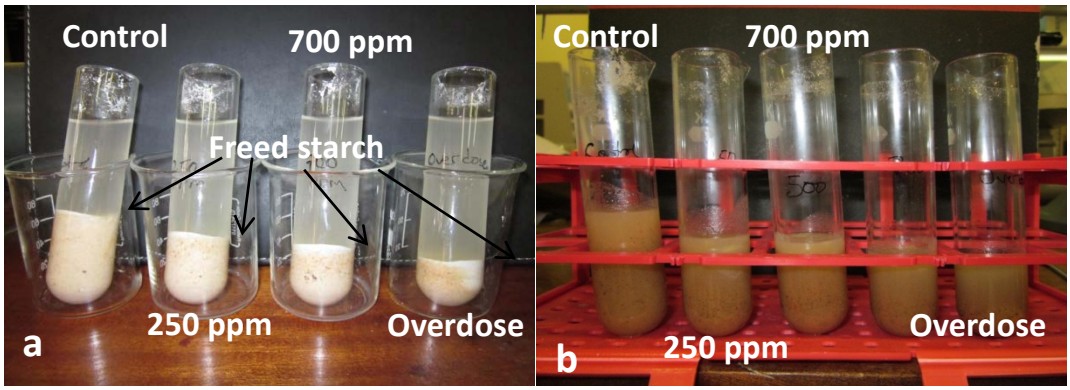


Figure 4.1.1: Effects of different Viscozyme enzyme concentrations on the amount of starch released and on the volume of insoluble solids of washed cassava slurry after incubating for 2 weeks. **a:** Before starch hydrolysis; **b:** After starch hydrolysis

Table 4.1.2: Effects of Viscozyme enzyme concentration on the volume and percentages of insoluble total solids content and particle size of cassava slurry after washing to remove soluble solids after two weeks incubation before starch removal

Treatments	Total solids volume before starch removal (cm^3)[2]	Starch content (g/100 g slurry dwb)	Total solids volume after starch removal (cm^3)	Insoluble fibre content (g/100 g slurry dwb)	Sieved Particle Size Fractions (%)		
					>500 (µm)	>250 - ≤500 (µm)	≤250 (µm)
Control	19.5	77.7[a] ±0.5	26.0	8.11	49.0	28.0	22.9
250 ppm	16.2	82.8[b]±0.3	21.1	6.83	39.2	13.7	47.1
500 ppm	nd[1]	nd	19.5	5.90	nd	nd	nd
700 ppm	13.0	81.9[b]±1.6	17.9	5.19	nd	nd	nd
Overdose of enzyme	9.8	78.6[a]±0.8	14.6	4.20	44.0	18.7	37.3

[1]not determined; dwb: Dry weight basis; Mean values in the same column but with different letters are significantly different ($p<0.05$); [2]Sufficient sample was not available to repeat the analysis in between the treatments.

The enzymatic treatment of the cake also resulted in a reduction in the particle size of the fibre materials (Table 4.1.2). The enzyme overdose sample had higher proportion of large particles than the 250 ppm treatment. These large particle size materials with

the enzyme overdose treatment may possibly be lignified materials that were not hydrolysed. Light microscopy of the cake insoluble solid materials remaining after enzymatic starch removal revealed considerable differences in the cell wall material appearance, as affected by the Viscozyme (Figure 4.1.2). The untreated control was characterised by clusters of parenchyma cells. In the case of the enzyme overdose treatment, most of the cell wall material had disappeared with the exception of the lignified material. This is because lignified materials contain few hydrolysable bonds and are poorly susceptible to hydrolytic enzymes (Marsden and Gray, 1986). These observations correspond with large reduction in the volume of solid material remaining in the enzyme overdose treatment (Figure 4.1.1b). Thus, the reason for greater fraction of large particle size material in the enzyme overdose treatment was due to more of the lignified materials (Figure 4.1.2). At a very low enzyme dosage of 250 ppm, considerable reduction in insoluble CWM was obtained with the release of the starch granules without any negative effect on the total starch content.

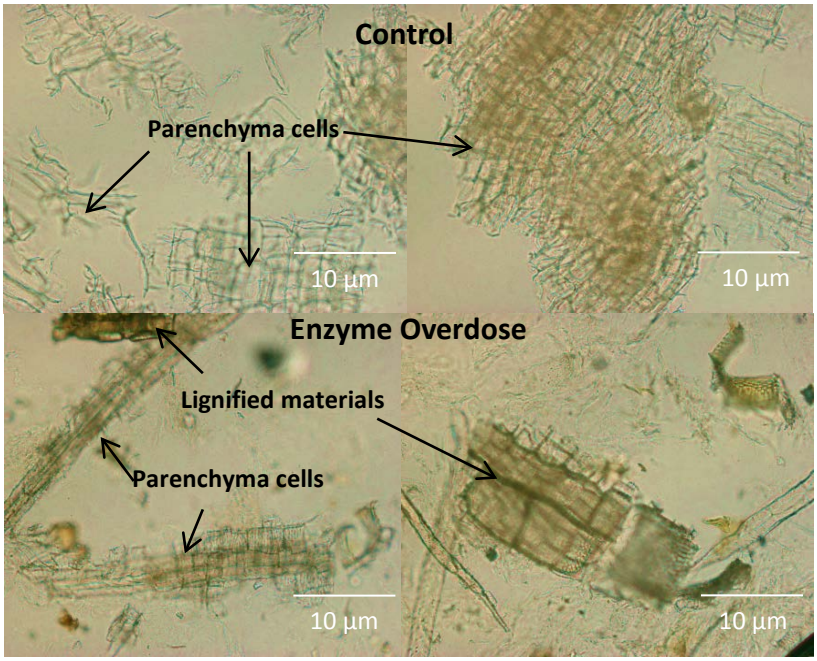


Figure 4.1.2: Light microscopy image of cassava insoluble solid residues after starch removal, showing the effects of Viscozyme treatment on the cell wall materials

4.1.3.2 Effects of Viscozyme and Ultraflo enzymes on cassava cake composition

The cassava cake used in this study had already fermented due to activity of lactic acid bacteria, and had a pH of 3.6. This pH was not suitable for the Ultraflo enzyme

preparation because it was below its optmum pH range. After two weeks incubation of the cassava slurry with Viscozyme and Ultraflo, a reduction in viscosity of the pastes was observed (Figure 4.1.3). The Viscozyme treatment had smoother and free flowing properties compared to the Ultraflo treatment and the untreated control. Also, the Viscozyme treatment resulted in greater reduction in slurry volume and an increased amount of freed starch granules than the Ultraflo treatment, after washing and centrifugation to remove the soluble solids (Figure 4.1.4).

Figure 4.1.3: Effects of treatment with Viscozyme and Ultraflo on cassava slurry viscosity after 1 week and 2 weeks incubation

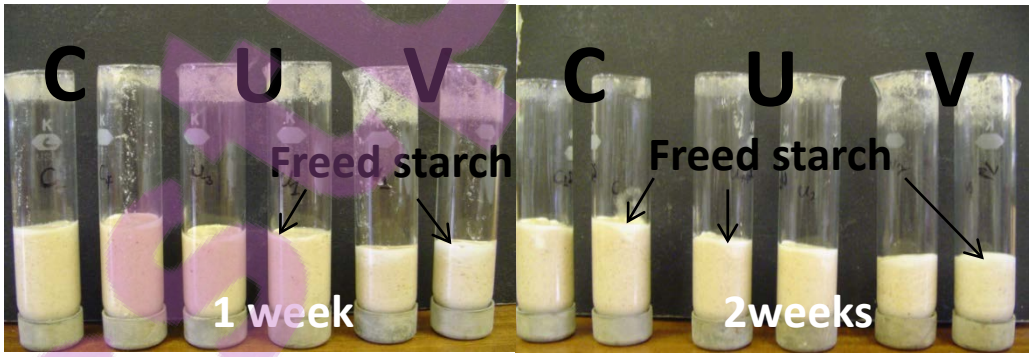


Figure 4.1.4: Effects of Viscozyme and Ultraflo on the cassava slurry volume after washing and centrifuging to remove the soluble solids. **C:** Control; **U:** Ultraflo; **V:** Viscozyme

Also, quantitatively, both the Viscozyme and Ultraflo treatments resulted in a considerable reduction in volume of the slurry (Table 4.1.3). The Viscozyme treatment reduction in the quantity of insoluble solids was consistent with the

reduction in slurry volume. This was presumed to be due to hydrolysis of the cell wall fibre material. However, the Ultraflo treatment reduction in insoluble solids volume did not correspond with the quantity of insoluble solids (Table 4.1.3). This may be due to the debranching activity of Ultraflo (Novozymes, 2008). It was observed that mould growth took place in both the Ultraflo and untreated control samples during incubation. Occurrence of mould growth was favoured by high water activity of the cake coupled with aerobic condition under which the cassava samples were incubated by using foil to cover the beakers during incubation. According to Yang, Willies and Wyman (2006), interference by obstacles in the enzyme path results in a decrease in the rate of enzymatic hydrolysis. Mould growth could have constituted an obstacle interfering with the hydrolytic activity of the Ultraflo coupled with the low pH of the fermented cassava cake used not optmum for the activity of the enzyme.

Also, it appeared that the amount of hydrolysis of the cell wall fibre material was greater in the Viscozyme treatment resulting in a lower proportion of large particle size fraction compared to the Ultraflo treatment (Table 4.1.3). This higher level of hydrolysis also resulted in less starch granules trapped within the cell wall materials (Figure 4.1.5a). Light microscopy of the insoluble solid materials remaining after starch removal using α-amylase also showed considerable differences between Viscozyme and Ultraflo in their effect on the cell wall material appearance (Figure 4.1.5b). Both the untreated control and Ultraflo treated samples were characterised by large clusters of parenchyma cells. With the Viscozyme treatment most of the cell wall material had disappeared, with the exception of dark staining lignified material. These effects can be attributed to their different hydrolytic activities. According to Novozymes (2008), Ultraflo has xylase and β-glucanase activities, whereas Viscozyme has combined activities of cellulase, β-glucanase, xylase, pentosanase and arabanase. The fibrous nature of cassava storage roots develops through massive cell division and differentiation of parenchyma cells of the secondary xylem (Sheffield, Taylor, Fauquet and Chen, 2006). The lignified cell wall materials in cassava root tuber are cross-linked phenylpropane units, which made up the xylem tissue and schlerenchymous fibres (Buschmann *et al*., 2002). The Viscozyme and Ultraflo treatments had slightly lower starch contents than the untreated control (Table 4.1.3). This may be attributed to hydrolysis of starch by side amylase activity in these enzyme preparations.

Table 4.1.3: Effects of Viscozyme and Ultraflo on cassava slurry volume, insoluble solids content, cake total starch content and particle size distribution after removal of soluble solids

Treatments	Slurry volume in centrifuge tube (cm^3)	Insoluble solids content (g/100 g $cake^2$)	Starch content (g/100 g insoluble $solids^3$)	Starch content (g/100 g $cake^2$)	Sieved Particle Size Fraction (%)		
					>500 (µm)	>250 - <500 (µm)	≤250 (µm)
Cassava cake[1]		39.7±0.2	81.4±0.5	32.3±0.3			
***Control (1 week)**	21.9	40.7^{b}±1.2	80.4^{a}±4.7	32.7^{abc}±0.8	21.1^{c}±4.0	9.3^{a}±1.0	69.7^{a}±5.0
***Control (2 weeks)**	19.5	41.2^{b}±1.4	84.4^{a}±1.7	34.8^{c}±0.5	21.7^{c}±0.9	8.8^{a}±3.4	69.6^{a}±2.5
***Ultraflo (1 week)**	17.9	39.4^{b}±0.6	80.2^{a}±4.2	31.6^{ab}±2.1	18.9^{bc}±5.6	8.3^{a}±0.5	69.9^{a}±9.3
***Ultraflo (2 weeks)**	17.1	40.0^{b}±1.4	84.6^{a}±1.4	33.9^{bc}±0.6	19.3^{bc}±1.5	10.8^{a}±4.1	69.9^{a}±2.6
Viscozyme (1 week)	14.6	36.0^{a}±0.4	85.4^{a}±0.2	30.8^{a}±0.4	7.6^{a}±4.7	9.4^{a}±3.0	83.0^{b}±1.7
***Viscozyme (2 weeks)**	13.0	38.4^{ab}±1.4	86.5^{a}±0.5	33.2^{abc}±1.0	11.2^{ab}±1.2	10.8^{a}±0.4	78.1^{ab}±0.8

[1]Cassava cake without enzyme treatment and without washing to remove soluble solids; [2]As is basis, [3]Dry weight basis; *Mould growth on the samples; Mean values in the same column but with different letters are significantly different (p<0.05)

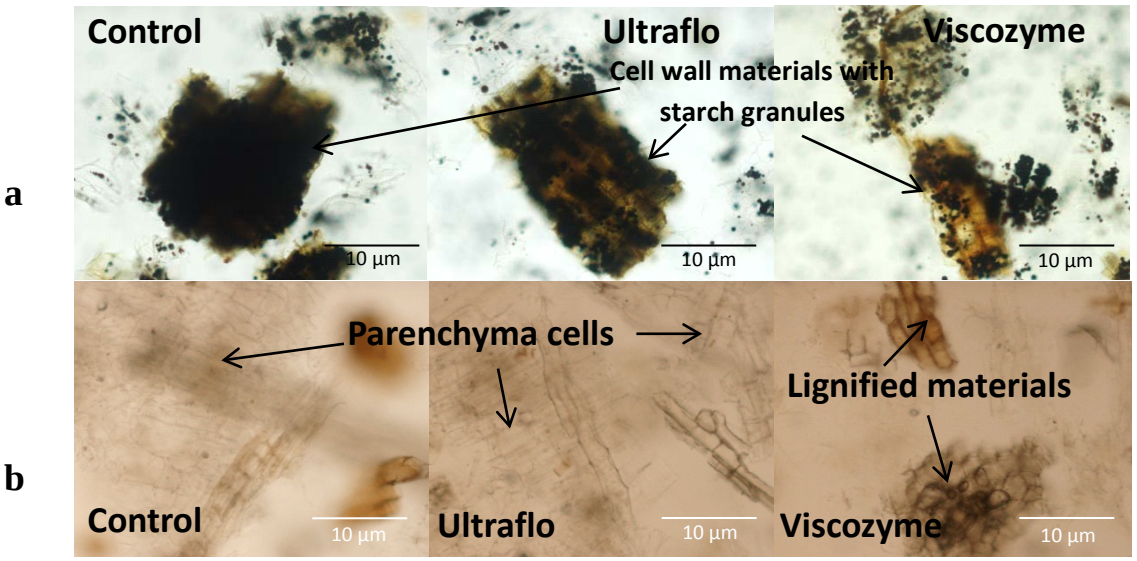


Figure 4.1.5: Light microscopy images showing the effects of Viscozyme and Ultraflo treatments on the cassava cake cell wall fibre materials after incubating for 2 weeks. **a:** Before starch hydrolysis; **b:** After starch hydrolysis

The quantity of insoluble cell wall fibre materials in the cake after enzymatic starch removal decreased with both the Viscozyme and Ultraflo treatments (Table 4.1.4). The Viscozyme treatment had the least amount of both insoluble and soluble fibre materials remaining. This is presumably due to a high level of hydrolysis of these non-starch polysaccharides. This corroborates that Viscozyme had more hydrolytic activity on the fibre materials than Ultraflo, as stated above. A substantial reduction in particle size of the fibre materials was also obtained with the Viscozyme treatment. It had the least large particle size fractions and highest proportions of smaller particle size fibre materials. It was observed that after starch hydrolysis with α-amylase, the Viscozyme treatment mash was free flowing, while the untreated control and the Ultraflo treatment mash were very viscous. This can be attributed to the significant reduction in the amount and particle size of the cell wall fibre materials.

Table 4.1.4: Effects of Viscozyme and Ultraflo on the insoluble and soluble fibre contents and particle size of cassava slurry after removal of the starch

Treatments	Insoluble fibre content (g/100 g solids[1])	Insoluble fibre content (g/100 g cake[2])	Soluble fibre content (g/100 g cake[3])	Sieved Particle Size Fraction (%)		
				≥500 (µm)	>250 - <500 (µm)	≤250 (µm)
***Control (1 week)**	8.0^{c}±0.4	3.3^{c}±0.2	4.7	75.5^{b}±0.6	13.9^{a}±2.0	10.5^{a}±1.3
***Ultraflo (1 week)**	6.4^{b}±0.2	2.5^{b}±0.1	5.3	68.5^{b}±2.2	15.9^{a}±1.2	15.9^{b}±3.3
Viscozyme (1 week)	5.3^{a}±0.6	1.9^{a}±0.1	3.3	55.9^{a}±3.5	24.6^{b}±1.3	19.5^{b}±4.5

[1]Dry weight basis; [2]As is basis; The soluble fibre content remaining was calculated as the difference between the total solids and insoluble solids contents; *: Mould growth on the samples; Mean values in the same column but with different letters are significantly different ($p<0.05$)

4.1.3.3 Effects of combining Viscozyme and Ultraflo on cassava cake composition

The pH of fresh cassava cake at day 0 was pH 6.4 and after 24 hr had dropped to pH 4.3 (Figure 4.1.6a). This drastic change in pH of the cassava cake may be due to activity of amylolytic lactic acid bacteria fermentation by LAB such as *Lactobacillus plantarium*, which has been isolated from cassava roots (Giraud, Brauman, Keleke, Lelong and Raimbault, 1991). After 24 hr, the rate of drop in pH of the cake slowed down considerably. The pH after incubation with the enzyme treatments was 3.7 for the Viscozyme treatment and 3.8 for both combined enzymes and the Ultraflo treatments, while that of untreated control was 4.2. In agreement with the pH values, the Viscozyme treatment had higher titratable acidity than the combined and Ultraflo treatments (Figure 4.1.6b). The untreated control had the lowest titratable acidity and remained the same throughout the incubation period. A probable explanation for further drop in pH with the enzyme treatment cakes is that the hydrolytic activity of the enzymes produced substrates such as sugars for the lactic acid bacteria.

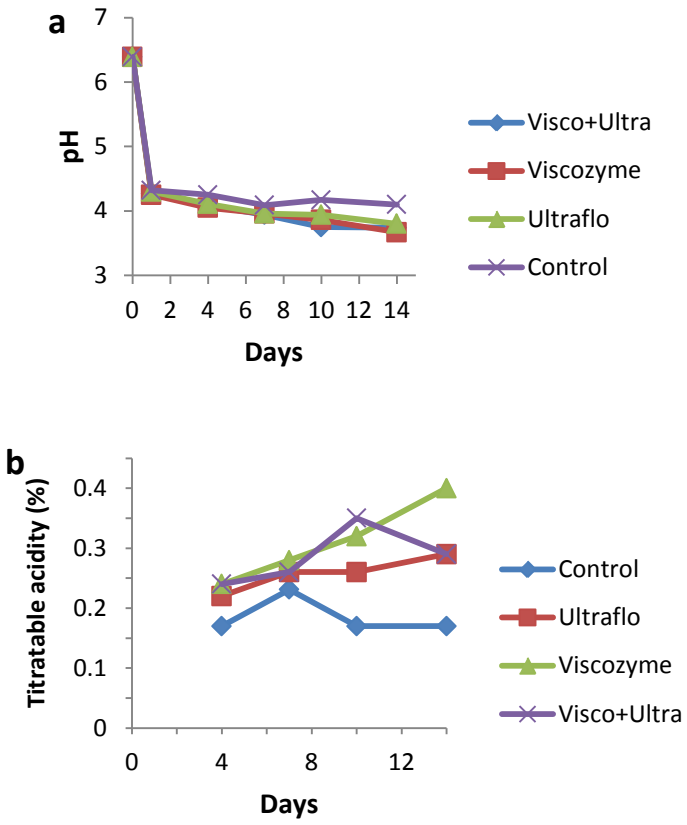


Figure 4.1.6: Effects of Viscozyme and Ultraflo treatments of cassava cake on the pH and titratable acidity of the cake. **a**: pH change over the period of incubation; **b**: Titratable acidity

Ultraflo and Viscozyme combined showed a strong complementary effect on cassava cake viscosity reduction (Figure 4.1.7). In agreement, the reduction in insoluble solids volume was greater with Viscozyme and Ultraflo combined treatment cake before and particularly after starch hydrolysis compared to separate treatments of the cake (Figure 4.1.8). These observations indicate that Viscozyme and Ultraflo combined were more effective in hydrolysing the cell wall fibre components of the cassava cake. The reduction in volume of the insoluble solids materials in separate Viscozyme and Ultraflo treatments, after starch removal by hydrolysing the cake with α-amylase, did not differ (Figure 4.1.8b). Notably, the effect of the Ultraflo treatment on the cassava cake differed in this experiment compared to the previous one. The Ultraflo treatment in this current experiment resulted in higher viscosity reduction of the cake both before and after starch removal, as observed. This may due to the influence of pH profile of the fresh cassava

cake used in this current experiment favoured the activity of the Ultraflo enzyme compared to low pH of the fermented cassava cake not optmum for the enzyme activity. According to Novozymes (2008), the performance of Ultraflo enzyme in terms of viscosity reduction is optimum at pH 4.9-5.2. As stated, the pH of the cassava cake at the beginning of incubation was pH 6.4 and at the end of incubation period the pH of the Ultraflo treatment was 3.8. This pH profile of the cake may have aided the activity of the Ultraflo.

