Flavonoid compounds of sorghum and maize bran and their inhibitory effects against alpha-amylase

BY

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DECLARATION

I, Ilriénne Johanna du Plessis, declare that the dissertation , which I hereby submit for the degree MSc Food Science, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:……

DATE:…….

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DEDICATION

I dedicate this work to my two grandchildren, Ulrike and Ewan Nederveen, born during the past two years who enrich my life tremendously and most of all, to my Saviour who gave me the strength and means to complete this study.

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ABSTRACT

Flavonoid compounds of sorghum and maize bran and their inhibitory effects against alpha-amylase

By

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Supervisor: Prof KG Duodu

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Diabetes mellitus is a chronic metabolic disease caused by insufficient insulin production by the pancreas or when the body loses its ability to utilise insulin effectively or both. This leads to an accumulation of glucose in the blood of diabetic people which is detrimental for their health in the long term. Due to an increase in prevalence, the disease is becoming a growing concern to health authorities worldwide, especially in developing regions where inadequate health care systems and poor socio-economic conditions exacerbates the situation. A potential way of preventing diabetes is to limit starch digestibility to control blood glucose levels. Sorghum and maize are important food cereals in many regions of the world and they contain various phenolic compounds, particularly flavonoids which can inhibit starch hydrolysing enzymes like α-amylase. Therefore these cereals could have potential anti-diabetic properties.

In this study, various extracts prepared from bran samples of white maize and white and red non-tannin sorghums were analysed for inhibitory activity against porcine pancreatic α-amylase using the Megazyme Ceralpha α-amylase assay kit. It was necessary to provide a basis for an understanding of the amylase enzyme inhibitory properties of the brans in relation to their phenolic content and therefore, their potential anti-diabetic properties. The total phenolic content of white maize and red and white non-tannin sorghum bran methanolic extracts was therefore determined, using the Folin Ciocalteu assay. The profile and concentration of flavonoids in extracts from the bran samples was determined using high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).

Red non-tannin sorghum bran and its extracts had higher inhibitory activity against porcine pancreatic α-amylase than bran and bran extracts from white maize and white non-tannin sorghum. Unextracted bran samples also inhibited the enzyme, indicating that the bran components inhibiting the enzyme did not need extraction and could exert inhibitory effects in situ. The bran of the red non-tannin sorghum varieties had significantly ($p<0.05$) higher levels of total phenolics (3.35 – 4.13 g CE/100 g) than that of the white maize (1.07-1.20 g CE/ 100 g) and white non-tannin sorghum varieties (0.99-1.15 g CE/ 100 g) as shown by results from the Folin Ciocalteu assay . Results from HPLC analysis showed that extracts from red sorghum varieties had significantly ($p<0.05$) higher levels of total flavonoids (166.8-269.8 mg/100 g) than extracts from white maize (18.7-24.8 mg/ 100 g) and white non-tannin sorghum (64.9-69.9 mg/100 g). Acidified organic bran extracts had higher total phenolics than non-acidified organic and water extracts. Results from LC-MS analysis showed that the acidified methanol extract from red non-tannin sorghum bran had the highest concentration of flavonoids with flavones (apigenin and luteolin) and flavanones (eriodictyol and naringenin) detected as the two main groups of flavonoids. In agreement with total phenolic and flavonoid content, this extract also had the highest α-amylase inhibitory activity. The water extract of the red non-tannin sorghum (Mr BUSTER), was the only water extract of all the grains that contained flavanones like eriodictyol, and was also the only water extract that showed inhibition against α-amylase. These observations indicate that the flavone and flavanone compounds identified in the extracts are important for inhibition of the α-amylase enzyme.

Nutraceutical-type preparations from red non-tannin sorghum bran could have applications in foods as anti-diabetic agents by inhibiting α-amylase activity and thus controlling postprandial glucose levels in people suffering from diabetes.

CHAPTER 1

1 Introduction and Problem Statement

Diabetes mellitus has been identified by health advocacy groups like the World Health Organization (WHO) as a world-wide illness of great concern (Wagman & Nuss, 2001). It has been projected by the WHO that the number of deaths as a result of non-communicable diseases including diabetes mellitus will increase by 15% globally between 2010 and 2020 (WHO, s.a.). The International Diabetes Federation (IDF) estimated that 366 million cases of diabetes were reported in 2011 causing about 4.6 million deaths and by 2030 the number of diabetes cases will increase to 552 million worldwide (IDF, 2011). The IDF also estimated that 183 million (50%) cases are undiagnosed (IDF, 2011). The highest prevalence of diabetes (80%) exists in low- and middle income countries with the highest number of people with diabetes between the ages 40 to 59 years. In their review, Abubakari and Bhopal (2008) predict that the largest proportional increase in diabetes cases will occur in developing regions like sub-Saharan Africa. The problem can be exacerbated in these regions, especially in the remote rural areas, due to ineffective and under-developed health care systems with inadequate resources and limited choice of medication (Bannon, 2011). Beside the health risk, the cost of diabetic health care world-wide was estimated at 465 billion US dollars in 2011 (IDF, 2011). People with diabetes need two to three times the health-care resources compared to people without diabetes, contributing to about 15% of national health care budgets (IDF, 2011).

Diabetes mellitus is a chronic metabolic disease caused by insufficient insulin production by the pancreas or when the body loses its ability to utilise insulin effectively or both. Two main types can be identified: insulin-dependent diabetes mellitus (IDDM) or Type 1 and non-insulin dependent diabetes mellitus (NIDDM) or Type 2 diabetes mellitus (Brody, 1999). Insulin is a hormone produced by the β-cells of the pancreas, and is responsible for controlling blood glucose levels by enabling tissue cells to take up glucose from the blood (IDF, 2011). Disruption in β-cell function is the main cause of Type 2 diabetes (Butler, Janson, Bonner-Weir, Ritzel, Rizza & Butler, 2003). Starch is the main source of energy in human diets, but diets containing highly digestible starches are not suitable for people with diabetes. Due to the compromised glucose metabolism of diabetic people, these carbohydrates are the main source of undesirable high plasma glucose concentrations after consumption of a meal containing starchy food (Reviewed by Butterworth, Warren & Ellis, 2011).

The enzyme α-amylase plays a major role in the hydrolysis of starch during digestion and is mainly responsible for breaking of the α-1,4-glucosidic bonds in the starch resulting in the formation of maltose and dextrin (Rooney & Pflugfelder, 1986). Therefore, a possible way of treating diabetes is by the inhibition of starch hydrolysing enzymes such as α-amylase (Wagman & Nuss, 2001). It is a

well-known fact that phenolic compounds like tannins are able to bind with proteins (Reviewed by Le Bourvellec & Renard, 2011; Haslam, 1974) and thus show significant inhibitory activity against α-amylase (Gonçalves, Mateus & de Freitas, 2011; Griffiths, 1986; Kandra, Gyémánt, Zajácz & Batta, 2004; McDougall, Shpiro, Dobson, Smith, Blake & Stewart, 2005). Various studies have shown that phenolic compounds in extracts from food sources including fruits (McDougall *et al.*, 2005), herbs (McCue & Shetty, 2004; McCue, Vattem & Shetty, 2004), tea and wine (Kwon, Apostolidis & Shetty, 2008) inhibit α-amylase activity. However, very little is known about whether phenolic extracts from cereal plant sources like non–tannin sorghum, can also inhibit amylase enzymes. There is therefore a gap in knowledge about the potential of these cereals to be utilised as food sources with anti-diabetic properties. Hargrove, Greenspan, Hartle and Dowd (2011) found that although the tannin-rich extracts of tannin sorghum inhibited α-amylase more, the extracts from non-tannin sorghum also inhibited the enzyme. This could be an indication that other phenolic compounds like flavonoids in the non-tannin sorghum extracts may have the ability to inhibit α-amylase. Similar findings were obtained by Lemlioglu-Austin, Turner, McDonough and Rooney (2012). Ju-Sung Kim, Hyun and Kim (2011) reported highest inhibitory activity of extracts from sorghum against α-glucosidase and α-amylase compared to extracts from proso and foxtail millet and this was attributed to the higher phenolic content of the sorghum extracts. However the phenolic compounds in the extracts were neither characterized nor quantified which provides an opportunity for further research. Furthermore, studies have shown that solutions of pure flavonoid compounds such as quercetin and luteolin, both found in sorghum (Reviewed by Awika & Rooney, 2004), are powerful inhibitors of porcine α-amylase (Tadera, Minami, Takamatsi & Matsuoka, 2006). This inhibiting effect is related to the chemical structure of the flavonoids (Tadera *et al.*, 2006; Lo Piparo, Scheib, Frei, Williamson, Grigorov & Chou, 2008).