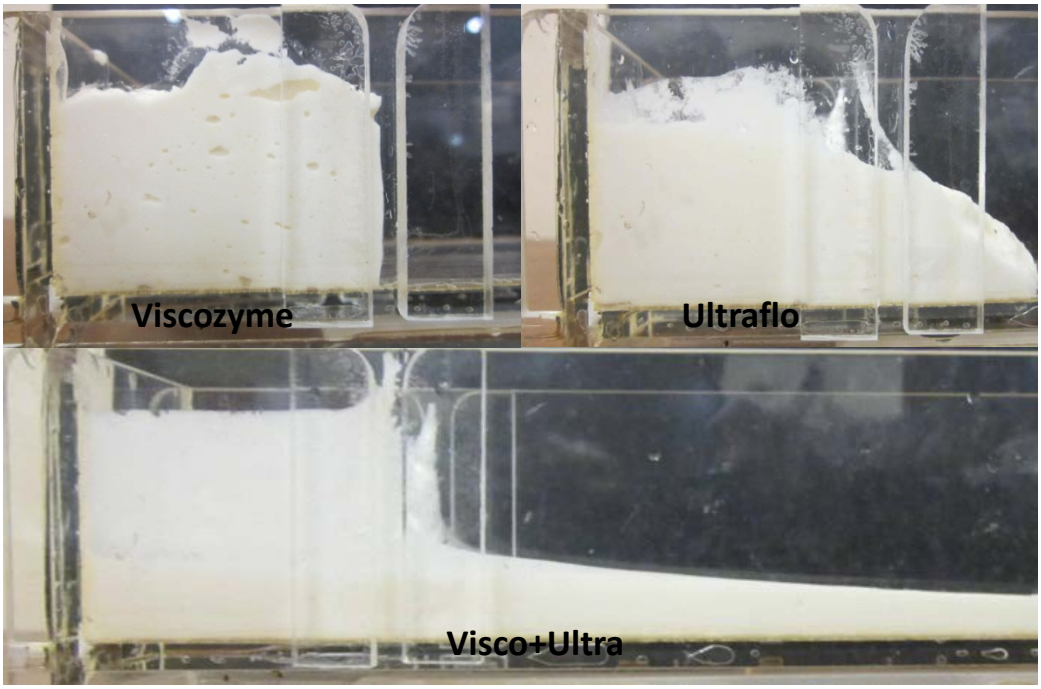


Figure 4.1.7: Effects of Viscozyme and Ultraflo treatments on cassava cake viscosity as determined using a Bostwick viscometer after incubation for 2 weeks

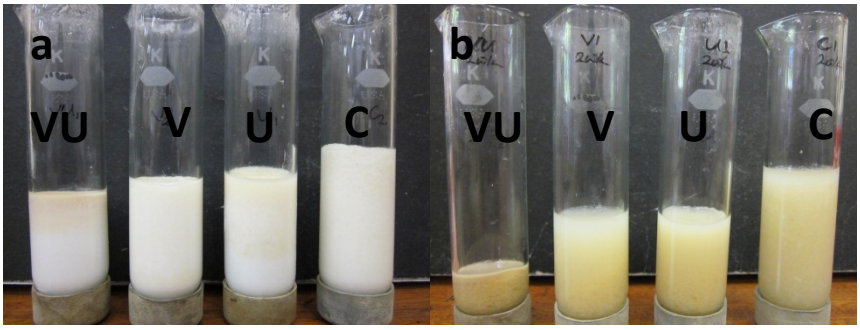


Figure 4.1.8: Effects of Viscozyme and Ultraflo treatments on the volume of insoluble solids of washed cassava slurry after incubating for 2 weeks. **a:** Before starch removal by α-amylase hydrolysis; **b:** After starch removal by α-amylase hydrolysis; **VU:** Visco+Ultra; **V:** Viscozyme; **U:** Ultraflo; **C:** Control

Table 4.1.5: Effects of combining Viscozyme and Ultraflo on the insoluble solids content, cake total starch content and particle size distribution after removal of soluble solids

Treatments	Slurry volume in centrifuge tube (cm^3)	Insoluble solids content (g/100 g cake[2])	Starch content (g/100 g insoluble solids[3])	Starch content (g/100 g cake[2])	Sieved Particle Size Fraction (%)		
					≥500 (µm)	>250 - <500 (µm)	≤250 (µm)
Raw cake[1]		42.5±0.1	84.6±0.8	36.0±0.2			
***Control (1 week)**	nd	46.6	82.4	38.4	54.9^{c}±0.5	2.5^{a}±0.1	42.6^{a}±0.7
Control (2 weeks)	27.4	42.6^{b}±0.9	87.0^{b}±0.3	37.1^{cd}±0.9	54.4^{c}±2.8	3.5^{ab}±0.5	42.0^{a}±2.5
Ultraflo (1 week)	nd	43.8^{b}±1.5	86.0^{b}±0.8	37.7^{d}±1.6	17.6^{b}±2.5	12.2^{d}±4.0	70.3^{b}±1.5
Ultraflo (2 weeks)	23.3	39.1^{a}±0.4	85.8^{b}±1.0	33.6^{b}±0.1	12.6^{a}±1.7	8.1^{bcd}±0.3	79.4^{c}±2.0
Viscozyme (1 week)	nd	43.4^{b}±0.2	81.9^{a}±0.1	35.5^{c}±0.1	12.5^{a}±1.6	8.9^{cd}±2.5	78.8^{c}±4.0
Viscozyme (2 weeks)	21.9	38.6^{a}±0.2	82.4^{a}±0.9	31.8^{a}±0.2	10.1^{a}±0.6	8.2^{bcd}±1.9	81.8^{c}±1.3
Visco+Ultra (1 week)	nd	38.4^{a}±0.5	85.9^{b}±0.2	33.0^{ab}±0.5	10.7^{a}±2.5	6.3^{abc}±3.2	83.2^{c}±5.7
***Visco+Ultra (2 weeks)**	19.2	38.1^{a}±0.4	82.8^{a}±0.4	31.6^{a}±0.5	9.0^{a}±0.5	6.7^{abc}±0.4	84.3^{c}±0.2

***Analysis not repeated; Mean values in the same column but with different letters are significantly different ($p<0.05$); [1]Cassava cake was not washed to remove soluble solids; [2]As is basis, [3]Dry weight basis; nd: Not determined.**

The combined Viscozyme and Ultraflo treatment resulted in the lowest total insoluble solids material left in the cake after washing and centrifuging to remove the soluble solids (Table 4.1.5). The combined treatment and the Viscozyme treatment also resulted in a significant reduction in the starch content of the cake. This may indicate that the Viscozyme enzyme preparation has amylase side activity. Particle size distribution did not differ significantly between the enzyme treatments, but all yielded less large (≥500 µm) and more small (≤250 µm) particle size materials than the untreated control. Light microscopy of the cake insoluble solid materials after incubation also show similar reductions in particle size of the cell wall fibre materials in all the enzyme treatments coupled with increased amounts of freed starch granules (Figure 4.1.9). These observations agree with the data in Table 4.1.5, and this gave a clear indication of combined hydrolytic activity of the enzyme preparations, which resulted in considerable particle size reduction of the cell wall fibre materials in the cake.

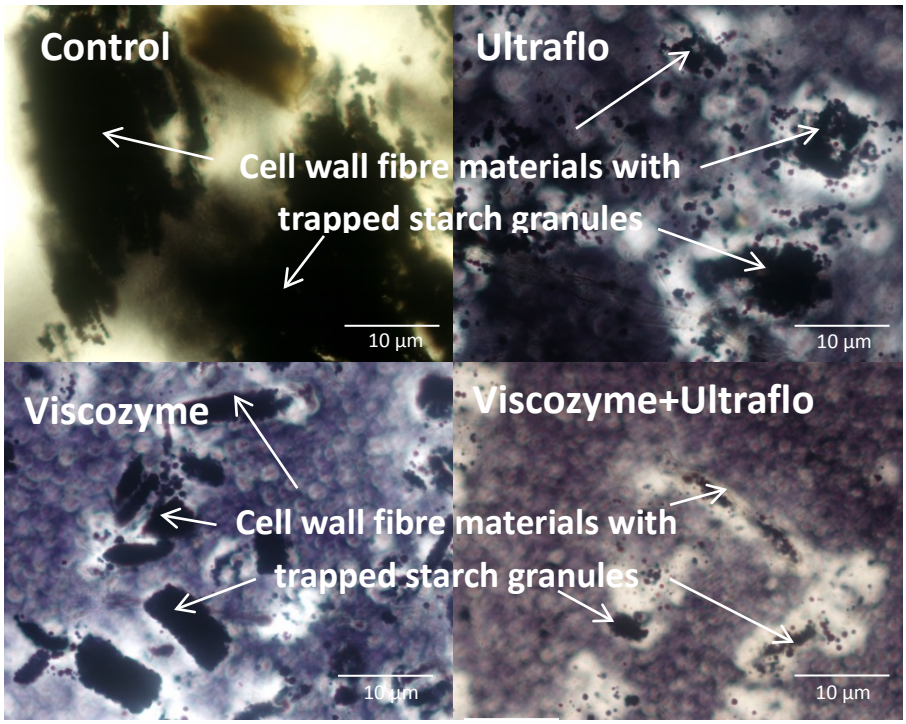


Figure 4.1.9: Light microscopy images of cassava cake showing the effects of Viscozyme and Ultraflo treatments on cell wall fibre materials after incubating for 2 weeks.

The combined Ultraflo and Viscozyme treatment resulted in a very substantial reduction in the amount of insoluble cell wall fibre materials of the cake after enzymatic starch removal (Table 4.1.6). This effect further reflected the combine activities of Viscozyme and Ultraflo in hydrolysing the fibre materials. Also, the enzyme treatment of the cake resulted in a significant reduction in the particle size of the fibre materials compared to the untreated control (Table 4.1.6). The combined Viscozyme and Ultraflo treatment had a higher proportion of large particles compared to their individual treatments. Light microscopy of the cake insoluble solids after removal of starch also revealed considerable differences in the cell wall material appearance (Figure 4.1.10). With the combined enzyme treatment, the cell wall fibre material had disappeared, except for the dark stained lignified material, whereas in the untreated control there were clusters of parenchyma cells. These observations again indicate that the large particle size materials in the combined enzyme treatment were lignified materials that were not hydrolysed, as explained before. The complementary effect of combining these enzyme preparations in reducing the cassava cake fibre materials may be due to the debranching activity of Ultraflo (Novozymes, 2008) aiding the hydrolytic activity of the Viscozyme.

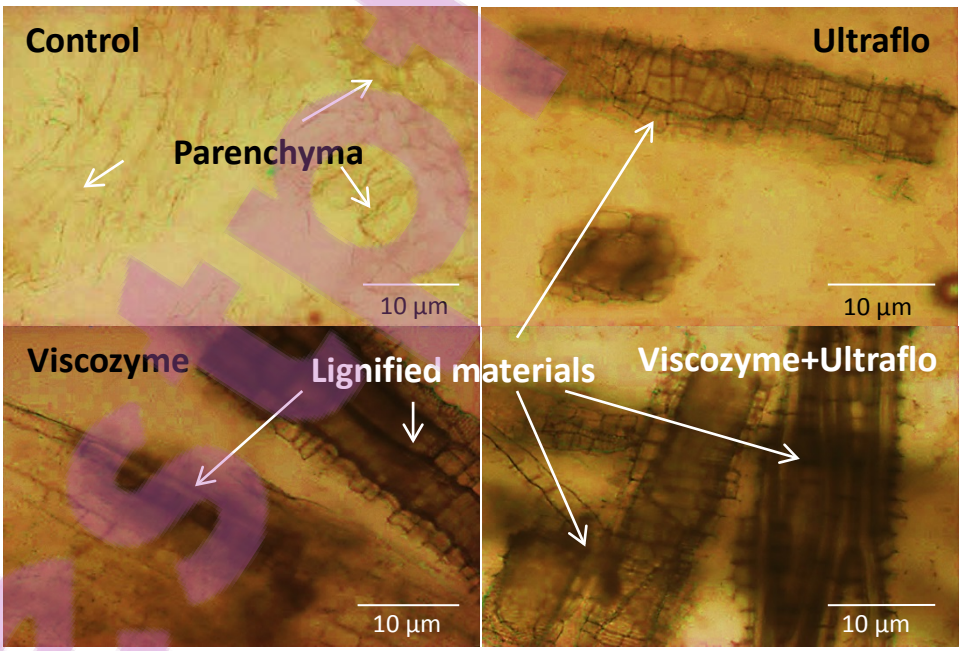


Figure 4.1.10: Light microscopy images of cassava insoluble solid residues after starch removal, showing the effects of Viscozyme and Ultraflo treatments on the cell wall materials.

Table 4.1.6: Effects of combining Viscozyme and Ultraflo on non-starch solids and insoluble fibre contents and particle size of cassava cake

Treatments	Slurry volume in centrifuge tube (cm^3)	Non-starch solids content (g/100 g cake[2])	Insoluble fibre (% solids[3])	Insoluble fibre (g/100 g cake)	Sieved Particle Size Fraction (%) ≥500 (µm)	>250 - <500 (µm)	≤250 (µm)
***Control (1 week)[1]**	nd	8.2	5.7	2.6	92.1^{c}±0.1	3.6^{ab}±1.3	4.0^{a}±1.7
Control (2 weeks)[1]	26.0	5.5^{ab}±0.0	7.2^{d}±0.1	3.0^{d}±0.1	92.1^{c}±1.1	2.4^{a}±0.7	3.0^{a}±0.8
Ultraflo (1 week)[1]	nd	6.1^{bc}±0.1	4.8^{c}±0.2	2.1^{c}±0.0	52.2^{b}±1.8	7.8^{bc}±0.5	39.9^{b}±2.7
Ultraflo (2 weeks)[1]	19.2	5.6^{ab}±0.5	3.6^{abc}±0.9	1.4^{ab}±0.3	44.9^{b}±2.2	8.1^{bc}±1.6	47.2^{b}±1.1
Viscozyme (1 week)[1]	nd	7.9^{e}±0.1	4.4bc±1.0	1.9^{bc}±0.4	29.3^{a}±2.1	12.5^{c}±4.8	57.5^{c}±2.1
Viscozyme (2 weeks)[1]	19.2	6.8^{d}±0.4	3.4^{ab}±0.1	1.3^{a}±0.0	29.0^{a}±1.3	9.4^{c}±0.3	62.4^{c}±0.8
Visco+Ultra (1 week)[1]	nd	5.4^{a}±0.0	3.0^{a}±0.0	1.2^{a}±0.0	33.8^{a}±0.3	8.0^{bc}±0.3	58.7^{c}±0.4
***Visco+Ultra (2 weeks)[1]**	8.2	6.6^{cd}±0.1	3.1^{a}±0.0	1.2^{a}±0.0	49.5	5.4	45.8

***Analysis not repeated; Mean values in the same column but with different letters are significantly different ($p<0.05$); [1]Cassava slurry washed to remove soluble solids; [2]As is basis; [3]Dry weight basis; nd: not determined**

4.1.3.4 Effects of enzymes treatment on cassava cell wall structural components

As analysed by GC, the monosaccharide composition of residual cassava cell wall materials (CWM) included arabinose, rhamnose, xylose, galactose and glucose (Table 4.1.7). Glucose was the major sugar unit of the CWM, and constituted about 85% of the residual material in the control (untreated). Similar data were reported by Salvador *et al*. (2000) with the cellulose fraction of the cassava CWM having 82% glucose. This suggests that cellulosic type material is the main component of cassava CWM. Xylose and galactose composition of the CWM were 3 and 8%, respectively. These levels were much lower than 27% xylose and 38% galactose reported by Salvador *et al*. (2000). A possible reason for this may be due to the activity of lactic acid bacteria present in cassava, resulting in partial hydrolysis of the soluble cell wall components. Likewise, thorough washing of the residual CWM in removing the soluble solids also contributed to the low levels of xylose and galactose reported in this present study.

There was a considerable reduction in glucose level in the enzyme treated residual CWMs compared to the control (Table 4.1.7). The combined Ultraflo and Viscozyme treatment had the lowest glucose level (5 and 8%). In the separate enzyme treatments, the Viscozyme treated residual CWM had slightly lower level of glucose than the Ultraflo treated CWM. There was increase in xylose level with decrease in glucose content of the enzyme treated residual CWMs compared to the control. This shows that Ultraflo and Viscozyme hydrolysed most of the cellulosic type components of the residual cell walls. The level of residual CWM not characterised increased with the enzyme treated residual CWMs. This CWM not characterised may be lignified CWM. As observed, hydrolysis of the non-starch polysaccharides components of the CWM resulted in concentration of the lignified materials. As stated, lignified materials are more resistant to hydrolytic enzymes (Marsden and Gray, 1986).

Table 4.1.7: Sugar composition of cassava cell wall material remaining after treatment with Ultraflo and Viscozyme

Treatments	Arabinose (%)	Rhamnose (%)	Xylose (%)	Mannose (%)	Galactose (%)	Glucose (%)	CWM Not analysed (%)
Control 1 week	1.8	1.4	3.4	0	7.5	134.5	0
Control 2 week	0.8	0.7	1.4	0	1.2	84.8	11.2
Ultraflo 1 week	1.1	0.8	3.3	0	2.3	13.0	79.5
Ultraflo 2 week	3.4	2.3	5.1	0	1.5	24.4	63.2
Viscozyme 1 week	0.4	0.3	4.6	0	1.1	21.6	72.1
Viscozyme 2 week	0.2	0.2	4.7	0	0.7	11.2	83.0
Visco/Ultra 1 week	0.1	0.1	2.0	0	0.6	5.3	91.8
Visco/Ultra 2 week	0.9	0.6	10.9	0	0.5	8.4	78.7

CWM: Cell wall material

Table 4.1.8 shows the relative percentage of sugars in the residual CWM. The enzyme treated samples had lower percentage of glucose compared to the control. However, glucose proportion was much higher in all the treatments than arabinose, rhamnose, and galactose, with the exception of the combined Ultraflo and Viscozyme after two weeks having higher level of xylose. Hydrolysis of the cellulolysic components of the CWM by Ultraflo and Viscozyme resulted in relative concentration of the hemicellulose component of the cell wall materials. In the separate enzyme treatments, Viscozyme treated CWM had a much lower proportion of arabinose and a higher proportion of xylose, while Ultraflo treated CWM had a higher proportion of arabinose and a lower proportion of xylose. This indicates that Ultraflo had more xylan degrading enzyme activity, while Viscozyme had more araban degrading enzyme activity. Mannose was not in any of the treatments. According to Salvador *et al*. (2000), about 3% mannose is present in cassava CWM. The absence of mannose in the characterised residual CWM could suggest its complete solubilisation and removal by washing with water.

Table 4.1.8: Relative percent of sugars in the remaining cell wall material after treatment with Ultraflo and Viscozyme

Treatments	Arabinose (%)	Rhamnose (%)	Xylose (%)	Mannose (%)	Galactose (%)	Glucose (%)
Control 1wk	1.2	1.0	2.3	0	5.0	90.6
Control 2wk	0.9	0.7	1.6	0	1.4	95.3
Ultraflo 1wk	5.2	4.0	15.9	0	11.3	63.6
Ultraflo 2wk	9.4	6.2	13.9	0	4.1	66.3
Viscozyme 1wk	1.4	1.0	16.3	0	4.0	77.3
Viscozyme 2wk	1.2	1.4	27.7	0	3.8	65.9
Visco/Ultra 1wk	1.1	1.7	24.6	0	7.5	65.2
Visco/Ultra 2wk	4.2	2.7	51.3	0	2.2	39.5

The proportion of identified glucosidic bonds in the residual CWM after hydrolysis with combined Ultraflo and Viscozyme was very low compared to the untreated control (Table 4.1.9). The relative percentage of β-(1-4) glucosidic linkages in all the treatments was much higher than β-glucosidic terminal and β-(1-6) glucosidic bonds. Combined activities of Ultraflo and Viscozyme enzymes on the cell walls indicated that both β-glucosidic terminal and β-(1-6) glucosidic bonds were completely hydrolysed with exception of the β-(1-4) glucosidic linkages in the residual CWM. The high proportion of glucosidic bonds in the untreated control CWM suggests that both the cellulosic type and hemicellulose components of the CWM were still intact. The Ultraflo residual CWM had a lower proportion of branched linkages and β-(1-4) glucosidic linkage than Viscozyme treated residual CWM. This may be related to the debranching activity of the Ultraflo (Novozyme, 2008). This implies that Ultraflo debranching action on the cell wall matrix polysaccharides gave Viscozyme more access to the cellulose component embedded in the cell wall structure. The proportion of α-(1-4) glucosidic linkages was much higher in the control CWM than the enzyme treated samples. Presence of α-(1-4) linkages in the residual CWM may be due to starch contamination resulting from starch granules still trapped within the CWM. The absence of β-(1-3) glucosidic bond indicates that cassava CWM does not contain β-glucan. The increase in unknown linkages with enzyme action is evidence of unhydrolysed lignified cell wall material.

Table 4.1.9: Relative percentage of different glycosidic linkages in cassava cell wall material remaining after treatment with Ultraflo and Viscozyme

Treatments	Beta-terminal (%)	Beta-1-6 (%)	Beta-1-4 (%)	Sum of beta linkages (%)	Alpha-terminal (%)	Alpha-1-6 (%)	Alpha-1-4 (%)	Sum of alpha linkages (%)	Unknown (%)
Control 1 week	5.9 (11.8)	0.6 (1.2)	43.5 (87.1)	50.0	1.3 (7.1)	0.2 (0.9)	17.4 (91.8)	18.9	31.1
Control 2 week	6.1 (12.9)	0.4 (0.9)	40.6 (86.1)	47.2	2.2 (12)	0.1 (0.8)	16.1 (87.2)	18.5	34.3
Ultraflo 1 week	0	0	10.4 (100)	10.4	0	0	1.7 (97.1)	1.7	87.9
Ultraflo 2 week	0.6 (3.9)	0.3 (1.9)	13.4 (94.4)	14.2	0	0	4.5 (100)	4.5	81.2
Viscozyme 1 week	0.5 (1.6)	0.7 (2.2)	29.4 (96)	30.6	0	0	9.2 (100)	9.2	60.2
Viscozyme 2 week	2.6 (7.2)	0.8 (2.3)	32.4 (90.4)	35.8	0.7 (5.8)	0	11.2 (94.2)	11.9	52.3
Visco/Ultra 1 week	0.1 (0.8)	0.5 (3.9)	13.3 (95.3)	14.0	0	0.2 (4.1)	4.6 (95.9)	4.8	81.2
Visco/Ultra 2 week	0	0	7.3 (100)	7.3	0	0	2.0 (99)	2.0	90.7

***Values in parentheses are relative percent of the glucosidic linkages**; ****Alpha-1-4 linkages due to starch contamination**

4.1.4 CONCLUSIONS

Enzymatic treatment of cassava root tubers with cellulolytic and hemicellulolytic enzyme preparations result in considerable structural and compositional modifications of cassava tuber cell walls. A combined enzyme preparation treatment has complementary effect in hydrolysing the parenchyma cell walls due to presence of multiple enzyme activities. This complementary activity results in a more hydrolysis of the cellulosic and hemicellulosic parenchyma cell walls. Hydrolysis of these cellulosic and hemicellulosic polymers effectively reduces water holding capacities of these CWMs. The remaining insoluble cell wall material lignified, the enzymes could not hydrolyse. Application of combined Viscozyme and Ultraflo treatment to cassava parenchyma cell walls can potentially improve wort filtration in brewing.