Sorghum is a tropical, drought-resistant cereal grown in developing regions like Africa and Asia (Leder, 2004). It is the world's fifth most important cereal, in terms of production (about 56 million tons) and area planted (FAOSTAT, 2012). Sorghum is a major source of dietary energy and protein for over 1 billion people in the semi-dry tropical areas of the world (Kent & Evers, 1994). It is processed into various foods such as grain rice-type products, baked products, porridges and beverages (Belton & Taylor, 2004) for human consumption. Maize is utilized as a staple food mainly in Latin-American and African regions. It is processed into food products including flour, grits, breakfast cereals, alcoholic beverages like whisky and corn starch and syrup (Kent & Evers, 1994).

The major groups of phenolic compounds found in sorghum are phenolic acids, flavonoids and condensed tannins (Reviewed by Dykes & Rooney, 2006). Maize only contains phenolic acids (Chiremba, Taylor, Rooney & Beta, 2012) and flavonoids (Styles & Ceska, 1977; Žilić, Serpen, Akıllıoğlu, Gökmen & Vančetović, 2012), but no tannins (Taylor & Dewar, 2001).

The inhibition of digestive enzymes such as α-amylase by flavonoids suggests that grains such as sorghums in general and non-tannin sorghums in particular and even maize, which are sources of flavonoids, may have important anti-diabetic properties. The use of non-tannin sorghum or maize either by inclusion in the diet or in the form of nutraceutical preparations could be considered as a potential strategy for controlling blood glucose levels and thereby possibly preventing the development of Type 2 diabetes without using expensive drugs.

CHAPTER 2

2 Literature Review

In this literature review an overview of diabetes mellitus with emphasis on type 2 diabetes will be given. The morphology and chemical composition of sorghum and maize with emphasis on starch chemistry, and phenolic content with emphasis on flavonoids content will be discussed. Starch digestion and its implications for diabetes, the possible interactions between flavonoids and starch-hydrolysing enzymes and the possible role these flavonoids could play in the prevention and control of type 2 diabetes will also be discussed.

2.1 Diabetes mellitus

2.1.1 Prevalence

According to the International Diabetes Federation (IDF) 366 million cases of diabetes were reported in 2011 causing about 4.6 million deaths and by 2030 the number of diabetes cases will increase to 552 million worldwide (IDF, 2011). It is been estimated that the highest prevalence of diabetes (80%) exists in low and middle income countries with the highest number of people with diabetes between the ages 40 to 59 years. An estimate of 183 million (50%) cases went undiagnosed (IDF, 2011). This might be due to the fact that signs of diabetes are not immediately obvious (Wagman & Nuss, 2001). In their review, Abubakari and Bhopal (2008) predicted that the largest proportional increase in diabetes cases will occur in developing regions like sub-Saharan Africa. The problem can be exacerbated in these regions, especially in remote rural areas, as a result of ineffective and under-developed health care systems with inadequate resources and limited choice of medication (Bannon, 2011).

2.1.2 Causes and characteristics of diabetes mellitus

Diabetes mellitus is a chronic metabolic disease caused by insufficient insulin production by the β-cells of the pancreas or when the body loses its ability to utilise insulin effectively or both (Butler *et al.*, 2003). The disease occurs mainly in two forms: insulin-dependent diabetes mellitus (IDDM) also known as Type 1 diabetes and non-insulin dependent diabetes mellitus (NIDDM) also known as Type 2 diabetes. Only 5 to 10% of people with diabetes have IDDM which may occur approximately at the age of 30. It can be characterised as an autoimmune disease and is caused by a loss of β-cells of the pancreas leading to termination of insulin production by the β-cells. On the other hand, NIDDM is more generally found in people over 30 years, accounts for more than two thirds of people with diabetes and is mostly associated with obesity due to the muscle tissue not responding to insulin. This

is the result of faulty cell signalling, reducing the effectiveness of the insulin, rather than a lack of insulin production (Brody, 1999).