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4.2 RESEARCH CHAPTER

Effects of treating milled tannin sorghum grain with dilute NaOH solution and soy protein on the beverage and brewing/bioethanol making quality of tannin sorghum

ABSTRACT

Utilisation of tannin sorghums is limited due to the condensed tannins interacting with other grain components. This interaction negatively affects the beverage and brewing/bioethanol making quality attributes of tannin sorghums. Type II and III tannin sorghums were selected for investigation of tannin inactivation by steeping the flours in 0.1, 0.2 and 0.4% NaOH solution. Tannin inactivation was also investigated by admixing with soy protein isolate. Sodium hydroxide steeping of the flour resulted in a substantial reduction in assayable total phenols and tannins. The effect of NaOH on the tannins in the Type III sorghum also resulted in 60-80% reduction in α-amylase enzyme inhibition by the tannins. Fermentability of the Type III sorghum by lactic acid bacteria improved with NaOH steeping. Soy protein isolate treatment of Type III sorghum resulted in 76% reduction in α-amylase enzyme inhibition by the tannins. Applying these treatments in a mashing process also resulted in a considerable reduction in starch liquefaction time with the Type III sorghum. The method of pre-treatment of milled Type III tannin sorghums in dilute NaOH could enable their utilisation, especially in bioethanol production. Application of NaOH treatment to tannin sorghum in the production of fermented matoho beverage is not necessary.

4.2.1 INTRODUCTION

Sorghum is unique compared to other major cereals in that some cultivars contain condensed tannins (proanthocyanidins or procyanidins) (Bullard, Garrison, Kilburn and York, 1980), which are polymeric phenolics. The condensed tannins in these sorghums confers on them agronomic benefits by protecting the grain from both bird and mould attack (Bullard and York, 1996; Waniska, 2000). However, food and brewing/bioethanol processing applications of condensed tannin sorghum types are limited. This is linked to the negative effects of the tannins on nutritional and functional properties of the products, resulting from interactions between the tannins and other grain components, particularly proteins (Emmambux and Taylor, 2003). The mechanism of tannin interaction with protein involves hydrogen bonding (Emmambux and Taylor, 2003) coupled with hydrophobic interaction (Oh, Hoff, Armstrong and Haff, 1980). Sorghum tannin interaction with proteins can be prevented by grain pre-processing steps such as chemical treatments (Beta, Rooney, Marovatsanga and Taylor, 2000).

Steeping whole tannin sorghum grain in dilute NaOH solution as is applied in malting reduces the negative effects of the tannins on malt enzyme activities (Dewar, Orovan and Taylor, 1997; Elmaki, Babiker and Tinay, 1999; Beta *et al*., 2000). Beta *et al*. (2000) suggested that the mechanism of inactivation of tannins when sorghum is steeped in NaOH is due to oxidative polymerisation, resulting from oxidation of the phenolic groups under moist conditions, as proposed by Porter (1992). However, dilute NaOH steeping of whole tannin sorghum grain was found not to be effective in inactivating tannins when the sorghum was then milled and used in brewing (Adetunji, 2011). This is because the tannins still negatively affected brewing quality attributes.

In brewing/bioethanol fermentation, soy flour is often used as a nutrient-rich supplement in improving yeast performance (Viegas, Sa-Correia and Novias, 1985). The negative effects of tannins on tannin-containing sorghums nutritional and functional properties could also be addressed by treatment with proline-rich proteins such as soy flour. According to Baxter, Lilley, Haslam and Williamson (1997), tannins are noted to have strong affinity for proline-rich proteins.

Matoho/Mageu is a traditional southern Africa non-alcoholic sorghum-based beverage product (reviewed by Parawira and Muchuweti, 2008), which is now being commercialised. This product is largely formulated using decorticated non-tannin sorghum flour and the manufacturing process may involve natural fermentation with lactic acid bacteria or acidification with food grade acids such as citric acid. Using tannin type sorghums in the production of matoho beverage could increase tannin sorghum utilisation.

Therefore, in this present work, application of NaOH and soy protein isolate flour treatments to milled tannin sorghum grain was investigated because sorghum is utilised mostly in flour form in brewing, bioethanol production and sorghum-based beverage products.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Materials

Two tannin sorghum grain cultivars and one non-tannin cultivar were used: PAN 3860 (Type III tannin, with red pericarp); White tannin sorghum (Gadam El Hamam-type) from Zimbabwe (Type II, with white pericarp) and MR Buster (Type I, non-tannin with red pericarp). Whole grain was milled using a hammer mill (Falling Number, Huddinge, Sweden) fitted with a 1.0 mm opening screen. The milled samples were stored in zip-lock type polyethylene bags at 6-8°C until analysis.

Commercial soy protein isolate products (SPI): XT 40, FP 950 and 783 IP, were kindly donated by Danisco SA (Johannesburg, South Africa). Commercial enzyme preparations: Termamyl SC (α-amylase), Cerezyme 2X Sorghum enzyme (cocktail of polysaccharide degrading and protease enzymes designed for brewing with up to 100% sorghum) and Fungamyl Brew Q enzyme (fungal α-amylase), were kindly donated by Novozymes SA (Benmore, South Africa).

A freeze dried-concentrated lactic acid bacteria (LAB) YO-E (F 8053) starter culture composed of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii*

subsp *bulgaricus*, for mageu-type (fermented) cereal beverages was kindly donated by Cape Food Ingredients (Johannesburg, South Africa).

Samples of Mageu (Original, Number 1) (Foodcorp, Pretoria, South Africa) a non-alcoholic maize beverage product, with different best before dates were purchased all at the same time and used as a cereal-based beverage reference standard. Commercial matoho products with different formulations were purchased in South Africa and Lesotho and used as references for the matoho products made from tannin sorghums.

4.2.2.2 Methods

4.2.2.2.1 Tannin inactivation by steeping in dilute NaOH solution

Distilled water containing 0.2% and 0.4% (w/w) NaOH (30 ml) was pre-heated to 50°C in a 500 ml stainless mashing beaker. Sorghum flour (10 g) was weighed into the mashing beaker and stirring commenced. Continuous stirring was maintained during steeping for 5 and 30 min. At the end of the steeping times, the pH was adjusted to pH 5.6-5.9 with 1.5 M HCl. The slurries at the end of steeping were freeze dried and the freeze dried flours stored in a zip-lock type polyethylene bags at 6-8°C until analysis.

4.2.2.2.2 Tannin inactivation with addition of SPI

Suspensions of SPI ranging from 50 to 1000 µg were prepared from stock solution of SPI (1 g/100 ml). Soy protein isolate suspension (100 µl) was added into a test tube containing 10 mg sorghum flour and this served as reaction mixture for α-amylase assay.

4.2.2.2.3 Mashing

Brewing type mashing was conducted using a BRF mashing bath (Brewing Research Foundation, Nutfield, UK).

The pre-treatment of the milled tannin sorghum was by (a) steeping in dilute NaOH solution and (b) admixing with SPI:

(a) NaOH pre-treatment of the mash was by weighing milled sorghum samples (100 g dry weight basis) in 150 ml 0.1%, 0.2% and 0.4% (w/w) NaOH on a flour basis in mashing beaker and pre-heated to 50 ºC. Continuous stirring was maintained during steeping for 5 and 30 min. Then the volume of the mash was made up to approx. 320 ml with distilled water containing 365 mg/L calcium chloride (130 ppm calcium) to give a

grist/liquor ratio of 1:3. Calcium ions were added to stabilize enzyme activity (Agu, 2006).

(b) Treatment with SPI was by admixing with milled tannin sorghum in a ratio 1 : 9 (100 g dry weight basis) into approx. 320 ml distilled water containing calcium chloride (approx. 130 ppm with respect to sorghum flour) to give a grist/liquor ratio of 1:3.

The pH was adjusted to pH 5.6-5.8 with a few drops of orthophosphoric acid. Mashing was carried out as described by Adetunji, Khoza, De Kock and Taylor (2013), with the following modifications. The concentration of Cerezyme 2X Sorghum enzyme added was 16 000 ppm with respect to milled sorghum grain. The mash was cooked at 94 °C for 45 min and cooled to 70 °C. Then, a further 5 mL of Cerezyme 2X Sorghum enzyme (16 000 ppm with respect to milled sorghum grain) was added and the mash rested for 15 min and then cooled to 58 °C.

4.2.2.2.4 Preparation of matoho beverage

Milled sorghum (70 g dry weight basis) was mixed with 1 L distilled water containing 0.1% and 0.2% (w/w) NaOH on a flour basis in a plastic beaker and pre-heated to 50 ºC. Continuous stirring was maintained for 30 min. At the end of the steeping time, the pH was adjusted to pH 5.6-5.9 with 1.5 M HCl.

The matoho was prepared as follows: Sorghum flour (70 g) and sucrose (70 g) were suspended in 1 L water with stirring. The gruel was then cooked on electric hotplate for 30 min, with continuous stirring to prevent lump formation. The cooked gruel was cooled down to ambient temperature (24ºC). Acidification of matoho without lactic acid fermentation was by adding food grade citric acid (2.5 g/L) to obtain a final pH of approx. 3.5 in the product. The matoho product was preserved by potassium sorbate (600 ppm per L) and stored at 4ºC.

Fermentation of matoho was by inoculating with 0.1 g freeze dried LAB starter culture/L and incubating at 42ºC for 24 hours to obtain a final pH of about 3.5. At the end of the fermentation period, the matoho was preserved by adding potassium sorbate and stored at 4ºC.

The fermentability of the cooked and uncooked tannin sorghum matoho was determined by inoculating with 0.1 g freeze dried LAB starter culture/L. The cooked tannin sorghum matoho was incubated at 42ºC for 24 h, while the uncooked tannin matoho was incubated at 42ºC for 48 h. The pH was monitored at intervals.

4.2.2.3 Analyses

4.2.2.3.1 Tannins and total phenols

Tannin content was determined by using the modified Vanillin-HCl method of Price, Van Scoyoc and Butler (1978) and expressed in g catechin equivalents (CE)/100 g. Total phenols content determined using the Folin-Ciocalteu method described by Waterman and Mole (1994) and expressed in g CE/100 g.

4.2.2.3.2 Alpha-amylase inhibition

Alpha-amylase inhibition was determined using the Megazyme Alpha-Amylase Assay Procedure (Ceralpha Method) (Megazyme International, 2011), with slight modifications. The reaction mixture was made up of BPNPG7 substrate (0.2 mL) and 10 mg freeze dried sorghum samples steeped in NaOH solutions and milled sorghum samples admixed with different concentration of SPI. The Termamyl SC α-amylase (300 ppm with respect to flour) in 0.1 M HEPES buffer (pH 6.9) was added to the reaction mixture.

4.2.2.3.3 Brewing/bioethanol quality attributes

Liquefaction time- was determined by measuring the duration from when the mash stopped stirring due to starch gelatinisation until the mash began to stir, with a magnetic stirrer bar alone (i.e. with additional manual stirring).

Wort Extract- by specific gravity and expressed in °Plato according to European Brewery Convention (1998) Method 6.6.

Wort FAN- by ninhydrin colorimetry according to European Brewery Convention (1998) Method 8.10.

Wort fermentable sugar spectrum- by HPLC according to European Brewery Convention (1998) Method 8.5 Fermentable Carbohydrates. A high-performance liquid chromatograph (HPLC) fitted with refractive index detector and Rezex RHM-

Monosaccharide column: 300 x 7.8 mm, Rezex H+ (8%) Monos. (Phenomenex, Torrance, CA) was used.

4.2.2.3.4 Matoho beverage physicochemical quality attributes

pH and titratable acidity- was determined by using a pH meter and titratable acidity according to GEA Niro analytical method A 19 a (GEA Niro, 2006) and expressed in percentage lactic acid.

Viscosity- in terms of flowability was determined using a Bostwick viscometer. Matoho (100 g) was poured into the Bostwick cell with the bridge closed. The bridge was opened completely to allow the sample to flow down the trough section for 30 seconds and the length covered was recorded in mm.

Total solids content- was determined by drying 100 ml beverage at 103ºC for 24 h (AOAC, 2002).

4.2.2.4 Statistical analyses

All experiments were repeated at least once because the experiments were repeatable. Data were analysed by one-way analysis of variance (ANOVA) in relation to the effect of treatment on tannin properties of each sorghum types. Significant differences among the means were determined by Fisher's least significant difference (LSD) test at $p<0.05$.

4.2.3 RESULTS AND DISCUSSION

As stated, red tannin (Type III), white tannin (Type II) and red non-tannin sorghum cultivars were investigated. This was with the aim of developing a method of inactivating condensed tannins in sorghum flour. Table 4.2.1 shows the results of a preliminary brewing mashing study to determine the potential of pre-steeping the flour in dilute NaOH. With the untreated tannin sorghum flour (rested in water), the mash turned to a very stiff porridge without any extract being produced. This was attributed to inactivation of the α-amylase by the condensed tannins. Pretreatment of the tannin sorghum flour in NaOH solution resulted in liquefaction of the starch. The NaOH treated tannin sorghum flour samples mashed with α-amylase had an extract yield close to that obtained from the non-tannin sorghum flour rested in water. This is presumably due to interactions between

the condensed tannins and NaOH resulting in inactivation of tannins. These findings suggested that NaOH pretreatment of flour had potential as a process for inactivating tannins.

Table 4.2.1: Effects of NaOH treatment of sorghum flour on mash liquefaction time, wort volume and extract yield

Sorghum types	Treatment	Liquefaction time (min)	Wort volume (ml)	Extract °Brix	Extract (%)
Type III tannin sorghum (PAN 3860)	***Control**	Was not liquefied	0	0	0
	0.1% NaOH[1]	75	290	15.6	64.7
	0.2% NaOH[1]	45	313	17.0	71.7
Red non-tannin sorghum (MR Buster)	***Control**	5	320	17.4	73.7
	0.1% NaOH[1]	5	335	18.7	80.5
	0.2% NaOH[1]	5	340	18.8	81.0

***Sorghum flour rested in water for 1 hr, [1]Sorghum flour rested in NaOH solution for 1 hr and then adjusting the pH to 5.**

4.2.3.1 Effect of NaOH steeping on tannins and total phenols

A more comprehensive experiment showed that steeping in NaOH solution resulted in a reduction in the level of assayable tannins and total phenolics of the tannin sorghum types (Table 4.2.2). Steeping the milled high tannin (Type III) sorghum in water resulted in a substantial reduction in assayable tannin and total phenolic contents. With increasing NaOH and steeping time there was a progressive further decrease in assayable tannin and total phenol contents. Steeping in 0.4% NaOH for 30 min resulted in an approx. 76% and 48% reduction in assayable tannins and total phenols, respectively. Steeping the milled low tannin (Type II) sorghum in water and NaOH solution resulted in a proportionally somewhat smaller reduction in assayable tannin and total phenol contents. However, a similar trend with regard to the effects of NaOH concentration and steeping time on the level of assayable tannins and total phenol was obtained. Steeping in 0.4% NaOH for 30

min resulted in an approx. 51% and 41% reduction in assayable tannins and total phenols, respectively. As expected, tannins were barely detected in the red non-tannin sorghum (Type I). However, steeping in 0.4% NaOH resulted in an approx. 38% reduction in total phenol content. These results were similar to those of Beta *et al*. (2000), who found a considerable reduction in the assayable tannin content of whole sorghum grain when steeped in dilute NaOH solution. Babiker and El Tinay (1992) noted that the level of reduction in assayable tannin content in sorghum is dependent on alkali concentration and steeping time, as observed in this present study.

Table 4.2.2: Effects of NaOH steeping of sorghum flours on tannin and total phenol contents

Sorghum types	Treatments	Tannin content (g/100 g dwb)		Total phenol content (g/100 g dwb)	
		5 min steeping	30 min steeping	5 min steeping	30 min steeping
Type III (red tannin)	**Raw flour**	4.53^{c}		1.34^{e}	
	Water	Not determined	1.83^{b}(-59.6)	Not determined	0.92^{cd} (-31.3)
	0.2%	1.99^{b} (-56.1)	1.66^{b} (-63.4)	0.94^{d} (-29.9)	0.90^{bc} (-32.8)
	0.4%	1.62^{b} (-64.2)	1.11^{a} (-75.5)	0.86^{b} (-35.8)	0.70^{a} (-47.8)
Type II (white tannin)	**Raw flour**	1.07^{c}		0.75^{e}	
	Water	Not determined	0.84^{bc} (-21.5)	Not determined	0.53^{d} (-29.3)
	0.2%	0.75^{abc} (-29.9)	0.74^{abc} (-30.8)	0.51^{cd} (-32.0)	0.48^{bc} (-36.0)
	0.4%	0.63^{ab} (-41.1)	0.52^{a} (-51.4)	0.46^{ab} (-38.7)	0.44^{a} (-41.3)
Type I (red non-tannin)	**Raw flour**	0.25^{b}		0.21^{c}	
	Water	Not determined	0.06^{a} (-76.0)	Not determined	0.22^{c} (+4.5)
	0.2%	0.05^{a} (-80.0)	0.04^{a} (-84.0)	0.22^{c} (+4.5)	0.17^{b} (-19.0)
	0.4%	0.03^{a} (-88.0)	0.01^{a} (-96.0)	0.17^{b} (-19.0)	0.13^{a} (-38.1)

Mean values in the same block within each sorghum types with different letters are significantly different ($p<0.05$); Values in parentheses are percentage increase or decrease; n= 2.

4.2.3.2 Effects of NaOH steeping of sorghum flour on brewing/bioethanol quality attributes

The level of tannin inactivation in milled sorghum obtained by NaOH treatment in a model brewing/bioethanol production system was determined by α-amylase inhibition

(Table 4.2.3). Steeping in water reduced α-amylase inhibition in the Type III tannin sorghum, but to a much lesser extent than the reduction in assayable tannin content approx. 23%, as against 60% (Table 4.2.2). With increase in NaOH concentration, tannin inhibition of α-amylase was reduced considerably. Steeping in 0.4% NaOH solution for 5 min resulted in an approx. 83% reduction in α-amylase inhibition. This high level of reduction in inhibitory activity of tannins in the NaOH treated samples may be attributed to interaction between NaOH and tannins. Both NaOH concentration and steeping time reduced tannin inhibition. However, the white tannin (Type II) and the red non-tannin sorghum flour did not have any inhibitory activity against α-amylase. In the case of the Type II sorghum, this is probably related to the low level of extractable tannins in this type of sorghum. In Type II sorghums, tannins are bound within the cell walls of the testa layer (Earp, McDonough, Awika and Rooney, 2004). In the case of the red non-tannin sorghum, tannins were absent (Table 4.2.2).

As observed in the preliminary work, the NaOH treatment substantially reduced the liquefaction time of the Type III tannin milled sorghum mash (Table 4.2.3). The rate of mash liquefaction increased considerably with increase in NaOH concentration and steeping time. Steeping in 0.4% NaOH solution resulted in an average 57% reduction in mash liquefaction time. The Type III sorghum mash treated in NaOH solution for 30 min liquefied faster than the mash treated for 5 min. This improvement in liquefaction time of the NaOH treated mash can be attributed to the reduction in α-amylase inhibition, by the NaOH chemically altering the condensed tannins. The Type II tannin sorghum mash liquefied at the same rate as the non-tannin sorghum. Again, this is probably related to the low level of extractable tannins in Type II tannin sorghum.

Sodium hydroxide pretreatment had significant effects on the wort free amino nitrogen (FAN) of the two tannin sorghum types (Table 4.2.3). With NaOH treatment of the milled Type III sorghum, there was an increase in FAN content with increase in NaOH concentration. Steeping in 0.4% NaOH resulted in an average 14% increase in FAN. The increase in FAN is presumably due to an effect of the NaOH on the tannins, which limited the interaction between the tannins and the proteins that results in the formation insoluble tannin-protein complexes (Emmambux and Taylor, 2003). However, the level

of wort FAN from the Type III tannin sorghum was very low compared to the other sorghum types. This low wort FAN from Type III tannin sorghum is similar to previous findings (Adetunji *et al*., 2013). This may be attributed to effect of milling enabling tannin interaction with protein forming insoluble complexes, hence reducing the amount of available protein in the milled sorghum grain.

In contrast, NaOH treatment of Type II tannin sorghum resulted in a reduction in wort FAN with increase in NaOH concentration, up to approx. 24% with steeping in 0.4% NaOH. With the red non-tannin sorghum there was no effect of NaOH treatment on FAN. The reduction in FAN in the Type II tannin sorghum with NaOH steeping may be due to denaturation of protein by the NaOH. Alkali treatment of protein results in losses of some amino acids and alteration of others (Nashef, Osuga, Lee, Ahmed, Whitaker and Feeney, 1977). Such reactions may have altered protein structure, thereby limiting it susceptibility to enzymatic hydrolysis.

Table 4.2.3: Effects of NaOH treatment of sorghum flour on α-amylase inhibition, mash liquefaction and wort free amino nitrogen (FAN)

Sorghum types	NaOH (%)	α-amylase inhibition (%)		Liquefaction time (min)			FAN (mg/l)		
		5 min steeping	30 min steeping	5 min steeping	30 min steeping	Mean	5 min steeping	30 min steeping	Mean
Type III (red tannin)	**Raw flour**	74.2^{d}		Not applicable	NA	NA	NA	NA	NA
	Water	Not determined	56.9^{c}(-23.3)	90	85	87.5^{b}	22.5	21.2	21.9^{a}
	0.1	ND	ND	80	70	75.0^{b} (-14.3)	24.3	23.0	23.7^{ab} (+7.6)
	0.2	19.3^{b} (-74.0)	22.1^{b} (-70.2)	50	45	47.5^{a} (-45.7)	25.2	25.2	25.2^{b} (+13.1)
	0.4	12.6^{a} (-83.0)	19.7^{b} (-73.5)	45	30	37.5^{a} (-57.1)	25.2	25.6	25.4^{b} (+13.8)
Type II (white tannin)	**Raw flour**	0.0^{a}		NA	NA	NA	NA	NA	NA
	Water	ND	1.3^{a}	10	10	10	40.6	41.9	41.3^{b}
	0.1	ND	ND	10	10	10	37.5	41.5	39.5^{b} (-4.4)
	0.2	0.8^{a}	2.1^{a}	10	10	10	37.5	33.1	35.3^{ab} (-14.5)
	0.4	0.7^{a}	1.5^{a}	10	10	10	29.6	33.6	31.6^{a} (-23.5)
Type I (red non-tannin)	**Raw flour**	0.0^{a}		NA	NA	NA	NA	NA	NA
	Water	ND	1.0^{a}	10	10	10	39.3	45.9	42.6^{a}
	0.1	ND	ND	10	10	10	45.0	45.0	45.0^{a}
	0.2	0.0^{a}	1.0^{a}	10	10	10	34.9	47.2	41.1^{a}
	0.4	0.0^{a}	0.0^{a}	10	10	10	37.5	49.4	43.5^{a}

Mean values in the same block or column within each sorghum types with different letters are significantly different ($p<0.05$); *Raw flour was without steeping in water or NaOH solution; NA: Not applicable; ND: Not determined; Values in parentheses are percentage increase or decrease.

4.2.3.3 Effects of inactivating tannins with SPI on brewing/bioethanol quality attributes

Soy protein isolate was investigated as a means of inactivating the tannins. This is because, as stated, soy flour is used as yeast food supplement in improving yeast performance during brewing/bioethanol fermentation process (Viegas *et al*., 1985). As shown in Figure 4.2.1, there was a reduction in inhibition of α-amylase which was directly proportional to SPI concentration relative to milled Type III tannin sorghum in the reaction mixture. A concentration of 1 mg SPI per 10 mg milled tannin sorghum resulted in 17% inhibition of the α-amylase compared to 72% inhibitory activity without SPI addition. Tannin inhibition of α-amylase enzyme was reduced to approx. 76% by admixing SPI with milled tannin sorghum, which falls within 74% and 83% reduction in tannin inhibitory activity by steeping milled tannin sorghum in 0.2 and 0.4% NaOH solution for 5 min, respectively (Table 4.2.3). This reduction in tannin inhibitory activity of α-amylase can be linked to the SPI interacting with the tannins.

Treatment of milled Type III tannin sorghum with SPI also dramatically reduced mash liquefaction time (Table 4.2.4). In fact, there was 94% reduction in liquefaction time compared to 57% reduction by steeping in 0.4% NaOH solution (Table 4.2.3). Generally, treatment of Type III tannin sorghum with all the three SPI products resulted in a considerable improvement in brewing/bioethanol quality attributes compared to the control (Table 4.2.4). There were significant differences in the effects of specific SPI products on the brewing/bioethanol quality attributes. Treatment with product FP 950 resulted in the highest extract, while product XT 40 gave the lowest total fermentable sugars, less than the control.

There was substantial increase in wort FAN with the SPI treatment. This may be due to the high level of soluble proteins in these SPI products. The substantial differences in FAN between the different SPI products may relate to difference in soluble protein content. This can be linked to differences in composition in terms of their peptide amino acid sequences and functionality (DuPont, 2014). According to the manufacturer, SPI products are soluble in neutral to slightly acidic pH condition. Variations in FAN content correspond with the levels of FAN determined in the SPI products (Table 4.2.5).

The improvement in the brewing/bioethanol quality attributes of milled Type III tannin sorghum can be attributed to the interaction between tannins and soy protein, thereby limiting tannin interaction with the brewing enzymes. This interaction between tannins and soy protein is presumably due to the ability of sorghum condensed tannins to complex with proteins (Emmambux and Taylor, 2003). This tannin-protein interaction is stabilised by hydrogen bonding between the phenolic OH group and the peptide carbonyl group (Hagerman and Butler, 1980). Condensed tannin interaction with proteins is noted to be specific and selective in manner (Hagerman and Butler, 1981). Proteins rich in proline have high affinity for condensed tannins (Hagerman, Rice and Ritchard, 1998; Emmambux and Taylor, 2003). This is because polypeptides with high proline content are strong hydrogen bond acceptors (Hagerman and Butler, 1981). The proline content of SPI is quite high, ranging between 4.5-5.0 g/100 g (Titi Tudorancea Bulletin, 2014).

Inactivation by soy protein treatment of milled Type III tannin sorghum similar to the level obtained by NaOH treatment required admixing SPI with milled sorghum in a ratio of 1:10 (w/w). This implies that a huge quantity of soy protein would be required and hence this method of tannin inactivation is probably not economical. Therefore, only the NaOH method was investigated further.

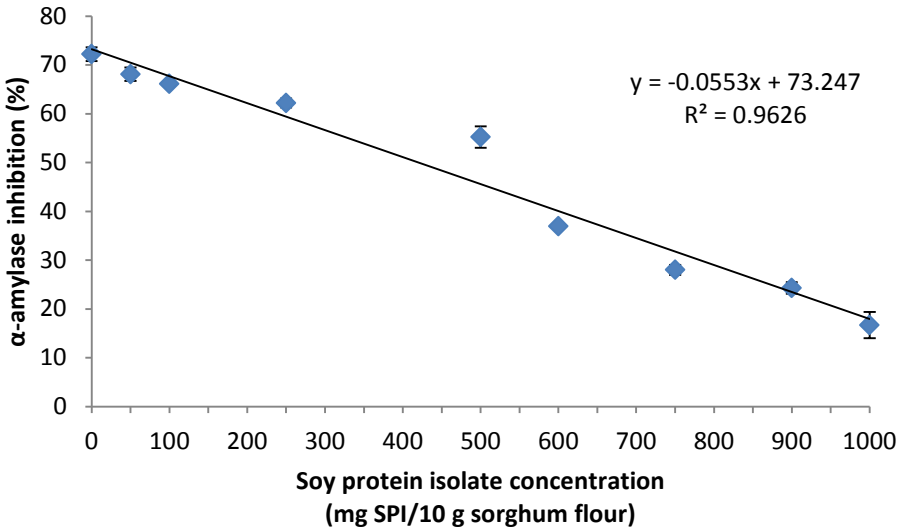

Figure 4.2.1: Effect of soy protein isolate (XT 40) on Type III tannin sorghum inhibition of α-amylase; Error bars indicate standard deviations (n=2).

Table 4.2.4: Effects of admixing soy protein isolate with sorghum flour on liquefaction time and brewing/bioethanol quality attributes

Treatment	Liquefaction time (min)	Extract (%)	FAN (mg/l)	Total fermentable sugars (g/100 ml)
Control Type III*	90	46.7[a]	21.4[a]	7.7[b]
XT 40+Type III**	5	66.5[b]	117.8[c]	5.6[a]
FP 950+Type III**	5	71.1[c]	219.2[d]	11.5[c]
783 IP+Type III**	5	65.5[b]	58.2[b]	11.9[c]

Mean values in the same column with different letters are significantly different (p<0.05); *Only sorghum flour; **1:9 ratio of SPI to sorghum flour.

Table 4.2.5: Soluble proteins in soy protein isolate products suspended in water

SPI products	FAN (g/kg)
XT 40	3.0
783 IP	2.5
FP 950	6.0

4.2.3.4 Effects of sorghum type and NaOH treatment on matoho physicochemical properties

Mageu made from maize meal is a well-established commercial cereal-based beverage product, and this served as reference standard in this study. The matoho products made from decorticated milled non-tannin sorghum grain are new commercial products based on a traditional Lesotho fermented beverage (Gadaga, Lehohla and Ntuli, 2013). The mageu and the matoho products were characterised in terms of their physicochemical attributes for comparison (Table 4.2.6). In this study, tannin sorghum matoho products were made in order to determine how tannin sorghum grain and NaOH treatment affect matoho physicochemical properties, with reference to commercial matoho products.

The pH of the mageu samples ranged between 3.5 and 3.6, while the total titratable acidity was approx. 0.2 % lactic acid equivalent. The pH and titratable acidities of the matoho varied somewhat from 3.5 to 3.9 and between 0.25 and 0.36 % lactic acid. Both were significantly higher than those of the mageu products, except for the Seqhaqhabola matoho where the pH was same. As indicated, the matoho products, with the exception of Seqhaqhabola, were naturally fermented with lactic acid bacteria. Seqhaqhabola matoho

was acidified with citric acid. This shows that the natural fermentation process did not result in as great reduction in pH as acidification with citric acid.

The viscosity of the mageu samples varied substantially, from free flowing to very viscous as the product became old. This may be attributed to low storage temperature of the mageu products (approx. 6 ºC). The viscosity of the matoho products varied similarly from free flowing to viscous. The Seqhaqhabola matoho was most free flowing despite it having the highest solids content. The variation in the viscosity of the matoho products may relate to the effect of pH and titratable acidity. The acidification of the Seqhaqhabola product with citric acid may have resulted in direct acid interaction with gelatinised starch. According to a study by Hirashima, Takahashi and Nishinari (2004), addition of acid to cornstarch paste to a pH ≤3.5 result in a reduction in viscosity. This interaction may have resulted in hydrolysis of the starch glycosidic bonds leading to reduction in viscosity.

In relation to mageu particle size, there was a substantial increase in larger particles (>500 µm) with age of the mageu samples. It was observed that the solid material coagulated and separated out in the oldest mageu sample. This may have contributed to the increase in viscosity with age of the product. These effects are probably due to starch retrogradation, which result from storage at 4ºC (Perdon, Siebenmorgen, Buesher and Gbur, 1999).

The total solids content of the mageu samples was approx. 7%, which corresponds with the maize meal content stated on the carton. The matoho products had total solids content of 13-14%. An explanation for high total solids in the matoho products is that a higher quantity of sucrose was included in the formulation of the matoho product compared to the mageu, which was formulated with artificial sweetener as indicated on the carton.

The matoho products varied widely in terms of colour. The Seshoai product was much darker with the lowest *L*-value, while the Mabele product was lighter with the highest *L*-value. The lowest *L*-value of the Seshoai may be due to the observed presence of dark specks. However, the degree of redness in terms of *a*-values for the matoho samples was not significantly different. In comparison to the mageu, the lower *L*-values of the matoho

sample are attributable to the fact that they were made from red sorghum and mageu is made from maize.

Table 4.2.6: Physicochemical attributes of commercial mageu and matoho beverage products

Beverage products	Best before date	pH	Titratable acidity % (Lactic acid)	[1]Viscosity (mm)	Total solids (g/100 ml)	Particle size distribution (%)			Colour		
						>500 (µm)	>250 - <500 (µm)	≤250 (µm)	L	a	b
Mageu No.1	**28 Jan 2014**	3.52^{ab}	0.20^{a}	11.3^{a}	7.5^{a}	54.0^{b}	16.0^{a}	30.0^{a}	ND	ND	ND
	03 Feb 2014	3.47^{a}	0.23^{b}	16.0^{b}	7.7^{a}	32.8^{ab}	19.3^{a}	48.0^{ab}	ND	ND	ND
	13 Feb 2014	3.58^{bc}	0.20^{a}	26.8^{c}	7.8^{a}	28.0^{a}	21.1^{a}	50.7^{b}	ND	ND	ND
Mabele		3.87^{e}	0.25^{bc}	14.6^{ab}	13.6^{c}	ND	ND	ND	50.9^{b}	2.83^{b}	4.97^{c}
Monepola		3.63^{cd}	0.27^{cd}	15.7^{b}	13.0^{b}	ND	ND	ND	47.2^{ab}	2.23^{ab}	3.10^{b}
Seshoai		3.68^{d}	0.28^{d}	11.6^{a}	13.2^{bc}	ND	ND	ND	44.8^{a}	2.00^{a}	1.99^{a}
Seqhaqhabola*		3.52^{ab}	0.36^{e}	24.9^{c}	14.2^{d}	ND	ND	ND	46.9^{ab}	1.71^{a}	2.80^{b}

***Chemically acidified with citric acid without natural fermentation; n=2, Mean values with different letters in the same column are significantly different ($p<0.05$); ND- Not determined. [1]Viscosity was measured based on the degree of flow of the product as it relate to the level of starch gelatinisation.**

Application of tannin sorghum types in the formulation of matoho resulted in some changes in the physicochemical characteristics of the product (Table 4.2.7). Both the fermented and citric acid acidified tannin matoho products had pH and titratable acidities similar to the commercial products. Similarly, the pH of citric acid acidified products was lower than that of the fermented products. The viscosity of these tannin sorghum matoho samples varied slightly from less viscous to viscous, in the same range with commercial matoho. These tannin sorghum matoho products had total solids content of 13-14%, as with the commercial products. High total solid of the matoho beverage can be evidently linked to the high quantity of sugar in the formulation, contributing to the total solids in the matoho product.

With regard to the effect of type of tannin sorghum, the Type III tannin sorghum matoho samples were slightly less viscous compared to the Type II tannin sorghum matoho (Table 4.2.7). Sodium hydroxide treatment had a varying effect on the viscosity of the matoho products made from the two tannin sorghum types. With the Type III sorghum matoho, there was an increase in viscosity with increase in NaOH concentration. Sodium hydroxide treatment of Type II sorghum resulted in decrease in viscosity with increase in NaOH concentration. A possible explanation for the low viscosity of the Type III sorghum matoho is attributable to higher level of extractable tannins in Type III sorghum (4.5 g/100 g). This may have resulted in high level of tannin complexing with the protein and starch components in the flour. Barros, Awika and Rooney (2012) findings on tannin interaction with starch showed a decrease in setback viscosity of the starch. This effect of tannin interaction with protein and starch forming insoluble tannin-protien/starch complexes may have limited starch granule expansion. The effect of NaOH treatment on the viscosity of Type III tannin sorghum matoho products can be linked to the NaOH reacting with tannins limiting the interaction between tannins and other grain components. In the case of the Type II sorghum matoho, its viscosity similar to that of the non-tannin matoho can be linked to its low level of extractable tannins (Table 4.2.2). The reduction in viscosity of Type II tannin sorghum matoho with NaOH treatment may be due to increased tannin interaction with the protein and starch resulting from the release of its bound tannins.

The tannin sorghum matoho products were considerably darker than the non-tannin sorghum matoho product (Table 4.2.7). The non-tannin sorghum matoho in this study had a higher *L*-value, similar to the commercial matoho products (Table 4.2.6). With regard to *a*-value, the tannin sorghum matoho products were considerably more red than the non-tannin sorghum product, and the commercial matoho products. The low *L*-value of the tannin sorghum matoho products is due to the presence of tannins. This can be attributed to the condensed tannins in these sorghum types, which affect physicochemical attributes such as colour of plant-derived foods and beverages (Cheynier, 2005). Sodium hydroxide treatment resulted in a slightly darker matoho product with increase in NaOH concentration. A possible explanation regarding the effect of NaOH treatment on the colour of these tannin sorghum matoho products may be linked to extractability of the phenolic compounds. This is because NaOH treatment has been noted to increase the extraction of polyphenol compounds (Feng, McDonald and Vick, 1988; Asenstorfer, Wang and Mares, 2006). Sodium hydroxide treatment may have also induced polymerisation reaction of the phenolic compounds, which can lead to formation of brown pigments (Andres-Lacueva, Monagas, Khan, Izquierdo-Pulido, Urpi-Sarda, Permanyer and Lamuela-Raventos, 2008).

Table 4.2.7: Effects of sorghum types and NaOH treatment on physico-chemical attributes of tannin sorghum matoho products acidified with citric acid

Sorghum types	Treatment	pH	Titratable Acidity (% lactic acid)	Total solids (g/100 ml)	Viscosity (mm)	Colour		
						L	a	B
Type I	**Water**	3.46	0.31	ND	13.6^{a}	46.1^{b}	2.28^{a}	3.00^{b}
Type II	**Water**	3.46	0.30	12.5	13.2^{a}	37.8^{a}	3.98^{b}	0.60^{a}
	0.1% NaOH	3.40	0.32	13.3	13.6^{a}	38.2^{a}	4.27^{bc}	0.92^{a}
	0.2% NaOH	3.49	0.29	12.9	14.5^{b}	36.3^{a}	4.21^{bc}	0.95^{a}
Type III	**Water**	3.48	0.29	12.7	17.9^{d}	37.4^{a}	4.72^{bcd}	3.29^{b}
	0.1% NaOH	3.47	0.30	13.0	15.7^{c}	37.6^{a}	5.23^{d}	3.81^{b}
	0.2% NaOH	3.50	0.30	13.6	15.1^{bc}	36.5^{a}	4.87^{cd}	3.16^{b}

n=2, Mean values with different letters in the same column are significantly different ($p<0.05$)

With regard to fermentability of tannin sorghum matoho products, there was no distinct difference between the NaOH treated and the control (Figure 4.2.2). At the end of fermentation, both the NaOH treated and control had approx. the same pH of 4.0. Likewise, the two tannin sorghum probably had a similar pH at the end of fermentation.

The titratable acidity of 0.2% NaOH treated Type III sorghum matoho was slightly higher at the end of fermentation. The untreated Type II sorghum matoho had the highest titratable acidity. The lack of a clear trend with regard to the effect of NaOH on fermentability may be due to the cooking step and the addition of sugar. The sugar may have provided some form of protection for the LAB from the tannins, especially in the untreated sample. Sugar reduces tannin binding capacity due to it masking tannin phenolic hydroxyls (McGrath, Kaluza, Daiber, Van der Riet and Glennie, 1982). The cooking step may have induced interaction between tannins and sucrose and other grain components such proteins and starch.

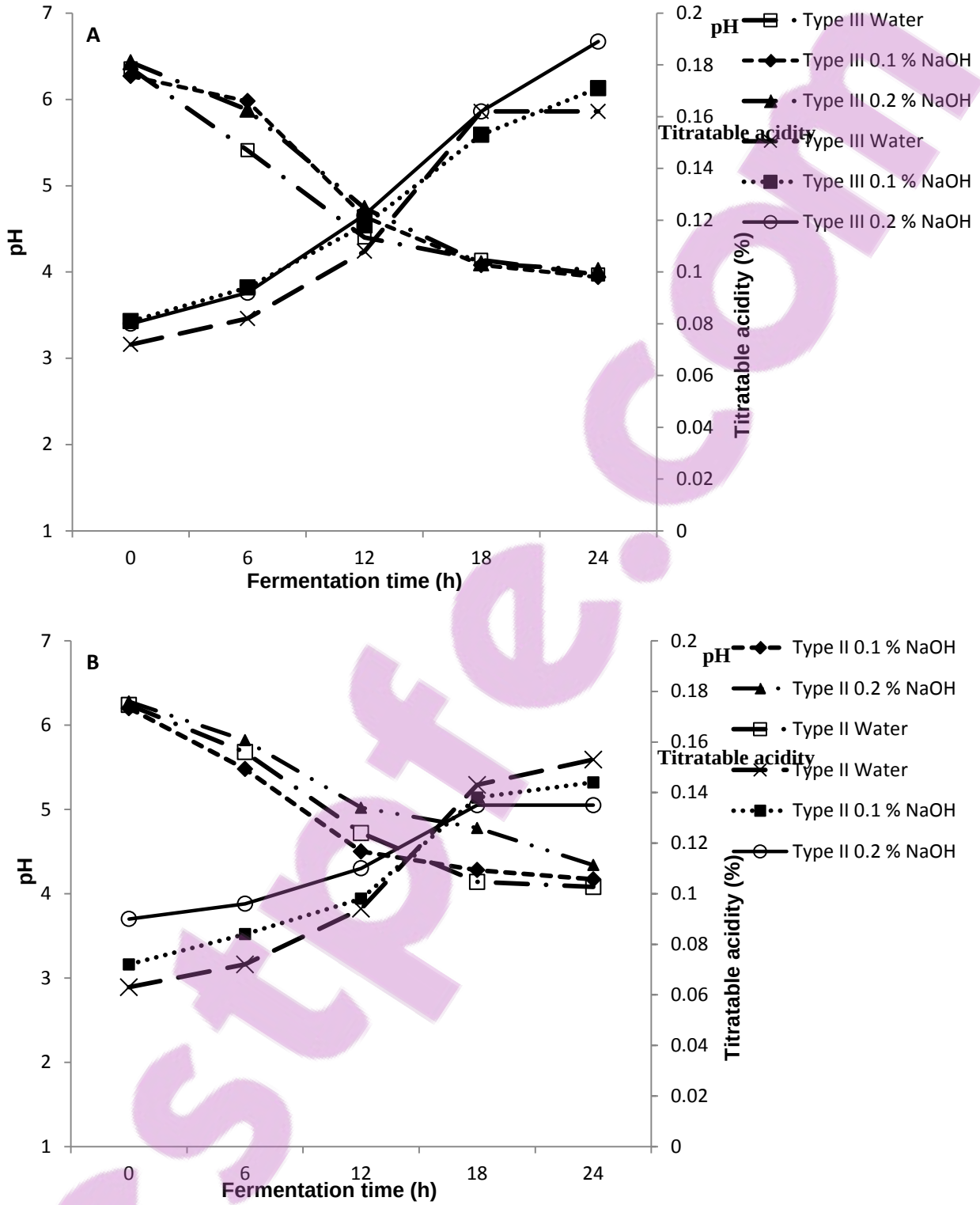

Figure 4.2.2: Effects of NaOH steeping of milled Type II and III tannin sorghums on the fermentation of their matoho beverage over 24 hr. **A**-Type III; **B**-Type II; n = 1.

In view of the above effects of NaOH treatment on the fermentability of milled Type II and III tannin sorghum, fermentation was carried out without a cooking step and with and without the addition of sugar. Fermentation of uncooked Type III sorghum flour with sugar added also did not show a distinct trend with regard to an effect of NaOH treatment (Figure 4.2.3A). However, fermentation of uncooked Type III sorghum without sugar clearly showed the effect of NaOH treatment (Figure 4.2.3B). NaOH treatment resulted in better fermentation of the Type III sorghum flour compared to the control. This is shown by the lower pH of the NaOH treated samples. This is an indication of NaOH reacting with tannins, limiting tannin inhibition of the LAB. This implies that the added sugar did protect LAB from being inactivated by tannins (Figure 4.2.2).

However, fermentation of uncooked Type II sorghum flour both with and without added sugar showed a clear trend (Figure 4.2.4). In contrast to effect of NaOH treatment in the Type III tannin sorghum, fermentability of uncooked Type II sorghum flour decreased with increase in NaOH concentration. This is shown by the 0.2% NaOH treated sample having a higher pH than the 0.1% NaOH treated and the untreated flour samples. The untreated Type II sorghum flour was effectively fermented by LAB as indicated by its lower pH at the end of fermentation. The implication of this is that NaOH treatment of Type II sorghum negatively affects the LAB. The effect of NaOH treatment may have resulted in the release of bound tannins in the Type II tannin sorghum, thereby enabling tannin interaction with the LAB.