Insulin is a hormone produced by the β-cells of the pancreas, responsible for stimulating glucose transport into various cells like adipose cells and muscle. After a meal containing sugar or starch, the level of plasma glucose is elevated resulting in an increased entry of glucose into these cells. In the case of NIDDM, insulin resistance occurs as a first step in the development of the disease, resulting in the tissue cells responding abnormally to insulin. As insulin resistance develops further, the pancreas start to compensate for this by increased secretion of plasma insulin resulting in more elevated levels of plasma glucose. Finally the β-cells start to fail, due to a decrease in β-cell mass as a result of increased apoptosis. It is suspected that this decreased β-cell mass results in insulin deficiency leading to IDDM. It is however very difficult to establish this effectively, because pancreatic tissue from humans usually only becomes available at an autopsy. By then the pancreas may have already undergone substantial autolysis. As a result, reliable clinical information about autopsy cases are often very difficult to obtain (Brody, 1999; Butler *et al.*, 2003).

2.1.3 Symptoms and risks of diabetes mellitus

Early symptoms of diabetes include extreme thirst, excessive food consumption, extreme urination, weight loss and blurred vision (Brody, 1999). Possible risks include reduced activity, obesity (Shaw, Sicree & Zimmet, 2010), under nutrition during pregnancy leading to genetic alterations in glucose metabolism (Reviewed by Pinney & Simmons, 2010) and oxidative stress due to over nutrition, resulting in apoptosis of β- cells (Reviewed by Donath & Shoelson, 2011). Long-term diabetes may lead to loss of eye sight, lower limb amputations, renal failure, doubled chance of contracting cardiovascular disease, nerve damage, pregnancy complications, impotence in males and higher risk of tuberculosis (Brody, 1999; IDF, 2011).

2.1.4 Prevention and treatment of diabetes mellitus

The main problem for people with IDDM or NIDDM is the inability to control their postprandial plasma glucose levels. This problem may be addressed by the use of drugs and dietary adjustments (Brody, 1999). The astronomic cost of diabetic health care world-wide (IDF, 2011), as well as inadequate health care in remote and rural areas (Bannon, 2011), creates a need for research in finding more cost-effective ways to control the escalating incidence of diabetes mellitus.

Diabetes medication is aimed at reducing plasma glucose levels or inhibition of hyperglycaemic spikes. One of the principles on which diabetic drugs is based, involves the limitation of spikes in postprandial glucose levels by the inhibition of starch hydrolysing enzymes like α-amylase and α-glucosidase. This seems to be very effective in the treatment of the disease (Wagman & Nuss, 2001).

Studies indicate that food components like polyphenols, for example, the anthocyanins from the flavonoid group in cereals may have potential health benefits with regard to prevention and control of diseases like diabetes (Ju-Sung Kim *et al.*, 2011). Tsuda, Horio, Uchida, Aoki and Osawa (2003) found that anthocyanins present in purple corn contributed to the prevention of obesity and diabetes in rats. Studies have shown that flavonoids such as quercetin and luteolin, both found in sorghum extracts (Reviewed by Awika & Rooney, 2004), are powerful inhibitors of porcine α-amylase (Tadera *et al.*, 2006).

The presence of flavonoids in some sorghum varieties (Reviewed by Awika & Rooney, 2004) as well as the fact that sorghum is the world's fifth most important cereal, in terms of production (about 56 million tons) and area planted (FAOSTAT, 2012), makes it a very suitable food source to investigate in the quest for a solution in combating and controlling diabetes.

2.2 Sorghum and maize grain morphology

Although similar in many ways, some differences occur in the structural and chemical characteristics of sorghum and maize as shown in Table 2.1.