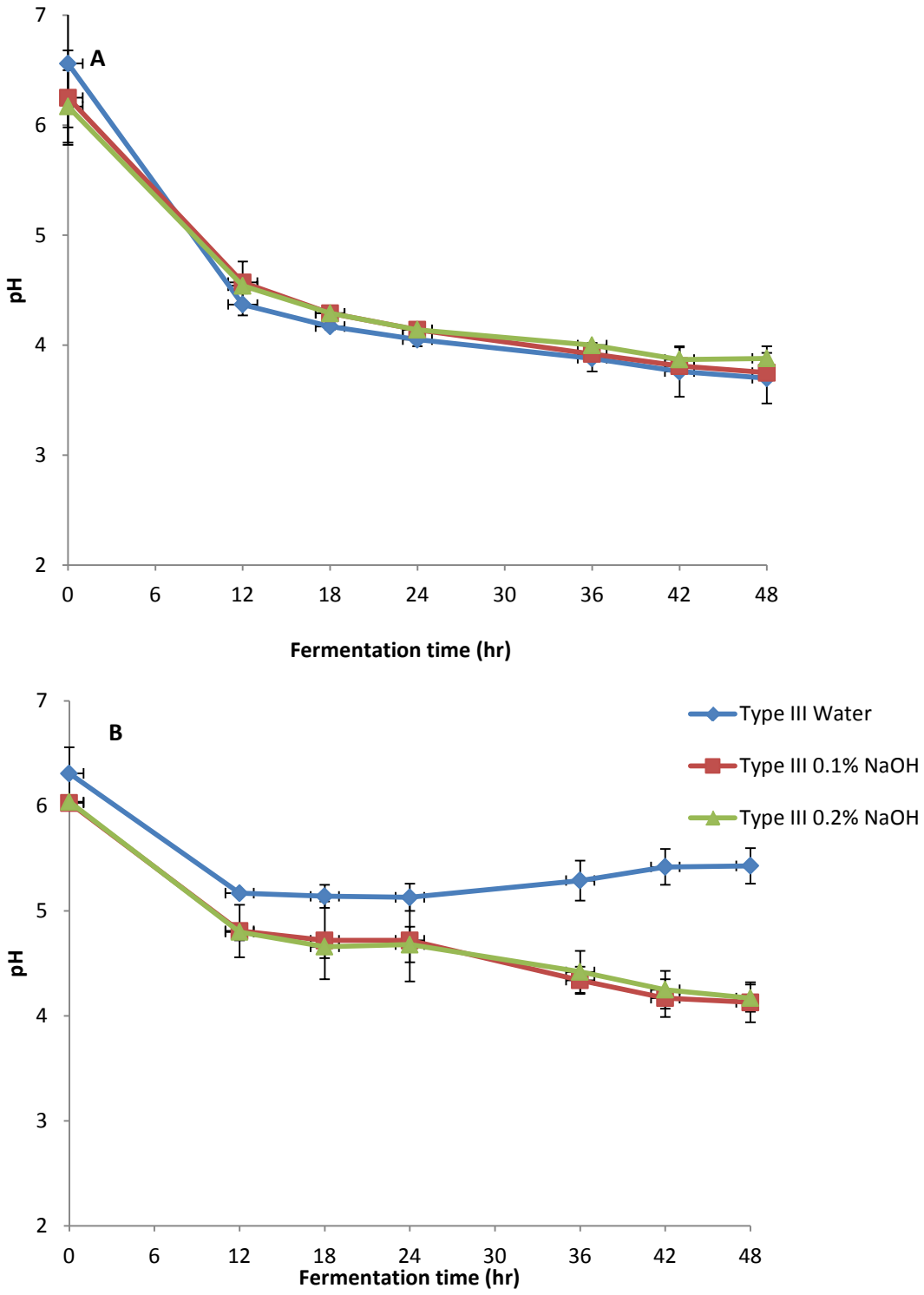

Figure 4.2.3: Effects of NaOH steeping of milled Type III tannin sorghum flour on fermentation of their uncooked matoho over 48 hr. **A**- Fermented with added sucrose; **B**- Fermented without sucrose added; Error bars indicate standard deviations (n=2).

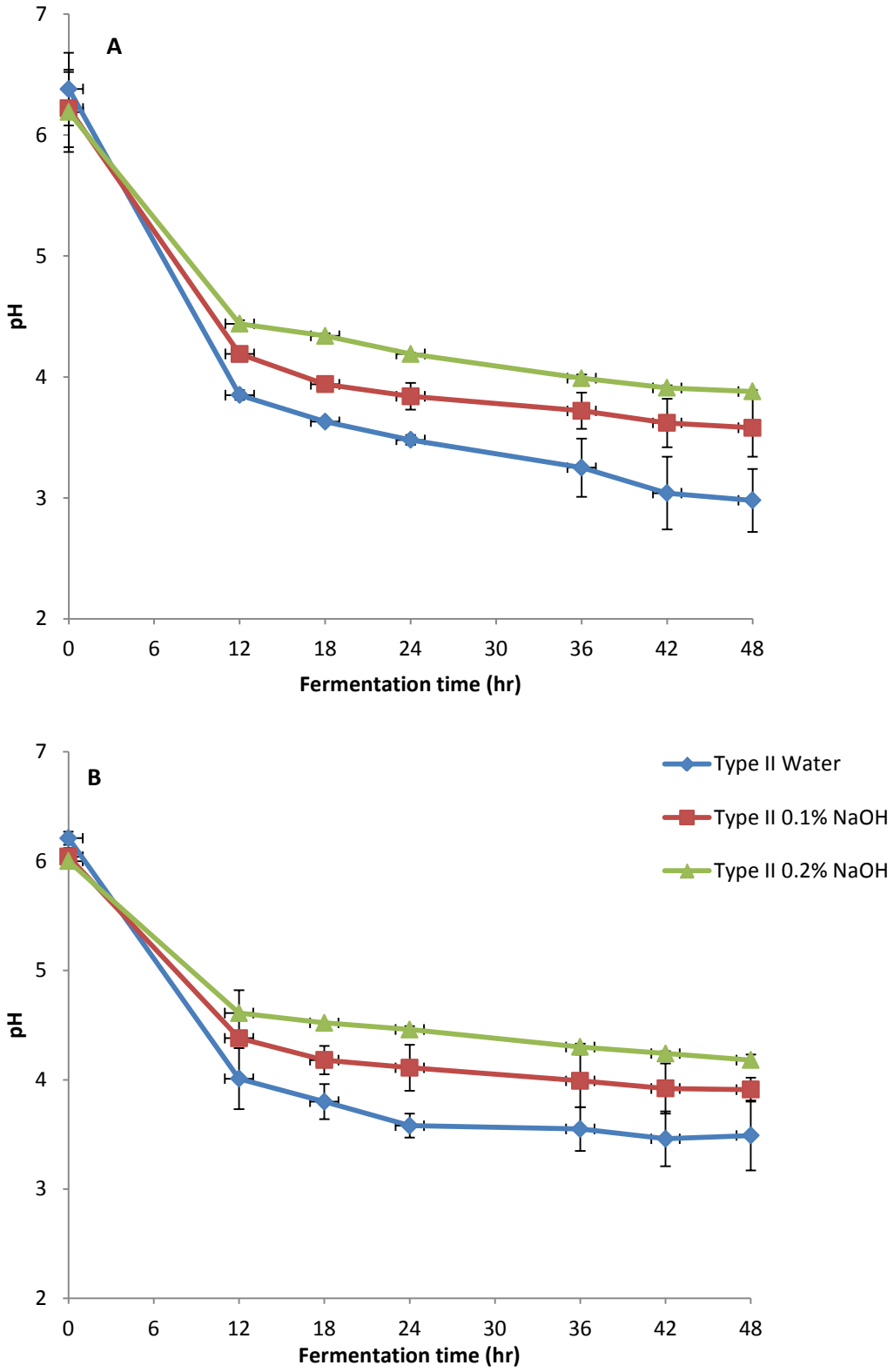

Figure 4.2.4: Effects of NaOH steeping of milled Type II tannin sorghum on fermentation of their uncooked matoho over 48 hr. **A**- Fermented with added sucrose; **B**- Fermented without sucrose added; Error bars indicate standard deviations (n=2).

4.2.4 CONCLUSIONS

Steeping milled tannin sorghum grain in dilute NaOH solution substantially reduces assayable tannin content. With Type III sorghum this results in a substantial improvement in brewing/bioethanol quality attributes, in particular reduced α-amylase inhibition. However, with Type II tannin sorghum, α-amylase is not inhibited and there is no improvement in brewing/bioethanol attributes with NaOH treatment. Admixing soy protein isolate with Type III tannin sorghum flour also considerably improved brewing/bioethanol quality attributes. Application of NaOH treatment to tannin sorghum in matoho production is not necessary. This is attributable to inclusion of sugar in the formulation of the product offering protection to LAB against tannins. Therefore, tannin sorghum types can be potentially utilised in the production of matoho beverage product with unique quality attributes. Application of NaOH pre-treatment method to milled Type III tannin sorghums could enable their utilisation, especially in bioethanol production. This technology will be more applicable in bioethanol production and not in brewing of beverage due to problem of heavy metals in low grade and cheap NaOH. Heavy metals such as mercury are present as contaminants in raw sodium hydroxides.

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4.3 RESEARCH CHAPTER

Understanding the chemistry of NaOH inactivation of sorghum condensed tannins and the effect of sonication on tannin extraction in tannin sorghum types

ABSTRACT

Structural and chemical complexity of tannins present in tannin sorghum limits its effective charactarisation. The effect of sonication on assayable tannin was investigated in different types of tannin sorghum cultivars. The chemistry of reaction between sorghum tannins and NaOH was also investigated using normal-phase HPLC and MS techniques. Sonication resulted in >60% increase in assayable tannins in Type II sorghum cultivars, as against less than 20% increase in Type III tannin sorghums. Application of sonication to Type II tannin sorghum therefore enables its tannins extractability. The effect of NaOH treatment on tannin profiles analysed by HPLC, indicated a general trend of increasing proanthocyanin/procyanidin size with increasing NaOH concentration and steeping time, coupled with a reduction in total area of peaks resolved. LC/MS only identified procyanidins ranging from dimers to hexamers in the NaOH treated Type II flour. These procyanidins were not identified in the Type III sorghum. The chemical reaction between tannins and NaOH indicated polymerisation, resulting in the formation of highly polymeric tannins in the Type III sorghum tannins. The effects of NaOH treatment in the Type II sorghum possibly involve the release of its bound tannins, with a further polymerisation to highly polymeric tannins.

4.3.1 INTRODUCTION

Generally, tannin property in sorghum is an important factor in the selection of sorghum types for food and industrial utilisation because of interaction between condensed tannins and other grain components such as starch and protein (Taylor and Duodu, 2009). The findings in Chapter 4.2 indicate that condensed tannin inhibitory activity against α-amylase differs in the two tannin sorghum types. This appears to be linked to the level of their extractable tannins as affected by the tannin structural characteristics (Asquith, Izuno and Butler, 1983). Condensed tannins, also known as proanthocyanidins, are typically oligomeric or polymeric polyphenols consisting of linked flavan-3-ol units (reviewed by Dykes and Rooney, 2006). Condensed tannin complexation interaction with protein is largely attributable to the properties of the phenolic nuclei in tannin molecules (reviewed by Haslam, 1996).

Condensed tannin complexation interaction with other substances such as cell wall results in the formation of insoluble polymeric complexes (Rohr, Meier and Sticher, 2000). This poses a great challenge in extraction of tannins for quantitative and qualitative analysis (Hellström and Mattila, 2008). As a result, an effective technique of tannin extraction that will allow for characterisation of sorghum tannins is necessary.

Tannin complex interaction with other grain components also negatively affects the processing functionality and nutritional properties of food and beverage products from tannin sorghums. Chapter 4.2 findings clearly showed that the Type III sorghum tannins have high negative effects on sorghum processing functionality. Apparently, Type II sorghum tannins have no negative effects on processing functionality. To address these negative effects, NaOH pretreatment of tannin sorghum has been applied (Reichert, Fleming and Schwab, 1980; Dewar, Orovan and Taylor, 1997; Beta, Rooney, Marovatsanga and Taylor, 2000). In the development of a NaOH steeping process for sorghum flour, it was found that NaOH steeping resulted in a considerable reduction in assayable tannins (Chapter 4.2), similar to that found NaOH steeping with whole-grain tannin sorghum by Beta *et al*. (2000). In addition, this treatment also resulted in a considerable decrease in tannin inactivation of α-amylase activity (Chapter 4.2). According to Beta *et al*. (2000), the effects of NaOH treatment in reducing assayable

tannin content may be due to tannin binding to some components in the grain or chemical interaction between tannin and the alkali leading to the tannins being less reactive. However, the mechanism of the chemical reaction between condensed tannins and alkali is not known for certain (Waichungo and Holt, 1995).

According to Laks, Hemingway and Conner (1987), reactions between proanthocyanidins and strong alkali (pH>9) in the presence of oxygen involved epimerisation at the Carbon-2 coupled with structural rearrangement to form catechinic acid type structures. This is because condensed tannin interaction via radical reactions in the presence of traces of oxygen brings about its C-ring opening and structural rearrangement (Kennedy, Munro, Powell and Porter, 1984). This may be the basis of the mechanism of tannin inactivation by alkali, with the prevailing alkaline condition promoting oxidative polymerisation, as proposed by Porter (1992).

In this study, the effect of sonication on tannin extraction from Type II and III tannin sorghum types was investigated. The chemical reaction between tannins and NaOH was investigated by normal-phase (NP) HPLC and LC/MS techniques to better understand the mechanism involved in tannins inactivation by NaOH.

4.3.2 MATERIALS AND METHODS

4.3.2.1 Materials

The following tannin sorghum grain cultivars were used: NS 5511, PAN 3860 and PAN 8629 (Type III, with red pericarp); Feterita (Sudan) and White Tannin Gadam El Hamam-type (Zimbabwe) (Type II, with white pericarp). The whole sorghum grain samples were milled using a hammer mill (Falling Number, Huddinge, Sweden) fitted with a 1.0 mm opening screen. The whole grain flours were stored in a zip-lock type polyethylene bags at 6-8°C until analysis.

4.3.2.2 Methods

4.3.2.2.1 Tannin extraction by sonication

Extracts were made by weighing 0.5 g flour samples into centrifuge tubes containing 25 ml 1% conc. HCl in methanol. Extraction was carried out by sonication at approx. 16

Watt (rms) (60˚C) for 10 min using an ultrasonic cell disruptor (Misonix, New York). After sonication, sonicated samples were rested at ambient temperature (24°C) for 50 min. The suspension was centrifuged at 1470 g for 15 min and the clear supernatant decanted. Extraction without sonication was carried out for 20 min at ambient temperature (24°C), with vigorous shaking at 5 min intervals. A clear supernatant was obtained by centrifugation for 10 min at 1200 g. The effect of sonication on the level of extractable condensed tannins in the Type II and III tannin sorghums was assayed by the modified Vanillin-HCl method.

4.3.2.2.2 Tannin inactivation by steeping in dilute NaOH

NaOH solutions (30 ml) 0.1 0.2 and 0.4% NaOH of flour (w/w) were measured into a 500 ml stainless steel beakers and pre-heated to 50°C. Distilled water (30 ml) was measured into a 500 ml stainless steel beaker and also pre-heated to 50°C. Sorghum flour (10 g) was weighed into the beakers and stirring commenced. Steeping was carried out for 5 and 30 min, with continuous stirring. At the end of the steeping periods, the pH was adjusted to pH 5.6-5.9 using 1.5 M HCl. The slurries at the end of steeping were freeze dried and the freeze dried flour stored, as described.

4.3.2.2.3 Preparation of tannin extracts

Tannin extracts for normal-phase HPLC analysis were prepared from the freeze dried sorghum samples. The HPLC procedure of Awika, Dykes, Gu, Rooney and Prior (2003) was used with slight modifications. One g sample was extracted in 10 mL acetone/acetic acid/water (70:1:29, v/v). The samples were sonicated at approx. 16 Watts (rms) for 10 min using the ultrasonic cell disruptor and left to stand at ambient temperature (24°C) for 50 min. The suspension was centrifuged at 1470 *g* for 15 min and a clear supernatant decanted. The extracts were filtered (0.45 µm) before HPLC analysis. The following standards were used: Catechin, procyanidin B1 and procyanidin B2 from Sigma-Aldrich (Johannesburg, South Africa). They were run separately and in combination.

Extracts for LC/MS analysis was carried out as described above with slight modifications. During extraction borosilicate glass wares were used throughout to avoid polyethylene contamination. The filtered extracts were stored in amber glass vials.

4.3.2.3 Analyses

4.3.2.3.1 Tannin content

Tannin content was determined by using the modified Vanillin-HCl method of Price, Van Scoyoc and Butler (1978) and expressed in g catechin equivalents (CE)/100 g.

4.3.2.3.2 Normal-phase HPLC

The HPLC system consisted of a binary pump, fluorescence detector, autosampler and column oven (Shimadzu, Kyoto, Japan). The separation of the proanthocyanidins to their oligomers and polymers were carried out on a Phenomenex (Torrance, CA) 5 µm Luna silica column (250 x 4.6 mm) at 28°C using a 10 µL injection volume. The binary mobile phase consisted of (A) dichloromethane and (B) acidified aqueous methanol (methanol/acetic acid/water; 95:2:3 v/v). Gradient was 15% B, 0-3 min isocratic; 15-55% B in A, 3-40 min; 55-100% B in A, 40-60 min; 100% B, 60-67 min isocratic; 100-15% B in A, 67-73 min; followed by 10 min re-equilibration of the column before the next run. The flow rate was 0.6 mL/min. Fluorescence detection was at an excitation wavelength of 276 nm and emission wavelength of 316 nm.

4.3.2.3.3 Liquid chromatograph- mass spectrometry (LC/MS)

The chromatographic analyses were performed using a Waters Synapt G2 system comprising a Waters Acquity Ultra-Performance Liquid Chromatograph (UPLC), equipped with a binary pump system (Waters, Milford, MA). The UPLC system was coupled to a Quadrupole Time of Flight mass spectrometer (QToF-MS, Waters) using an electrospray ionization (ESI) source and a photodiode array (PDA) detector (Waters). Separation was done on a Waters high strength silica column (150 x 2.1 mm, 1.7 µm). The mobile phase consisted of 2% (v/v) aqueous formic acid (solvent A) and acetonitrile (solvent B). Gradient elution was done according to the following program: 95% A from 0 to 0.5 min; 56% A from 0.5 to 20 min; 0% A from 20 to 21 min; 0% A from 21 to 22 min; 95% A from 22 to 25 min. An injection volume of 3 µL and a flow rate of 0.35 ml/min were used. Ionization was in negative mode with a capillary voltage of 3 kV and cone voltage of 15 V. Identification was done by comparing MS/MS fragmentation data and UV spectra with phenolic compounds reported in literature. Leucine enkaphelin (molecular weight 555 Da) was used as lock mass. Data were acquired using MassLynx v. 4.1 software (Waters).

4.3.2.4 Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine the effects of sorghum types, sonication and interaction and measured variables with mean separation by Fisher's Least Significance Difference (LSD) test using Statistica software for Windows, version 12 (StatSoft, Tulsa, OK).

4.3.3 RESULTS AND DISCUSSION

The effect of sonication on the level of extractable condensed tannins in the Type II and III tannin sorghums was quantified by the modified Vanillin-HCl method. To understand the effect of alkali treatment on condensed tannins properties, NP-HPLC was used for profiling the proanthocyanidin compositions based on the degree of polymerisation. Identification of the tannin molecules present in the raw and alkaline treated sorghum flour samples was carried out by LC/MS.

4.3.3.1 Effects of sonication on assayable tannin in milled sorghum grain

As shown in Table 4.3.1, both sorghum type and sonication had significant effects ($p<0.001$) on assayable tannin content. There was also a significant interaction effects ($p<0.001$) between the sorghum type and sonication on tannin content. With the Type II tannin sorghums, sonication resulted in >60% increase in tannin content. In contrast, the Type III tannin sorghums, there was <20% increase in tannin content. In relation to the specific cultivars, sonication resulted in a significant increase in tannin content of Feterita, Tannin White and PAN 8629 ($p<0.05$). Sonication had no significant effect on the NS 5511 and PAN 3860 ($p>0.05$). Thus, the sonication only resulted in a substantial increase in extraction of assayable tannins with the Type II tannin sorghum.

These effects are possibly due to the differences in tannin deposition between Type II and III sorghums, as described by Earp, McDonough, Awika and Rooney (2004). Type II tannin sorghums possess the recessive spreader gene *s*, whereas the Type III has a dominant spreader gene *S* (Rooney, 2000). Non-expression of the spreader gene in the Type II sorghum may be responsible for most of the tannins in Type II to be deposited and bound within the vesicles of the testa layer. Another possible explanation for the differences in extractability of tannins between the Type II and III tannin sorghums is the

different structural characteristics of their tannins. According to a study by Asquith *et al.* (1983), more acid-labile extractable tannins are in the Type II tannin sorghums compared to the Type III tannin sorghum type. It was explained that the presence of acid-labile bonds such as glycosidic or ester bonds enables linkage of the tannins in Type II tannin sorghums to other components of the grain. This acid-labile structure of tannins in the Type II sorghum limits accessibility of extraction solvent to the tannin (Asquith *et al.*, 1983).

Sonication, as explained by Vinatoru (2001), is based on the application of ultrasound in enhancing solvent extraction of bioactive compounds. The principle involves the generation of cavitation bubbles due to local pressure fluctuations. The collapse of these cavitation bubbles results in the release of a large amount of mechanical energy in form of elastic waves that brings about disruption of the intermolecular interactions between materials. This process may have led to disruption of the testa cell wall structure enabling accessibility of the extraction solvent to the tannins in the Type II tannin sorghums. However, the expression of the spreader gene *S* in the Type III causes tannins to be deposited along the cell walls of the testa and some in the pericarp (Dykes and Rooney, 2006). In addition, the Type III sorghum tannins have a less acid-labile structure and are not bound within the cell wall of the testa layer (Asquith *et al.*, 1983). This may explain the reason for much lower increase in assayable tannin with the Type III sorghums with sonication.

Table 4.3.1: Effects of sonication and tannin sorghum types on assayable tannins measured by the modified Vanillin-HCl method

Sorghum type	Cultivar	Tannin content (CE g/100 g d.b.)			% increase in assayable tannin
		Not sonicated	Sonicated	Overall mean	
Type II	**Feterita**	0.40[a]	1.14[b]	0.77	64.9
	Tannin White	1.18[a]	3.19[b]	2.18	63.0
Type III	**NS 5511**	2.70[a]	2.98[a]	2.84	9.4
	PAN 3860	2.52[a]	2.94[a]	2.73	14.3
	PAN 8629	4.73[a]	5.82[b]	5.27	18.7

Mean values with different letters in the same row different significantly ($p<0.05$); Effect of sorghum type ($p<0.001$); Effect of sonication ($p<0.001$); Interaction effect ($p<0.001$); n = 2.

4.3.3.2 Normal phase HPLC and LC/MS of polyphenols from NaOH treated sorghum

To understand the effect of NaOH treatment on Type II and III sorghum tannins, normal-phase HPLC and LC/MS were applied to the acidified aqueous acetone extracts from the freeze-dried sorghum samples. The monomer, oligomer and polymer regions indicated in Figure 4.3.1 were estimated based on the monomeric and dimeric standards used and the Sumac (Type III tannin) sorghum normal phase HPLC profile reported by Dlamini, Dykes, Rooney, Waniska and Taylor (2009). The effects of treatments and steeping time on the chromatographic profile are also depicted quantitatively in Table 4.3.2 in terms of monomer, dimer, oligomer and polymer peak areas, and total peak area resolved.

With the Type III tannin sorghum samples, the total area of peaks resolved and eluted distinctively from the column were much lower for the NaOH treated samples compared to the raw grain and water steeped samples (Table 4.3.2). Steeping the Type III tannin sorghum in water resulted in a considerably increase in total area of peaks resolved as compared to the raw grain. This effect is similar to the effect of traditional wet cooking of Type III tannin sorghum porridge, which results in an increase in the peak areas of tannin profiles compared to the raw flour (Dlamini *et al*., 2009). The effect may be due to the release of some of these phenolic compounds that are water soluble (Shelembe, Cromarty, Bester, Minnaar and Duodu, 2012). Steeping the Type III tannin sorghum in

dilute NaOH resulted in a large reduction in the number of monomer peaks and the monomer absolute peak area at all NaOH concentrations when compared to steeping in water (Figure 4.3.1 and Table 4.3.2). With the Type III sorghum steeped for 5 min in the 0.4% NaOH, there was an increase in the number of peaks and their areas in the oligomeric and polymeric regions compared with steeping in water. Steeping the Type III sorghum for 30 min in all three NaOH concentrations resulted in the complete disappearance of the peaks in the polymeric region. In terms of the relative peak areas, when Type III sorghum was steeped for 5 min there was a reduction in relative peak area of the monomers with increasing NaOH concentration, while the relative peak areas of oligomers and polymers increased. With steeping for 30 min a similar trend in relative peak areas was only seen in the monomer and oligomer regions at higher NaOH concentration because the polymeric peaks were not detected.

With the raw and water steeped Type II tannin sorghum the resolved total peak areas were far lower than for the Type III sorghum (Table 4.3.2). When the Type II tannin sorghum was steeped in NaOH for 5 min, there was complete disappearance of peaks in the polymeric region for the higher NaOH concentrations of 0.2% and 0.4%. Steeping the Type II sorghum in NaOH for 30 min also resulted in a substantial reduction in the polymer peak at the higher NaOH concentrations of 0.2% and 0.4% (Figure 4.3.1). In contrast to the Type III sorghum, the Type II sorghum steeped for 5 min showed an increase in relative peak areas of its monomers and dimers with increase in NaOH concentration, while oligomer relative peak areas decreased substantially at 0.4% NaOH concentration (Table 4.3.2). Steeping Type II sorghum for 30 min also resulted in an increase in relative peak areas of monomers, dimers and oligomers, while the polymers decreased with increase in NaOH concentration.

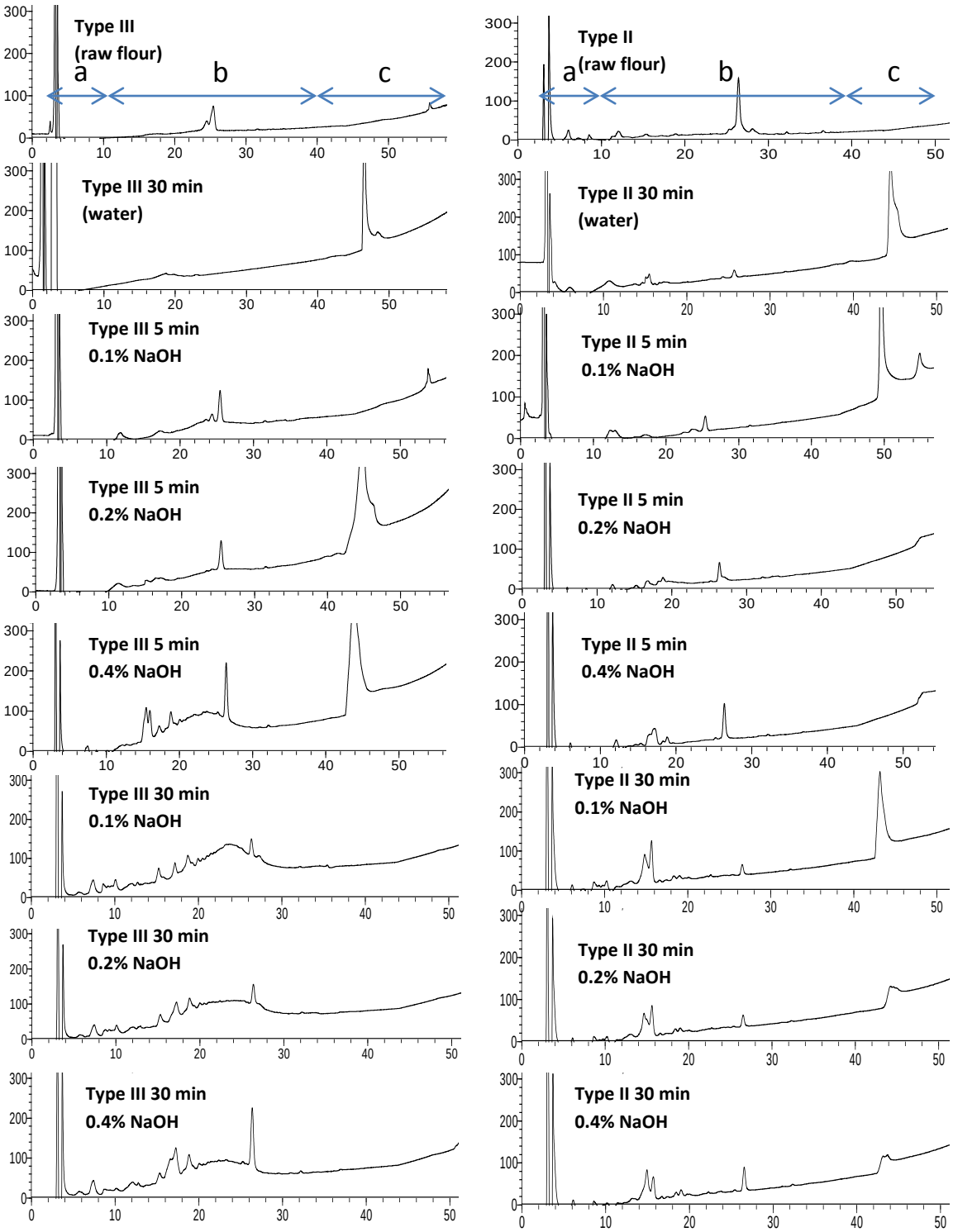

Figure 4.3.1: Effects of NaOH steeping of Type II and III tannin sorghum flours on the chromatographic profiles of their tannins. (a) Monomer and dimer peaks, retention time 3-10 min; (b) Oligomer peaks, retention time 10-40 min; (c) Polymer peaks, retention time 40-60 min.

Table 4.3.2: Effects of NaOH steeping on the sum of absolute peak areas and relative percentage of peak areas detected by HPLC for each regions

Sorghum types	Treatment	Absolute peak areas				
		Monomers	**Dimers**	**Oligomers**	**Polymers**	**Total**
Type III (red tannin)	**Raw flour**	67423(67.6)	404(0.4)	30961(31.0)	928(0.9)	99716
	Water 30 min	249596(96.6)	ND	ND	8853(3.4)	258449
	0.1% NaOH 5min	36245(88.9)	ND	3848(9.4)	670(1.6)	40763
	0.2% NaOH 5min	40301(56.1)	ND	3699(5.2)	27811(38.7)	71811
	0.4% NaOH 5min	8424(20.9)	462(1.1)	8310(20.6)	23120(57.3)	40316
	0.1% NaOH 30min	15138(75.0)	1490(7.4)	3569(17.7)	ND	20197
	0.2% NaOH 30min	8337(54.6)	1097(7.2)	5825(38.2)	ND	15259
	0.4% NaOH 30min	7327(38.2)	1156(6.0)	10720(55.8)	ND	19203
Type II (white tannin)	**Raw flour**	3407(30.0)	1455(12.8)	6477(57.1)	ND	11339
	Water 30 min	18705(53.9)	613(1.8)	3082(8.9)	12317(35.5)	34717
	0.1% NaOH 5min	3544(13.6)	406(1.6)	2704(10.4)	19442(74.5)	26096
	0.2% NaOH 5min	3282(44.9)	1310(17.9)	2720(37.2)	ND	7313
	0.4% NaOH 5min	3674(81.1)	699(15.4)	155(3.4)	ND	4528
	0.1% NaOH 30min	1676(8.4)	265(1.3)	7436(37.3)	10561(53.0)	19938
	0.2% NaOH 30min	3957(29.9)	1612(12.2)	5124(38.7)	2542(19.2)	13235
	0.4% NaOH 30min	4498(32.5)	1602(11.6)	5956(43.1)	1771(12.8)	13827

***Raw flour was without steeping; **Values in parentheses are relative percentage; ND: No peaks detected.**

Identification of the phenolic compounds was based on comparing their mass spectral data with data from literature (Lazarus, Adamson, Hammerstone and Schmitz, 1999; Hammerstone, Lazarus, Mitchell, Rucker and Schmitz, 1999; Xu, Liu, Li, Tu and Chen, 2011) and the polyphenol database Phenol Explorer (Rothwell, Perez-Jimenez, Neveu, Medina-Ramon, M'Hiri, Garcia-Lobato, Manach, Knox, Eisner, Wishart and Scalbert, 2013). Quantification of the compounds could not be carried out due to lack of higher oligomeric and polymeric standards. As shown in Table 4.3.3, there were differences based on the specific group and/or type of phenolic compounds between the two sorghum types. With the Type III sorghum in all the treatments (raw flour, water steeped and

NaOH treated flours) only non-procyanidin phenolic compounds were identified. These included members of the phenolic acid, anthocyanin, 3-deoxyanthocyanin, flavanone, flavone, flavonol (and various glycosyl derivatives of these) groups and the flavanonol taxifolin. With increasing NaOH concentration there was an increase in the number of these non-procyanidin phenolic compounds. This could be due to NaOH steeping increasing the extraction of these non-procyanidin compounds. The effect of NaOH treatment has been reported to result in increase in phenolic extractability (Feng, McDonald and Vick, 1988; Asenstorfer, Wang and Mares, 2006). The peaks that eluted last (Table 4.3.4) could not be identified, due to their masses not corresponding to the molecular masses of phenolic compounds reported in literature. However, they were presumably proanthocyanidins. It is noteworthy that with the Type III sorghum the NaOH treated samples yielded fewer fragment ions than the water treated sample, which in turn yielded fewer fragment ions than the raw grain. The likely explanation is that water and NaOH further polymerised the proanthocyanidins/procyanidins.

In contrast to the Type III sorghum, with the Type II sorghum, procyanidins were identified in the extracts (Table 4.3.3). In the raw flour, procyanidin pentamer (DP5) and procyanidin dodecamer (DP12) were identified. Type II sorghum steeped in water had oligomeric procyanidins, ranging from procyanidin dimer to procyanidin hexamer, as well as procyanidin dodecamer. These procyanidins were also identified in the NaOH treated samples. However, procyanidin dimer (DP2) and tetramer (DP4) were absent in the 0.4% NaOH treatment. The fact that oligomeric procyanidins were found in the Type II sorghum but not the Type III sorghum may simply be a consequence of differences in the bound properties of their tannin, the Type II sorghum tannins are characterised predominantly with acid-labile structure (Asquith *et al*., 1983). This acid-labile structure enables Type II sorghum tannins to be strongly bound with cell wall components. Apart from the procyanidins, the same groups of non-procyanidin phenolic compounds were identified in the Type II sorghum samples as in the Type III sorghum samples, except for flavonol which was not identified in the Type II samples. The number of these non-procyanidin phenolic compounds identified decreased with increase in NaOH concentration in the Type II sorghum.

Concerning the presumed procyanidin/procyanidin fragment ions which eluted last and could not be identified (Table 4.3.4), in contrast to the Type III sorghum, the highest number of fragment ions were from the water steeped Type II sorghum and similarly lower numbers of fragment ions were from the raw grain and NaOH treated samples. This is possibly a result of a combination of the steeping treatment releasing the bound tannins in the Type II tannin sorghum proanthocyanidins/procyanidins and polymerisation of them by the NaOH. As stated, the tannins in Type II tannin sorghum are characterised by having a high degree of polymerisation and being bound with the testa cell walls (Asquith *et al*., 1983).

Based on the above findings, it appears that NaOH treatment of both milled Type III and Type II sorghums resulted in further polymerisation of the proanthocyanins/procyanidins into large polymers which could not be resolved by normal phase HPLC. Such polymerisation rendered some tannin molecules too large to be measured by the vanillin HCl reagent. The structural property of sorghum condensed tannins in terms of acid-labile structure seems to affects tannin reactivity, which also influences tannin extractability (Asquith *et al*., 1983). This may be the basis for the Type II tannins not having inhibitory activity on the α-amylase protein.

Table 4.3.3: Retention time and mass spectral characteristics of proanthocyanidin and other phenolic compounds identified in extracts from raw and NaOH treated Type II and III sorghum flour steeped for 30 min

Type III sorghum

Ret. time (min)	$[M-H]^-$ (m/z)	MS/MS Fragment ions (m/z)	Proposed compound	Raw flour	Water	0.2%	0.4%
6.18	253	253, 161, 133, 135, 196	Caffeoylglycerol	-	-	-	+
6.72	449	147, 157, 161, 281, 337, 359	Eriodyctyol hexosyl	+	-	-	+
7.77	289	137, 165, 151, 125, 237	Catechin/epicatechin	-	-	-	+
7.97	287	145, 160, 161, 208, 269, 287	Cyanidin	-	-	+	+
11.02	301	301, 165, 161, 150, 147, 141	Peonidin	+	+	+	+
11.30	303	151, 163, 179, 303	Taxifolin	-	+	+	+
12.65	285	191, 165, 150, 122	Sakuranetin				+
12.66	285	116, 133, 161, 267, 285	Luteolin	-	-	+	+
13.16	415	415, 253, 161, 179, 135, 133	Apigeninidin-5-glucoside	-	-	-	+
13.47	287	100, 136, 151, 163, 285, 287	Cyanidin	+	+	-	+
13.50	285	285, 133, 151	Luteolin	+	+	+	+
14.80	399	161, 163, 253, 399	Feruloyl-methylaldaric acid	-	-	-	+
15.35	429	161, 179, 225, 233, 429	Feruloyl-caffeoyl-glycerol	-	-	-	+
15.57	269	269, 151, 159, 117, 191, 145	Luteolinidin	+	+	+	+
15.81	285	42, 147, 184, 285	Luteolin	-	+	-	-
16.28	299	159, 175, 183, 299	4-hydroxybenzoic acid 4-O-glucoside	-	-	+	-
18.70	429	159, 183, 223, 429	Feruloyl-caffeoyl-glycerol	-	+	+	+
18.81	431	51, 125, 147, 187, 244	Apigenin glucoside	+	-	-	-
19.75	399	54, 175, 183, 283, 399	Feruloyl-methylaldaric acid	+	+	+	+
20.88	415	159, 175, 183, 280, 339	1,3-dicaffeoylglycerol	+	-	-	-

Non-proanthocyanidin compounds in: - Raw flour: 8; Water: 8; 0.2% NaOH: 9; 0.4% NaOH: 16

Table 4.3.3 continued: Type II sorghum

Ret. time (min)	$[M-H]^-$ (m/z)	MS/MS Fragment ions (m/z)	Proposed compound	Raw flour	Water	0.2%	0.4%
4.18	577	577, 407, 289	Procyanidin dimer DP2	-	+	+	-
4.28	865	865, 695, 575, 407, 289	Procyanidin trimer DP3	-	+	+	+
4.50	576	864, 577, 1153, 407, 289	Procyanidin tetramer DP4	-	+	+	-
4.84	720	1145, 864, 575, 449, 407, 289, 285, 161, 125	Procyanidin pentamer DP5	+	+	+	+
4.99	864	1065, 864, 575, 407, 289, 125	Procyanidin hexamer DP6	-	+	+	+
5.39	864	1065, 864, 575, 407, 289, 125	Procyanidin hexamer DP6	-	+	+	+
4.65	465	303, 125	Delphinidin 3-O- hexosyl	+	+	-	+
10.53	577	1156, 867, 579, 559, 433, 287, 151	Procyanidin dodecamer DP12	+	+	+	+
6.18	253	253, 175, 161, 133, 135, 196	Caffeoyl glycerol	+	+	+	+
6.72	449	125, 137, 147, 151, 157, 161, 287, 289, 405	Eriodictyol hexosyl	+	-	-	-
7.72	883	269, 271, 297, 405, 433, 595, 721, 883	Tetrahydroxylflavan-glucosyl-eriodictyol glucoside	+	+	+	+
8.57	433	151, 271, 287, 433	Naringenin glucoside	+	+	+	+
8.80	303	125, 151, 163, 179, 285, 303	Taxifolin	+	+	+	-
11.15	721	721, 559, 433, 405, 297, 271, 151	5,7,3',4'-tetrahydroxylflavan-5-O-β-hexosyl-4,8-eriodictyol	+	+	+	+
12.77	287	100, 136, 151, 163, 285, 287	Cyanidin	-	+	+	-
13.15	415	415, 253, 161, 179, 135, 133	Apigeninidin-5-glucoside	+	+	+	+
13.50	285	116, 133, 161, 267, 285	Luteolin	-	+	+	+
15.62	269	269, 151, 159, 117, 191, 145	Luteolinidin	+	+	+	+
16.27	299	159, 175, 183, 299	4-hydroxybenzoic acid 4-O-glucoside	+	+	+	-
19.09	431	51, 125, 147, 187, 244	Apigenin glucoside	+	+	-	-

Proanthocyanidin compounds in:- Raw flour: 2; Water: 6; 0.2% NaOH: 6; 0.4% NaOH: 4

Non-proanthocyanidin compounds in:- Raw flour: 11; Water: 12; 0.2% NaOH: 10; 0.4% NaOH: 8

Table 4.3.4: MS/MS fragment ion signals of higher molecular weight compounds that could not identified by LC-ESI/MS of peaks that eluted at the end LC separation

Sorghum types	Ret. time (min)	MS/MS Fragment ions (m/z)			
		Raw flour	Water	0.2% NaOH	0.4% NaOH
Type III	21.31	1106, 1166, 1361, 1371, 1491, 1550, 1580, 1688, 1789	1342, 1371, 1491, 1579, 1680, 1789, 1797	1361, 1371, 1576, 1679, 1789	1371, 1577,1675, 1789
	21.35	1764			
Type II	21.31	1167, 1346, 1371, 1421, 1689, 1799	1349, 1371, 1421, 1587, 1686, 1797	1166, 1349, 1371, 1585, 1688, 1798	1371, 1421,1497, 1688, 1799
	23.58		1325, 1366, 1385, 1494, 1499, 1591, 1708, 1799		

4.3.4 CONCLUSIONS

Application of sonication considerably increases extractable tannins from Type II tannin sorghum types. Sonication enables better estimation sorghum tannin content and tannin characterisation. The probable mechanism of tannin inactivation by NaOH treatment is polymerisation, rendering the tannins too large to assay and in the case of the Type III sorghums tannins too large to interact with the α-amylase protein. In the case of the Type II sorghum, NaOH treatment possibly suggests release of its bound tannins and a further polymerisation to highly polymeric tannins.

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5. GENERAL DISCUSSION

This general discussion is divided into three sections. The first is a critical review of the methodology used in the study and the challenges faced in analysing the materials studied. The second section focuses on the major findings on cassava root tuber cell wall material hydrolytic enzyme treatment for improved beverage processing application and the findings with respect to NaOH flour steeping technology for tannin inactivation in tannin sorghum cultivars and a probable mechanism of tannin inactivation by NaOH. This section also focuses on the potential application of enzymatic treatment for improving cassava beverage utilisation and NaOH flour steeping technology for tannin sorghum utilisation in brewing/bioethanol production. The final section identifies future research needs.

5.1 Methodology: Critical review

As described in Chapter 4.1, enzymatic treatment of cassava tuber cell walls was studied with the aim of improving mash filtration in cassava brewing. Two different cassava samples were used in the study. For the first part of the study, cassava root tuber already processed into cassava cake was received from SABMiller, Mozambique. The challenge faced using this cassava cake that had already been prepared had to do with its low pH. The cassava cake had already fermented to a pH of about 3.6, below the optimum pH range required for the enzyme preparations investigated. The Ultraflo preparation has an optimum pH range between 5.0 and 7.0, with the following enzyme activities: xylase, pentosanase and glucanase (Novozyme, 2008). The Viscozyme preparation has an optimum pH range between 3.5 and 6.0, with the following enzyme activities: cellulase, xylase, pentosanase, glucanase and arabanase (Novozyme, 2008). The drop in pH of the cassava is caused by the activity of amylolytic lactic acid bacteria such as *Lactobacillus plantarium*, which has been isolated from cassava roots (Giraud, Brauman, Keleke, Lelong and Raimbault, 1991).

In view of the optimum pH conditions for the enzyme preparations used, the rate of change in pH of the cake was determined by using freshly milled cassava tubers obtained from a retail outlet. Fresh cassava tubers could not be received directly from Mozambique because an import permit for agricultural plant materials is required.

According to the South African Department of Agriculture, Forestry and Fisheries (2011), importation of agricultural products need to meet phytosanitary regulations based on Agricultural Pest Act No 36 1983 before an import permit can be issued. However, compositional data of the freshly milled cassava tubers were comparable with the received fermented cassava cake. For example, the starch content of the fermented cassava cake was about 81% and the freshly milled cassava tuber about 85%. Further, the freshly milled cassava root tubers were also from Mozambique and thus likely to be similar with respect to microflora. With regard to effect of pH, the change in pH of the cassava cake was monitored during the enzymatic treatment. This showed how critical the pH of the cassava cake was to the hydrolytic activity of the enzyme preparations on the cell wall materials. Enzyme treated fermented cassava cake had a higher residual solids content than the freshly milled cassava cake, which clearly indicates that the low pH of the fermented cassava cake limited the activity of the enzymes.

To understand the effect of hydrolytic enzymatic treatment on the cassava cell wall materials, the starch component of the cake was completely hydrolysed with α-amylase in a model mashing system, as assesed using iodine reagent. The degree of flow of the residual insoluble material after starch removal was determined using a Bostwick viscometer, according to Mouquet, Greffeuille, and Treche (2006). The assumption was that the degree of flow of the residual materials was related to their water holding capacity, which in turn was an indication of modification in structure and composition of the cell wall materials due to the hydrolytic treatment.