Table 2.1. Structural and chemical characteristics of sorghum and maize (Adapted from Taylor & Dewar, 2001)

Characteristic of kernel	Sorghum	Maize
Structural:		
Shape	Oval	Flattened
Size	Approx. 3 mm diameter	Several times larger
Naked grain (absence of hull/husk)	Naked grain	Naked grain
Mesocarp	Starchy	Not starchy
Ventral furrow	Absent	Absent
Germ	Large integral	Large integral
Endosperm clearly differentiated in corneous and floury parts	Differentiated	Differentiated
Endosperm cell walls remain intact during malting	Remain intact	Remain intact
Pigmented testa	Present in tannin sorghum	May be pigmented in coloured varieties, but no tannins present in maize
Chemical:		
Starch composition:	Similar	Similar
Phenolic content	Phenolic acids, flavonoids, tannins	Only phenolic acids and flavonoids

2.2.1 The grain caryopsis

The sorghum as well as the maize kernel (Figure 2.1) are both described as a naked caryopsis (Watson, 2003; Taylor & Dewar, 2001) and consist of three structural parts: the pericarp (outer layer), the germ (embryo) and endosperm (storage tissue). The ratio of these three components vary according to variety and environment (Serna-Saldivar & Rooney, 1995; Watson, 2003).