The residual solid materials after starch removal were further characterised, by determining their particle size distribution. This was carried out by wet sieving through 500 and 250 µm opening test sieves with a gentle spray of water. This method of particle size determination was used because it was simple. The particle size of the materials was assumed to be a reflection of degree of fragmentation of the cell wall materials, as a result of hydrolysis. A more accurate and precise quantitative method that could have been applied is Laser particle size analysis. As reviewed by Lee Black, McQuay and Bonin (1996), this technique is able to provide detailed information regarding particle diameter, shape and size distribution. However, the application of this technique is not

simple because the instrument requires to be specifically adapted for a particular application.

Light microscopy was used to qualitatively compare the effect of the enzymatic treatments on the structure of the cell wall materials. This method is very quick and simple. Generally, the major limitation of any microscopic technique is on the small sample size analysed, which may not give a true reflection of the structure of the cell wall materials.

The cassava cell wall materials (CWM) were analysed by GC-FID to characterise the effect of the enzymatic treatments on the composition and structure of the cell wall materials. Compositional analysis of the cell wall materials was carried out by hydrolysing them into their component sugars using acidified methanol, according to the procedure of Laine, Tamminen, Vikkula and Vuorinen (2002). Samples for structural analysis were first per-methylated using dimethyl sulphoxide (DMSO) (Ciucanu and Caprita, 2007). This is a methylation reaction that enables stability of ions and increases sensitivity for detection (reviewed by Ciucanu, 2006). Per-methylation is also required to determine the position of the glycosidic linked side chains (Ciucanu, 2006). After this treatment, the per-methylated materials were hydrolysed with acidified methanol and analysed by GC-FID.

A major challenge faced in analysing the residual CWM was the difficulty in hydrolysing them into their sugar components under both preparation conditions. This can be attributed to the CWM being lignified (Buschmann *et al*., 2002). Due to the effect of the hydrolytic enzymatic treatment coupled with purification of the residual CWM, these may have resulted in the removal of most of the hydrolysable components of the CWM. The residual lignified materials were poorly susceptible to hydrolysis under the preparation conditions applied. This implies that the residual CWM actualy analysed may not have been complete representative of the cassava CWM present. Pre-treatment of these CWMs with 12 M H_2SO_4 before hydrolysis with 1 M H_2SO_4 as recommended by Salvador *et al*. (2000) may have possibly enabled more complete hydrolysis.

Another weakness in the characterisation of the CWM was the use of flame ionisation detection (FID) technique. FID is a very old detection method, which may not have given a comprehensive analysis compared to MS detection. MS is suitable to apply for both structural and compositional elucidation of complex carbohydrates such as cell wall polysaccharides (Ciucanu, 2006).

Chapter 4.2 focused on improving tannin sorghum processing functionality with application in brewing/bioethanol and beverage. The effect of NaOH treatment of milled tannin sorghum on tannin functionality was evaluated in terms of enzyme inhibitory activity of tannins using a standard Alpha-Amylase Assay (Megazyme, 2011). The effect of NaOH treatment in reducing tannin inhibitory activity against α-amylase was directly related to the determination of starch liquefaction time using a model mashing system. Liquefaction time was measured in terms of the duration from when the mash stopped stirring due to starch gelatinisation until the mash began to stir by means of the magnetic stirrer bar alone. This was based on the assumption that the rate of reduction in mash viscosity to a free flowing mash can be related to the level of activity of α-amylase in hydrolysing gelatinised starch to dextrins and maltodextrins. With high tannin functionality, starch liquefaction time is prolonged due to high tannin inhibitory activity against α-amylase.

The effect of NaOH treatment on tannin functionality was also evaluated by measuring the assayability of the tannins using modified Vanillin-HCl assay. The substantial reduction in assayable tannins in this study may be as a result of steeping in dilute NaOH solution, which may have enable interaction tannin with other grain components. According to Naczk and Shahidi (2004), tannin interaction with other constituents results in the formation of insoluble complexes that limits their extraction. In this study, methanol acidified with 1% HCl was used to extract the tannins. The extraction procedure used may not have extracted all the tannins, thereby the extracts analysed may not have been representative of the actual levels of tannins present. Application of sonication would have been more effective in extracting the tannins, as applied by Awika, Dykes, Gu, Rooney and Prior (2003). Another factor that determines assayable tannins using this assay is the reactivity of the tannin polymers with vanillin. According

to Beta *et al*. (2000), the interaction of NaOH with phenolic compounds involves oxidation of phenolic groups resulting in the formation of highly polymeric compounds. With vanillin-HCl tannin assay, the structural complexity of tannin polymer subunits affects reactivity of its internal flavan-3-ol units with vanillin in the formation of colour complexes (Schofield *et al*., 2001). The formation of highly polymeric tannins due to reaction between tannins and NaOH may have also reduced assayable tannin measured using this assay. However, there is an indication that the level of assayable tannins measured by the Vanillin-HCl assay corresponded with the tannin functionaility determined by both Alpha-Amylase Assay and starch liquefaction time.

In Chapter 4.3, the effect of the NaOH treatment on the tannins was investigated using normal-phase HPLC. Generally, the structural and compositional complexity of condensed tannins poses a major challenge in terms of extraction, quantification and identification (Schofield *et al*., 2001). Accessibility of the extraction solvent in extracting tannins has been noted to be affected by their structure (Asquith *et al*., 1983). In view of this, sonication was investigated in the preparation of the tannin extracts. Sonicated extracts gave resolvable peaks compared to extraction without sonication as analysed using the normal-phase HPLC. The normal-phase HPLC technique used has been successfully applied in characterising tannins (Lazarus *et al*., 1999; Awika *et al*., 2003). The method separates and quantifies based on the degree of polymerisation, as the tannin molecules elute with increase in the degree of polymerisation (Gu *et al*., 2002). In the study, monomeric and dimeric compounds were easily identified by comparing their retention times with the external standards. The limitation encountered was that there are no commercially available tannin standards with DP>3. The range of tannin compounds with DP>2 were estimated based on the normal phase HPLC profile of Sumac (a Type III tannin) sorghum, as reported by Dlamini, Dykes, Rooney, Waniska and Taylor (2009).

In view of the drawback of the normal-phase HPLC in tannin analysis, MS was applied in an attempt to characterise the resulting effect NaOH on tannin properties. Mass spectrometry can give qualitative information in relation to both structural and molecular properties of higher molecular weight oligomeric tannin compounds (Hammerstone *et al*., 1999). Limitation of this technique also relates to the lack of oligomeric tannin standards.

The huge peaks with very large molecular masses eluted at the end could not be identified in comparison to tannin molecular weights reported in literatures. These peaks with massive molecular weights could be related to the effect of the NaOH treatment, presumably polymerising the tannin molecules to very large molecular weight compounds.

A qualitative and quantitative chromatographic technique that could have been used for analysis of the tannins and the effects of the NaOH treatment is size exclusion chromatography (SEC). SEC is based on separating compounds according to their size in the solution, with the separation taking place within the pores of the column packing material by a physical sorting process (Hagnauer, 1982). It is a well-accepted technique for the measurement of the molecular weight of polymeric materials (Barth, Jackson and Boyes, 1994). However, setting up SEC with existing HPLC instrumentation and finding compatible mobile phase/column packing material that can operate at room-temparature has been noted a major limitation (Kostanski, Keller and Hamielec, 2004). SEC could not be used in this study because it required specialised HPLC instruments that can operate at elevated temperatures to give satisfactory results (Kostanski *et al*., 2004).

5.2 Research findings

A major finding of this study was the synergistic effect of combining the Ultraflo and Viscozyme enzyme preparations in modifying the structure and composition of the CWM. This synergy can be attributed to the unique activities of the enzyme preparations complementing each other. According to Ahola, Turon, Osterberg, Laine and Rojas (2008), complementary activity of combining multiple enzyme activities relate to the different cleavage mechanisms that enable complete and effective enzymatic hydrolysis. The Ultraflo preparation has β-glucanase, xylanase and other pentosanase activities, while the Viscozyme enzyme preparation has β-glucanase, cellulase, xylanase, pentosanase (presumably more general pentosanase) and arabanase activities (Novozymes, 2008). As reviewed by Ghose and Bisaria (1987), hemicellulases comprise of complex array of enzymes that are endo and exo in nature. Combining the two enzyme preparations probably brought together endo and exo hemicellulolytic activities. The complementary nature of the exo- and endo- cleavage mechanisms therefore enabled

more complete hydrolysis of the xylan and other pentosan polymers. The cellulase activity in the Viscozyme preparation further contributed to the synergistic effect.

Qualitative and quantitative characterisation suggested that the residual CWM remaining after the enzyme treatment was lignified. This clearly relates to the complementary effects of the multiple enzyme activities in hydrolysing the thin parenchyma cell walls. As the thin parenchyma cell walls are not lignified (McCluskey, Allison, Duncan and Jarvis, 1984).

A possible mode of action of the cellulolytic and hemicellulolytic activities of the enzyme preparations on the parenchyma cell wall polymers is illustrated in Figures 5.1 and 5.2. The cassava tuber parenchyma cells that hold the starch granules are made up of cellulosic cell walls (Souza, Gomes-Filho and Campos, 1998). As illustrated in Figure 5.1a, general plant cell wall arrangement and structural organisation is based on the primary cell walls referred to as cellulosic cell walls (Maieves, De Oliveira, Bernado, Muller and Amante, 2012). The secondary cell walls are made up of hemicellulosic polymers of arabinose, xylose, other pentoses, hexoses, glucuronic acids and some deoxyl sugars (Saha, 2003). The intracellular spaces contain pectic polysaccharides, which serve as adhesive connecting the cellulosic cell walls in the middle lamella (Maieves *et al*., 2012). Cassava root tuber cell walls also contain soluble hemicellulose mucopolysaccharides, known as cassava tuber mucilage (CTM) (Charles *et al*., 2008). This CTM also constitutes part of the adhesive components of the intercellular spaces.

As illustrated in Figure 5.1b, complementary cleavage activities of the various xylanases, pentosanases and arabanase in the enzyme preparations completely hydrolyse the hemicellulosic polymers around the closely packed cellulose microfibrils. Possible minor enzyme activities in the two enzyme preparations act on the pectin polymers in the intercellular spaces holding the cellulosic cell walls together. All these activities result in opening of the compact structure of the cell walls due to the hydrolysis of the cell wall hemicellulosic polymers, pectin and mucillage layers, thereby exposing the cellulosic cell walls. With the cellulose microfibrils loosely packed, this enables access of the cellulase in the Viscozyme preparation to the cellulosic cell walls. This cellulase activity leads to

the fragmentation of the cell walls, hence releasing the trapped starch granules (Figure 5.2).

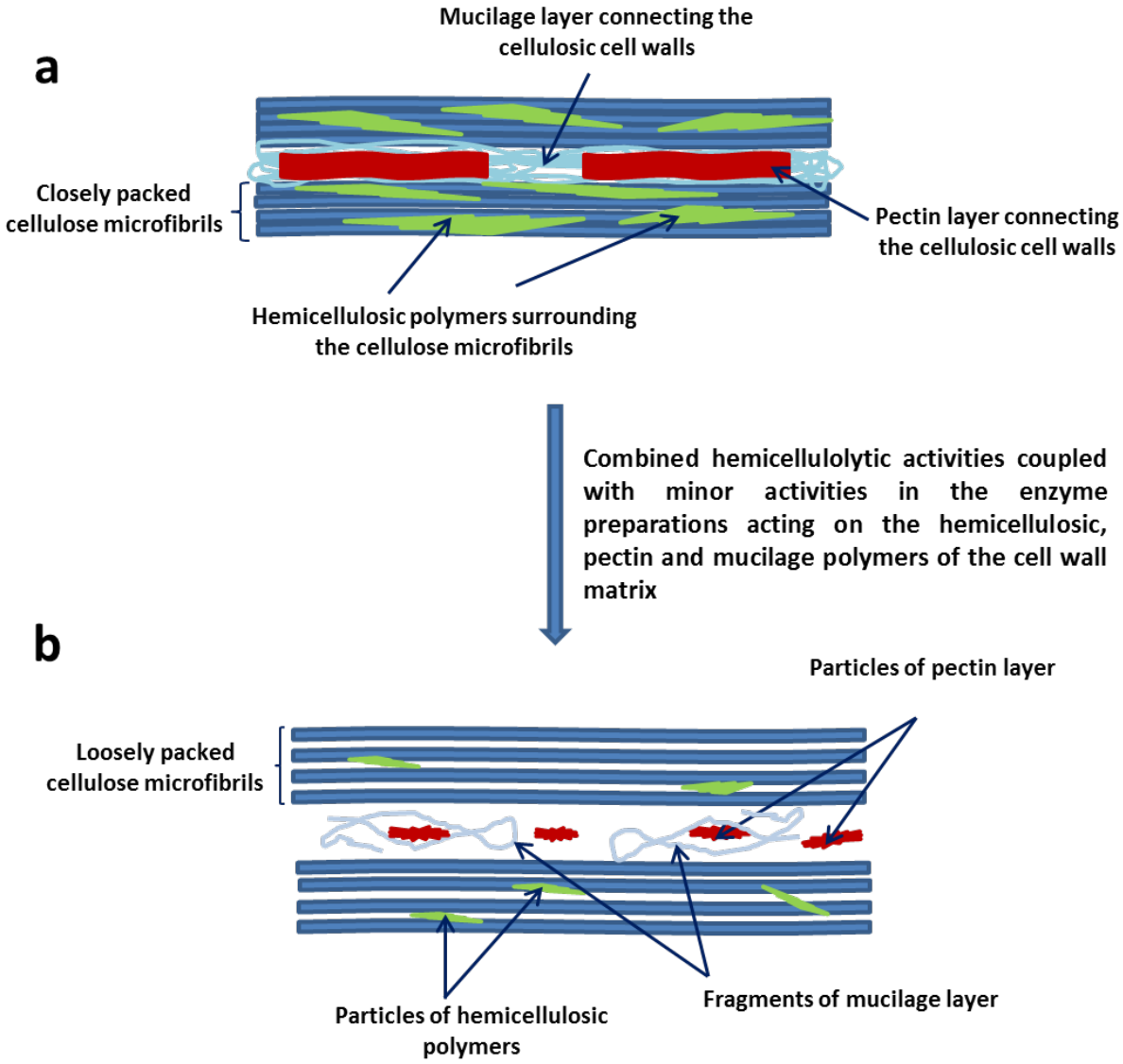

Figure 5.1: Schematic illustration of the proposed synergistic mechanism of the hemicellulolytic and other minor enzyme activities in the enzyme preparations on the structure and composition of the cassava parenchyma cell walls. **a:** Cell wall structural organisation before the enzymatic treatment; **b:** Hydrolysis of the mucilage, pectin and hemicellulosic polymers resulting in the opening of the compact cellulosic cell walls.

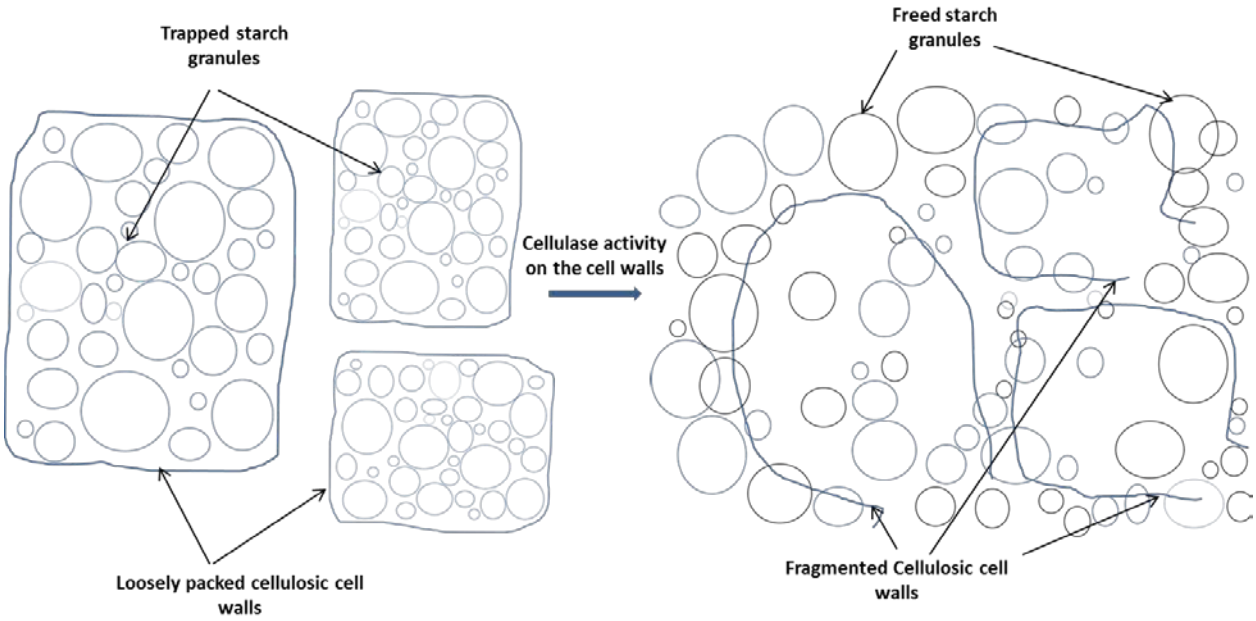

Figure 5.2: Schematic illustration of the effect of cellulase activity from the Viscozyme preparation on the loosely packed cellulose microfibrils, leading to the fragmentation of the parenchyma cell walls freeing the starch granules.

The findings on the tannin properties of the Type II and III tannin sorghums indicated significant differences in their functionality. The Type III sorghum condensed tannins had high α-amylase inhibitory activity, as compared to the Type II sorghum tannins which had no α-amylase inhibitory activity, i.e. the same as the Type I non-tannin sorghums. This difference can be attributed to differences in the structural characteristics of their condensed tannins. The Type II sorghum tannins are deposited within the cell wall of the testa layer, while the Type III sorghum tannins are deposited along the testa layer and the pericarp of the grain (Earp *et al.*, 2004). This is a result of the Type III tannin sorghums possessing spreader genes, whereas the Type II does not possess the spreader gene (Rooney, 2000). This genetic factor may possibly also have an impact on the bound structure of their condensed tannins. In the absence of spreader gene in Type II sorghum, its tannin molecules may have strongly bound with the testa layer cell walls. The influence of spreader gene in the Type III sorghum may have resulted in more extractable tannin molecules, due to more of less bound tannins. In view of this, the enzyme inhibitory activity of the Type III sorghum tannins could be due to free multiple binding sites of its phenolic groups due to its tannins less bound. Multiple binding sites

serve as a key factor that determines selectivity and specificity of condensed tannin interaction with proteins (Hagerman and Butler, 1981).

Sonication has previously been applied in optimising the extraction of phenolic compounds in sorghum (Awika *et al*., 2003) and in coconut shell powder (Rodrigues, Pinto and Fernandes, 2008). A major finding of this present study was that sonication resulted in a considerable increase in the assayable tannins in all the Type II sorghums and in the higher tannin Type III sorghum, whereas with the low tannin Type III sorghums there was no significant increase in their assayable tannin (Table 4.3.1). This effect of sonication on the level of assayable tannins further revealed differences in their tannin properties, as it relates to their structure.

The effect of sonication on the level of assayable tannin can be explained by two possible mechanisms. The first relates to the release of the bound tannins, thereby resulting in more extractable tannins. During sonication, mechanical energy generated could result in disruption of the structural linkages between the tannin and the cell wall components. Sonication also facilitates swelling and hydration, resulting in the enlargement of pores in the cell wall due to diffusion of the extraction solvent into the cell wall layers (Vinatoru, 2001). This would lead to an increase in mass transfer of the freed tannin molecules. Another possible effect of sonication relates to depolymerisation of the polymeric tannin molecules. Depolymerisation of the tannin polymers could increase tannin reactivity with vanillin in the assay, resulting in an increase in assayable tannins. This is because not all the internal flavan-3-ol units in the tannin polymer react with vanillin (Schofield *et al*., 2001). These two mechanisms may have complemented each other, thereby increasing the level of assayable tannins.

Based on the findings of this study, it is suggested that the combination of these two mechanisms of sonication resulted in the considerable increase in assayable tannins measured in the Type II sorghum. As stated, with the Type II sorghum, its tannins are characterised with acid-labile bonds such as ether and glycosidic bonds linking the tannin molecules to other cell wall materials (Asquith *et al*., 1983). Likewise, the Type II sorghum tannins are characterised with relative higher degree of polymerisation compared to the Type III tannin sorghums with low degree of polymerisation (Bullard *et*

al., 1981). The effect of sonication may have depolymerised the freed tannin polymers, resulting in increased level of assayable tannins in the Type II sorghum due to increase in tannin reactivity with vanillin.

The reduction in tannin reactivity with α-amylase is suggested to be linked to the reaction between tannin and NaOH resulting in the formation of structurally modified tannin polymers. It is evident that the resulting polymeric compounds were extractable, as there was an increase in the number and area of eluted peaks in the NaOH treated extract (Figure 5.3), as analysed by NP-HPLC. The extractability of these highly polymerised compounds was also evident with the MS analysis, as peaks eluted at the end have massive molecular masses (Table 4.3.4). According to Laks *et al*. (1987), condensed tannin preparations obtained by alkaline extraction exhibit lower reactivity with aldehyde. This effect is attributable to reaction between alkali and proanthocyanidin involving structural rearrangement of their pyran ring (C-ring) forming catechinic acid type structure (Hashida and Ohara, 2002). Therefore, the polymerisation of the tannin molecules by NaOH supports the suggestion by Beta *et al*. (2000) that the reaction between NaOH and tannin phenolic groups results in the formation of highly polymeric compounds.