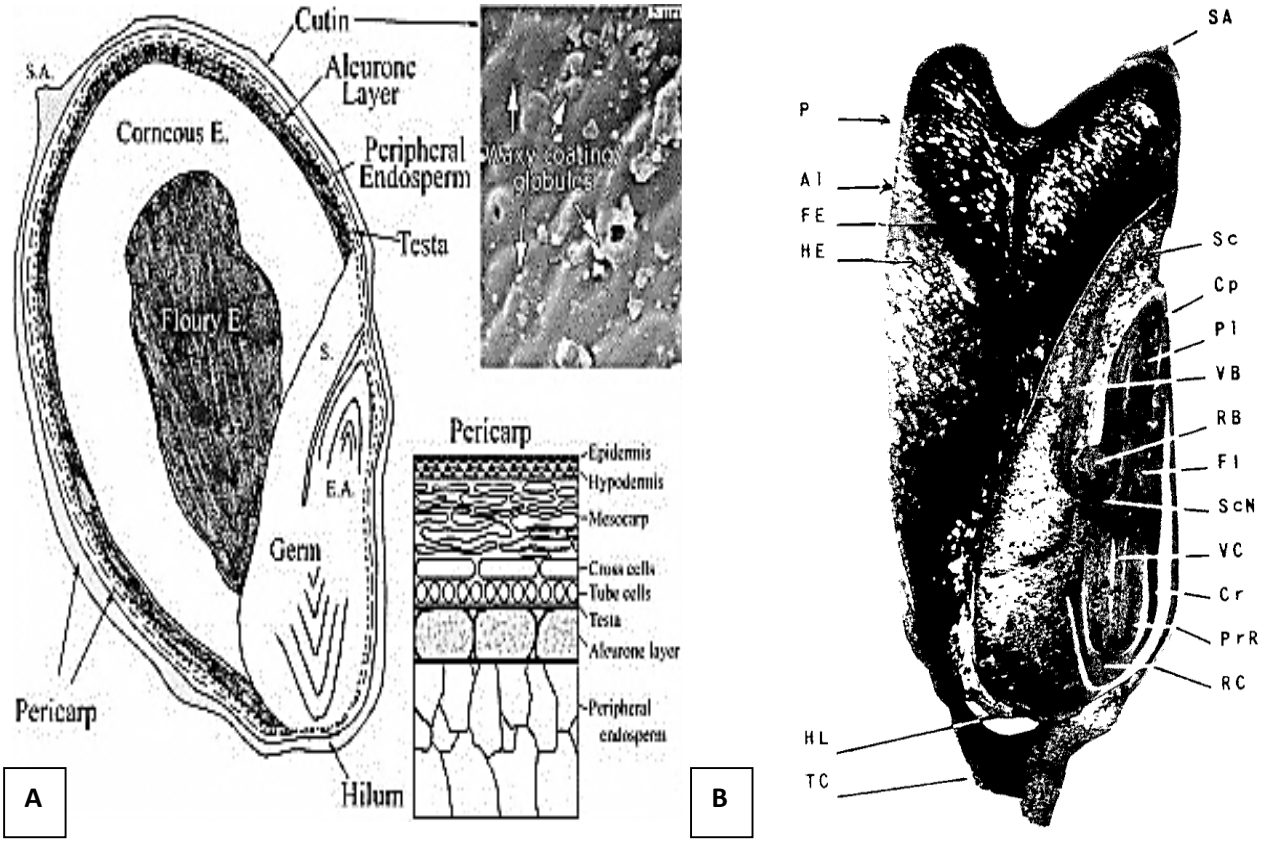


Figure 2.1 Schematic illustration of the sorghum kernel (A) and maize kernel (B) with a breakdown of the pericarp layers for sorghum. To the upper right of A is a view of the cuticle from the outside of the sorghum grain: S.A.=stylar area; E.A.=embryonic axis; S=scutellum (Earp, McDonough & Rooney, 2004). B illustrates a longitudinal section perpendicular to the face of the maize kernel: SA=silk attachment; P=pericarp; A=aleurone; FE=floury endosperm; HE=corneous endosperm; HL=hilar layer; TC=tip cap; Sc=scutellum; Sc – RC=collectively named 'the germ' (Adapted from Watson, 2003)

2.2.2 The pericarp

Similar to that of maize kernels,the pericarp contributes to 6.5 % of the whole grain sorghum kernel and consists of the epicarp and the mesocarp. In sorghum, the epicarp (Figure 2.1) consists of two to three layers of rectangular cells which may contain pigmented components and is usually covered with a layer of wax. The mesocarp of the sorghum contains starch granules (Serna-Saldivar & Rooney, 1995) which differentiates sorghum from other grains like maize (Watson, 2003). Phenolic compounds present in the pericarp of the sorghum kernel are responsible for pigmentation of the pericarp which is genetically controled by R,Y,B_1, B_2 and S genes. However, pigments or pigment precursors are found in nearly all sorghum types, regardless of the color (Hahn & Rooney, 1985).

Some maize varieties e.g. yellow and purple corn (Aoki, Kuze, Kato & Gen, 2002; Žilić *et al.*, 2012) may contain pigments (Taylor & Dewar, 2001). Colour differences in maize may be due to genetic differences in the pericarp, aleurone, germ and endosperm (Watson, 2003).

2.2.3 The testa

The testa (Figure 2.1) or seed coat of sorghum and maize derives from the ovule integuments(Kent & Evers, 1994; Serna-Saldivar & Rooney, 1995). The presence of a pigmented testa in sorghum is mainly controled by B_1 and B_2 genes and is usually an indication of the presence of high levels of tannins in Type II and Type III sorghum (Hahn & Rooney, 1985). Type II sorghums contain tannins in vesicles within the testa layer and in Type III sorghums most of the tannins are located along the cell walls of the testa (Reviewed by Dykes & Rooney, 2006). Although pigmentation may occur in maize, it does not contain condessed tannins (Taylor & Dewar, 2001).

2.2.4 The endosperm

The endosperm (Figure 2.1) of sorghum and maize forms the largest part of the grain (Kent & Evers, 1994). It contributes to 84.2% of the whole sorghum kernel (Serna-Saldivar & Rooney, 1995) and 82-84% of the maize kernel (Watson, 2003). The endosperm of both grains consists of the aleurone layer, peripheral, and differentiated corneous and floury endosperm (Kent & Evers, 1994; Taylor & Dewar, 2001). The cells of the aleurone layer are characterized by a thick cell wall and contain small starch granules and large amounts of protein (protein bodies), enzymes, ash (phytin bodies) and oil (spherosomes). Several layers of densely packed cells containing high amounts of protein and small starch granules are the main constituents of the peripheral endosperm. A blue autofluorescence can be detected in the cell walls of the pericarp, aleurone, and endosperm due to the presence of esters of ferulic acid (Tester, Karkalas & Qi, 2004).

Adjacent to the peripheral endosperm is the corneous or vitreous endopserm followed by the floury endosperm in the centre of the caryopsis (Serna-Saldivar & Rooney, 1995; Kent & Evers, 1994; Watson, 2003). The proportion of the peripheral, corneous and floury endosperm varies in different types of grains (Kotarski, Waniska & Thurn, 1992). In waxy grain types (high amylopectin) the peripheral endosperm is smaller than in normal grain types and consists of a less dense protein matrix with larger starch granules. The constituents of the corneous and floury endosperm include starch granules, protein matrix, protein bodies and cell walls containing β-glucans and hemi-cellulose (Serna-Saldivar & Rooney, 1995; Kent & Evers, 1994; Watson, 2003).

In both sorghum and maize, the protein matrix in the corneous endosperm is in uninterrupted contact with the starch granules and protein bodies lodged in the matrix. These protein bodies are characterized by a circular shape and vary in size 0.4 to 2.0 µm in diameter. The corneous endosperm has a

translucent or vitreous appearance and the starch granules have a polygonal shape, varying in size from 4 to 25 µm with an average size of 15 µm (Serna-Saldivar & Rooney, 1995; Kent & Evers, 1994; Watson, 2003).

The floury endosperm has a chalky appearance (Kotarski *et al.*, 1992) and consists of a discontinuous protein matrix, containing loosely packed, round-lenticular (biconvex) starch granules. The starch granules in the corneous endosperm are smaller and angular and the granules in the floury endosperm are more round and bigger (Serna-Saldivar & Rooney, 1995; Watson, 2003).

2.2.5 The germ

The germ (Figure 2.1) of sorghum (Serna-Saldivar & Rooney, 1995) and maize (Watson, 2003) consists of the embryonic axis, containing the new plant and the scutellum which upon germination forms the leaves and stems from the plumule part and the roots from the radicle part. It contributes to 9.4 % of the sorghum kernel (Serna-Saldivar & Rooney, 1995) and 10-12% of the maize kernel (Watson, 2003). The germ serves as reserve tissue and contains high amounts of oil, protein, enzymes and minerals (Serna-Saldivar & Rooney, 1995; Watson, 2003).

2.3 The chemistry of starch and phenolic compounds in sorghum and maize

Starch, as in all cereals, is the primary carbohydrate in sorghum (BeMiller & Huber, 2008) and maize (Boyer & Shannon, 2003). It contributes to 60 to 80% of normal non-waxy sorghum kernels (BeMiller & Huber, 2008). Starch consists of two forms of glucose polymers: amylose and amylopectin. The amylose and amylopectin are present in the highly organized granules found essentially in the sorghum (Serna-Saldivar & Rooney, 1995) and maize (Watson, 2003; Boyer & Shannon, 2003) starchy endosperm, embedded in a protein matrix. Amylose is a linear polymer and amylopectin is a highly branched polymer. The glucose units of amylose are linked with α(1→4) glycosidic bonds (Figure 2.2) (BeMiller & Huber, 2008). Amylopectin polymers (Figure 2.2) are larger polymers than amylose polymers with numerous branched chains (Figure 2.3) attached to only one reducing end group (BeMiller & Huber, 2008). The glucose units of amylopectin are also linked with α(1→4) glycosidic bonds, but branch points are due to 1→6 linkages (Coultate, 2009). The unbranched chains in the amylopectin (Figure 2.3) are referred to as A chains, the branched chains are referred to as B chains and the central chain containing the reducing group is referred to as the C chain (Rooney & Pflugfelder, 1986).

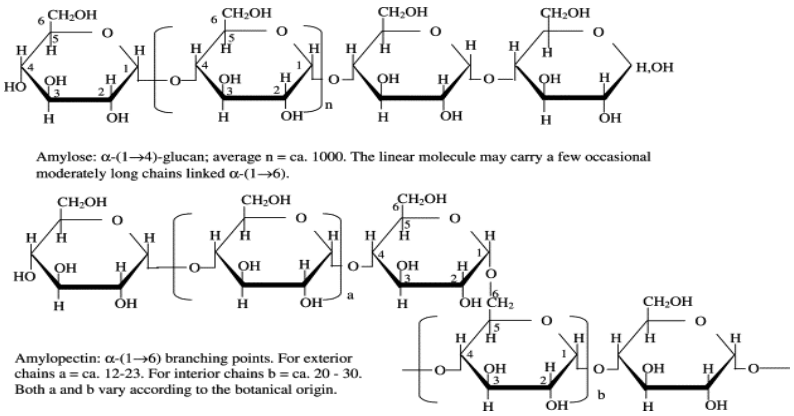


Figure 2.2. Chemical structure of amylose and amylopectin (Tester *et al.*, 2004)