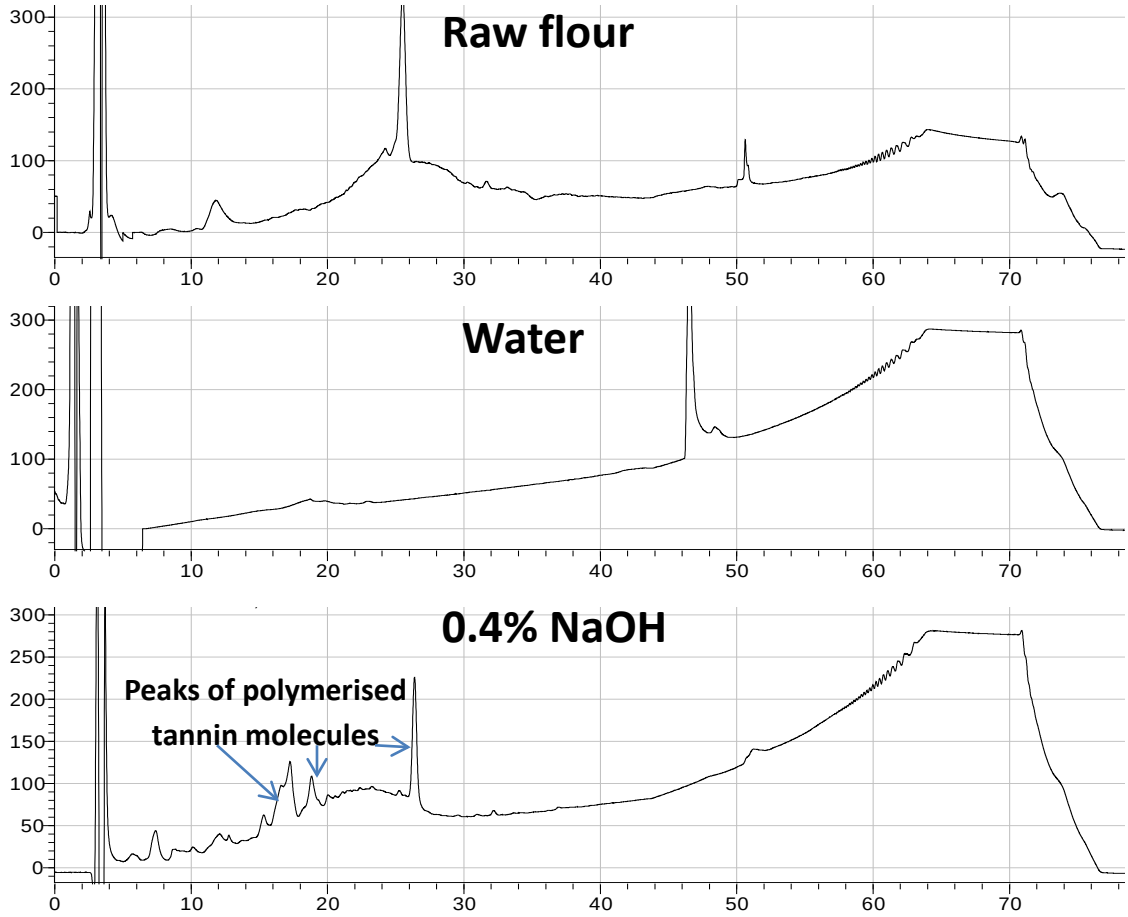

Figure 5.3: Normal-phase HPLC chromatograms indicating that NaOH treatment polymerised the tannin molecules in Type III sorghum.

To explain the possible mechanism responsible for tannin inactivation by NaOH, the effect of NaOH steeping resulting in the formation of polymeric tannin molecules is illustrated in Figures 5.4 and 5.5. As shown in Figure 5.4a, deposition of tannin molecules in Type III tannin sorghum is along the testa layer cell walls, suggested slightly bound tannin molecules. The Type II tannin molecules are shown to be deposited within the testa layer cell walls and strongly bound to the testa layer cell walls (Figure 5.4b). It is speculated that NaOH first resulted in cleavage of the linkage between the tannin molecules and the testa layer cell walls (Figure 5.4c). This is based on the assumption that alkali steeping leads to opening of the cell wall structure (Dewar *et al*., 1997). This effect of NaOH would release the tannin molecules bound to the cell walls.

This facilitated polymerisation of these tannin molecules, which was possibly initiated by the effect of NaOH ionisation in water (Figure 5.5).

It is suggested that polymerisation of the freed proanthocyanidin molecules involves cross-linking, leading to formation of more highly polymeric proanthocyanidin compounds. This is based on the probable mechanism of cross-linking of tannin molecules by formaldehyde in the formation of large polymers (McGrath, Kaluza, Daiber, Van der Riet and Glennie, 1982). This cross-linking reaction between the tannin molecules could be by oxidative coupling of the carbon at position 2 of the opened pyran ring (C-ring) with oxygen at position 7 of other tannin molecules (Figure 5.6). Cross-linking is initiated by the hydroxyl ions from NaOH ionisation in water. According to Kennedy *et al*. (1984), condensed tannins in alkaline solution undergo C-ring opening and rearrangement via radical reactions involving traces of oxygen. Formation of double ether bonds in A-type proanthocyanidins involving oxidative coupling between C2 and O7 is mediated by hydroxylation (Porter, 1988).

The reduction in reactivity of tannins against enzymes may be linked to a steric hindrance effect on the reactive sites of the polymerised proanthocyanidin molecules formed. This could be as a result of possible transformation in the stereochemistry of the polymerised proanthocyanidin molecules. In relation to cross-linking of the tannin molecules, the formation of a double linked ether bond coupling the C2 and O7 appears to shield the OH group at C3 (Figure 5.6). These structural modifications of the polymerised molecules may obscure the phenolic functional groups, thereby reducing the number of available reaction sites. Asano, Ohtsu, Shinagawa and Hashimoto (1984) noted that condensed tannin substitution pattern affects tannin-protein interactions.

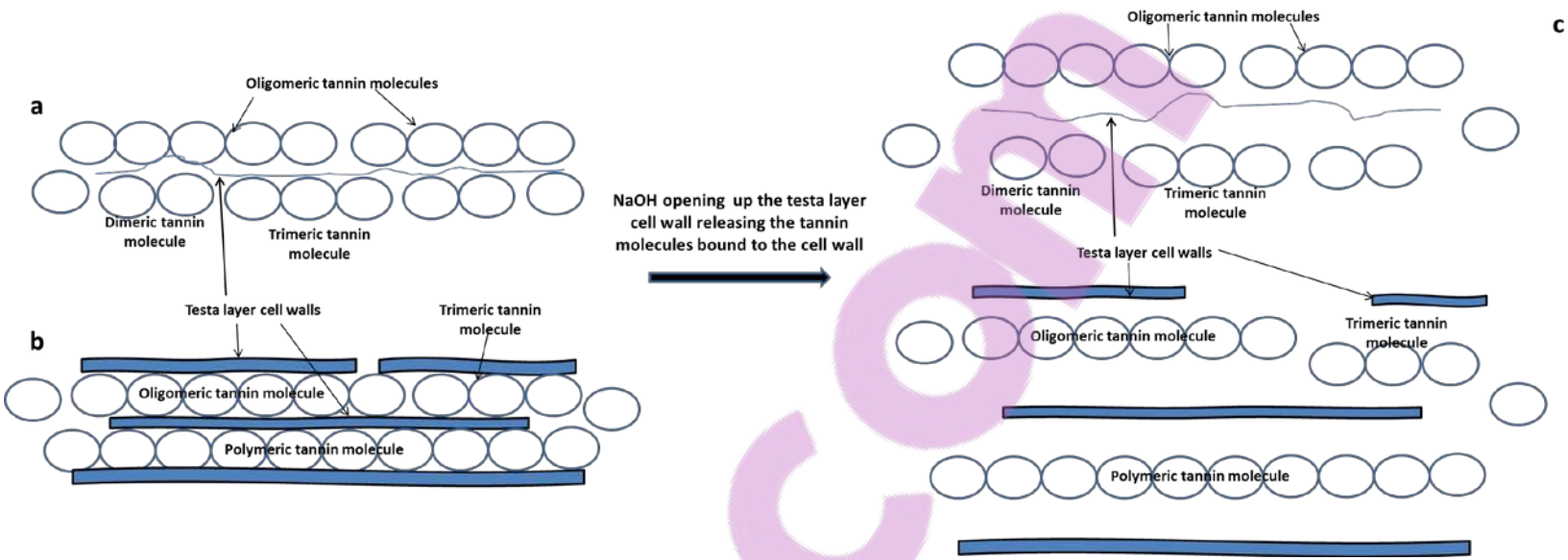

Figure 5.4: Illustration of the proposed effect of NaOH steeping on sorghum proanthocyanidins in freeing the tannin molecules from the testa layer cell walls. **a:** Tannin molecules in the Type III sorghum deposited along and slightly bound to the testa layer cell wall (Earp *et al*., 2004); **b:** Tannin molecules in the Type II sorghum deposited within and strongly bound to the testa layer cell wall (Earp *et al*., 2004); **c:** Release of the tannin molecules due to the effect of NaOH steeping opening up the testa layer cell wall structure.

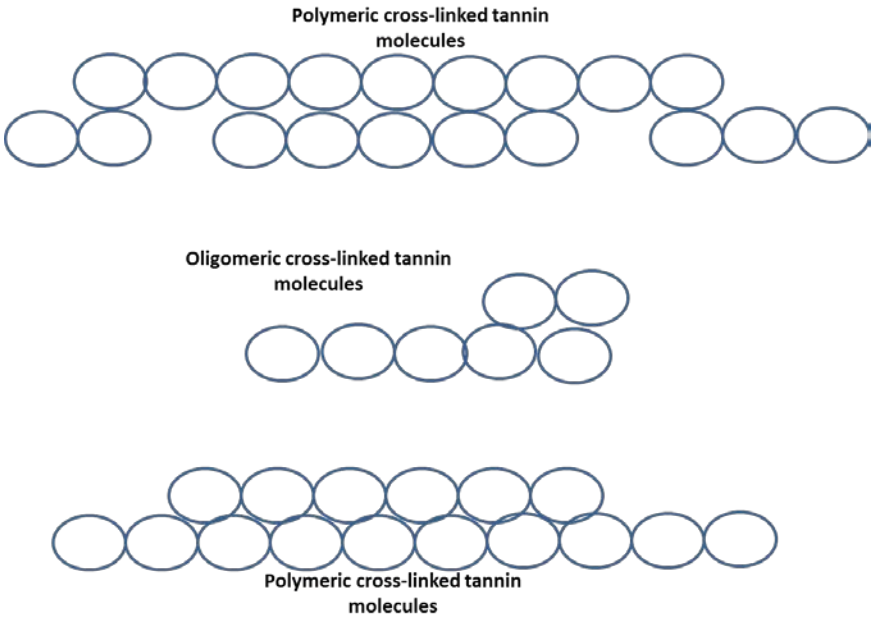

Figure 5.5: Illustration of the formation of higher oligomeric and polymeric tannin compounds through cross-linking of the freed tannin molecules released as shown in Figure 5.4, initiated by hydroxyl ions from NaOH ionisation.

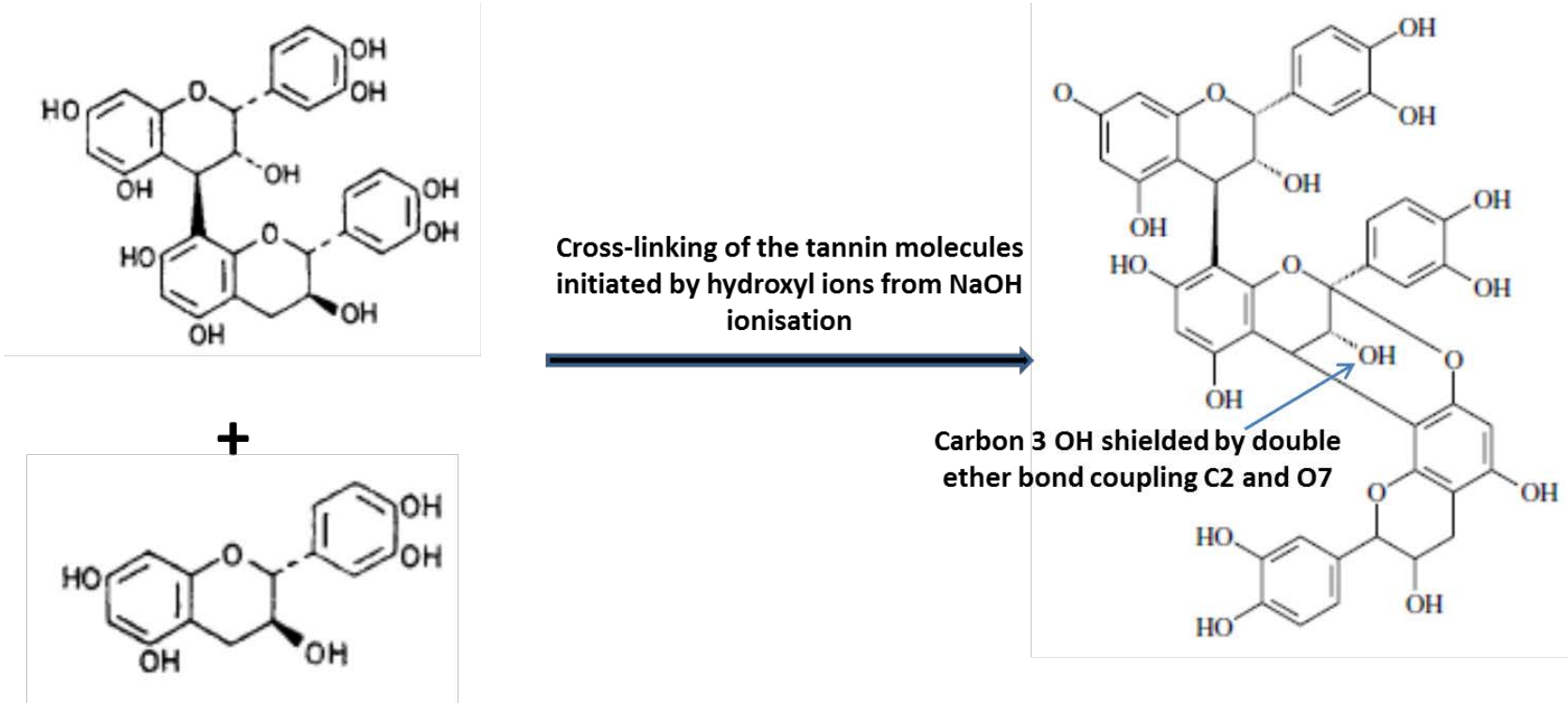

Figure 5.6: Possible polymerisation reaction initiated by hydroxyl ions from NaOH ionisation involving oxidative coupling of C2 and O7 in the cross-linking the tannin molecules, leading to formation of highly polymeric tannin molecules.

5.3 Potential application of cell wall and condensed tannin pre-treatments in cassava root tuber and tannin sorghum utilisation

The application of enzymatic pre-treatment technology using multiple cellulolytic and hemicellulolytic enzyme activities with complementary hydrolytic mechanisms will potentially improve processing functionality of cassava tuber cell walls. In addition, this enzymatic pre-treatment technology will reduce the level of cassava bagasse generation, as well aid in processing application of cassava bagasse in bioethanol production. Hence, adoption of this technology would facilitate increase in industrial utilisation of cassava root tuber as raw material in food and beverage processing.

With tannin sorghum, application of the NaOH pre-treatment technology to milled grains has potential to improve its processing functionality in brewing and bioethanol production. However, applying this pre-treatment technology in brewing may constitute a problem, in particular, using low grade and cheap NaOH that could be heavily contaminated with heavy metals. This is because heavy metals such as silver, mercury and nickel are source of contamination in raw sodium hydroxide (Avantor Materials, 2011). Its application in bioethanol production would be more appropriate because there would be less concern about presence of heavy metals. Bioethanol production utilising high tannin sorghum pre-treated with NaOH will be use as biofuel. According to Parekh, Khwaja, Khan, Naqvi, Malik, Khan and Hussain (2002), presence of heavy metals such as lead based on WHO guidelines not exceeding 0.15 g/L fuel is considered safe. This NaOH pre-treatment technology will potentially make tannin sorghum a suitable raw material for the bioethanol industry, while making the non-tannin sorghum more available as a human foodstuff. Therefore, adoption of NaOH pre-treatment technology to the Type III tannin sorghum would potentially create a market for its large-scale industrial use, providing a market for small-holder commercial farmers.

5.4 Future work

There is need for better characterisation of the cassava tuber cell walls before and after the enzymic treatment. Accurate characterisation of the CWM in terms of the proportions of the individual soluble and insoluble cell wall polymers is necessary. Adequate information on the cell wall components will help to further improve on this enzymatic pre-treatment technology. In view of this, a wider range of cellulolytic and hemicellolytic

enzymes with known cleavage mechanisms should be investigated to determine the most suitable enzyme preparations. Detailed characterisation of the microstructure and composition of the cell walls should be explored using transmission electron microscopy, and using scanning electron microscopy. These techniques should facilitate a better understanding of the cell walls structural organisation before and after the enzymatic treatment.

In relation to quantitative analysis, method development with regard to isolation of the cell wall components is required to prepare the samples for both compositional and structural quantitative analysis. In view of this, Solid-state Nuclear Magnetic Resonance (NMR) spectroscopy should be considered. This is because NMR spectroscopy is non-destructive technique that can resolve separate cell wall components and also capable of measuring relatives changes in the components (Gamble *et al*., 1996). This is in contrast to chromatography, where chemical solubilisation of the cell walls into their polymeric components is required. Such chemical solubilisation leads to hydrolysis and loss of the some covalent and most non-covalent bonds prior to characterisation of the cell walls (Jarvis and Apperley, 1990). NMR spectroscopy can also provide information on the molecular ordering of the cell wall polymer components (Newman, Davies and Harris, 1996). By using ^{13}C-NMR signal dispersion, signals from cellulose, pectic and other matrix polysaccharides can be resolved without overlapping (Jarvis and Apperley, 1990).

To understand the exact chemistry of the reaction between condensed tannins and NaOH, research should focus on characterising the structural linkages and configuration of the tannin polymers formed. With the Quadrupole Time of Flight mass spectrometry technique used in this study not being able to provide the necessary information, Matrix-Assisted Laser Desorption/Ionization Time of Flight mass spectrometry (MALDI-TOF MS) should be considered. MALDI enables non-destructive vaporisation and ionisation of both large and small biomolecules, as well as allowing for the measurement of compounds with high accuracy and sub-picomole sensitivity (Lewis, Wei and Siuzdak, 2000). According to Krueger *et al*. (2003), application of MALDI-TOF MS in proanthocyanidin characterisation is able to identify molecular weight differences based on the extent of hydroxylation, the type of interflavan linkages (A- or B-type) and

substitution pattern such as glucosylation. MALDI-TOF MS also makes it possible to assign specific stereochemistry to heteropolyflavan-3-ols of a higher degree of polymerisation by comparing the MALDI-TOF MS mass distributions with formulated predictive equations (Krueger *et al*., 2003). This would enable the determination of alterations in tannin structure based on their oxygenation and substitution pattern, as well as their stereochemistry. MALDI-TOF MS would also be useful in determining the effect of sonication during extraction on the structural characteristics of condensed tannins in the Type II and III tannin sorghums.

A further recommendation in relation to inactivating the tannins in Type III tannin sorghum would be to investigate food grade alkalis such as steeping in weak alkaline solution of calcium carbonate. More simply, admixing of Type III tannin sorghum with Type II tannin and non-tannin sorghum types should be considered in combination with application of this pre-treatment technology. Future study on tannin sorghum utilisation in the production of beverage products such as matoho should investigate the mechanism by which added sugar (sucrose) in the formulation inactivates tannins. Furthermore, both descriptive and consumer sensory analyses of utilising tannin sorghums in the production of matoho beverage product should be conducted. This will help to determine sensory properties of the product in order to facilitate its consumer acceptability.

6. CONCLUSIONS

Cellulolytic and hemicellulolytic hydrolysis of cassava parenchyma cell walls that are not lignified results in considerable structural and compositional modification of the cell walls. The direct effect of this enzymatic treatment on the cell walls leads to a substantial reduction in the volume and viscosity of the cassava cake. This enzymatic treatment of the cell walls also results in an increase in the level of freed starch granules. Treatment of the cell walls with multiple enzymes in combination leads to complementary activity to hydrolyse the parenchyma cell walls. The enzymes did not hydrolyse the lignified cell wall components. These findings indicate that the hydrolysable parenchyma cell wall components in cassava are responsible for the poor wort filtration in brewing with cassava. The hydrolysable cell wall components consist of cellulolysic and hemicellulosic polymers, as indicated by their sugar units. Application of this combined enzymatic treatment could substantially increase the use of cassava in brewing and in other beverage.

The substantial differences in the tannin functional properties of Type II and III tannin sorghums are attributable to the difference in the degree of their condensed tannins being bound to the testa cell walls. The Type I non-tannin sorghum and Type II tannin sorghum does not have amylase inhibitory activity. In the case of the Type III tannin sorghums, its high tannin amylase inhibitory activity is due to its extractable tannins. The higher level of extractable tannins seems to enable more interaction with protein/enzymes. However, both Type II and III tannin sorghums can be potentially be utilised in fermented non-alcoholic beverage products without need for application of pre-treatment technology. Inclusion of sugar (sucrose) in the formulation appears to provide some form of protection against tannin inhibitory activity especially in the Type III sorghum to the bacteria starter culture during fermentation.

Steeping milled Type III tannin sorghum grain in dilute NaOH solution effectively inactivates the tannins and significantly reduces the inhibitory activity of the tannins against α-amylase. This reduction in inhibitory activity relates directly with an improvement in starch liquefaction time during the brewing mashing process. The action of NaOH on the tannin molecules results in the formation of highly polymeric

compounds. The chemistry of reaction between tannin and NaOH is probably by cross-linking of the tannin molecules. Therefore, the mechanism of tannin inactivation by NaOH steeping is probably due to cross-linking of tannin molecules, resulting in the formation of highly polymeric tannin molecules that are less reactive with the α-amylase protein. Application of this technology to Type III tannin sorghums could increase their utilisation, especially in bioethanol production.

Application of the cassava and tannin sorghum pre-treatment technologies will potentially increase their industrial applications. The creation of new markets for these crops should help ensure sustainable food security through economic empowerment of the small-holder farmers in sub-Saharan Africa.

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8. APPENDIXES

Presentations:

Poster presentations-

Title: Development of methodology for tannin inactivation in sorghum for improved tannin sorghum utilization.

Authors: Adetunji, A.I., Duodu, K.G. and Taylor, J.R.N.

20th Biennial SAAFoST Congress, October, 2013 at CSIR, Pretoria, South Africa

Title: Inactivation of condensed tannins in sorghum flour by alkali treatment.

Authors: Adetunji, A.I., Duodu, K.G. and Taylor, J.R.N.

AACCI Annual Meeting, October, 2014 at Provindence, Rhodes Island, USA

Oral presentation-

Title: Development of methodology for tannin inactivation in sorghum flour for improved tannin sorghum utilization.

Authors: Adetunji, A.I., Duodu, K.G. and Taylor, J.R.N.

New Voices in Cereal Science and Technology, September, 2014 at SAGL, Pretoria, South Africa

Publication:

Manuscript accepted for publication in the journal Food Chemistry.

Title: Inactivation of tannins in milled sorghum grain through steeping in dilute NaOH solution

Authors: Adetunji, A.I., Duodu, K.G. and Taylor, J.R.N