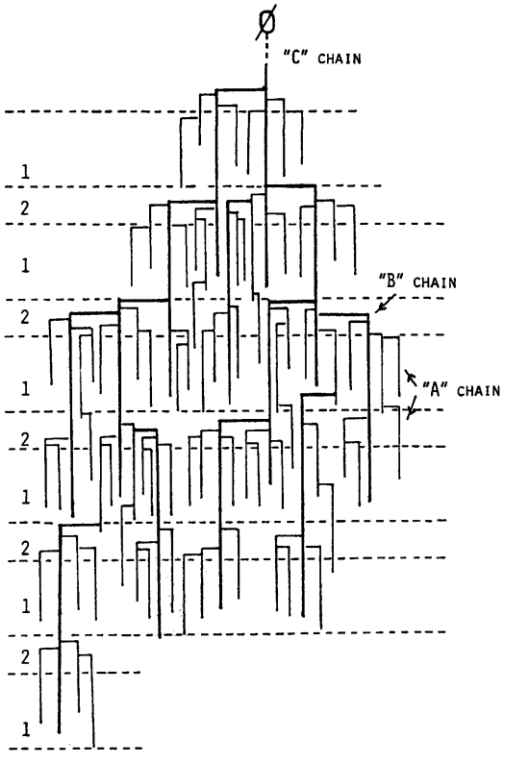


Figure 2.3. Illustration indicating the branched structure of amylopectin (Rooney & Pflugfelder, 1986)

Sorghum (Serna-Saldivar & Rooney, 1995) and maize (Watson, 2003) starch in the endosperm can be classified into waxy and non-waxy or normal starch according to amylose-amylopectin ratio. The ratio may vary between different varieties. Normal sorghum starch contains 23 -30% amylose and waxy types contain 5% (Serna-Saldivar & Rooney, 1995). Normal maize starch contains approximately 27% amylose and 73% amylopectin, waxy maize contains 100% amylopectin and high-amylose corn starch contains 50 to 75% amylose (Mauro, Abbas & Orthoefer, 2003).

2.4 Starch digestion

In the human diet, starch is generally the main source of digestible carbohydrates and as a result of digestion, also the main source of relative high plasma glucose concentrations after consumption of a meal containing starchy food (Reviewed by Butterworth *et al.*, 2011).

Humans produce a range of digestive enzymes responsible for the hydrolysis of starch through the digestive process. In the mouth, the saliva contains α-amylase which hydrolyses accessible starch consumed in the diet. As the starch remains in the mouth for such a short period of time, the enzymes present in the saliva make a very small contribution to starch hydrolysis (Tester *et al.*, 2004). In the stomach, amylase is inactivated by the presence of stomach acids and protein digesting enzymes and very little starch is hydrolysed in the stomach. It is only after the food reaches the duodenum that major starch hydrolysis takes place due to the secretion of pancreatic α-amylase into the lumen. Pancreatic juice, also secreted into the duodenum, neutralizes the pH in the duodenum, creating a favourable neutral pH (pH 6.9 – 7) for the pancreatic α-amylase to become effectively activated. The pancreatic α-amylase hydrolyses the α-(l-4) bonds in the starch polymers resulting in the production of maltose and other disaccharides. On the outer membranes of the intestinal cells, specific enzymes are responsible for the final hydrolysis of the maltose and other disaccharides to glucose molecules. After four hours all sugars and most starches are digested. Some glucose can be absorbed by the mucosal layer in the mouth, but most glucose is absorbed by the cells of the small intestine lining, through active transport, resulting in a rapid rise in blood glucose levels (Whitney & Rolfes, 2011).

The enzyme α-amylase, present in saliva and pancreatic juice (Brody, 1999), can be classified under the group of enzymes responsible for hydrolysis of carbohydrates, called glycosyl hydrolases or glycosidases (Reviewed by Davies & Henrissat, 1995). It is the pancreatic α-amylase which is mainly responsible for catalyzing the primary stages of starch hydrolysis (Figure 2.4) in the small intestine (Reviewed by Butterworth *et al.*, 2011; Tester *et al.*, 2004). Alpha-amylase can further be described as an endo-enzyme due to its random action within the starch polymer chain and not at the terminal glucose units of both polymers and branched points of the amylopectin. The action of alpha-amylase on starch leads to the formation of dextrins, monosaccharides and various disaccharides. Exo enzymes like β-amylase, on the other hand, (Figure 2.4) act on terminal glucose units. Beta-amylase in particular breaks down starch from the non-reducing end and catalyzes the hydrolysis of the second α-1,4 glycosidic bond, releasing two glucose units (maltose) at a time (Brody, 1999; Henrissat & Davies, 1997; Tester *et al.*, 2004).

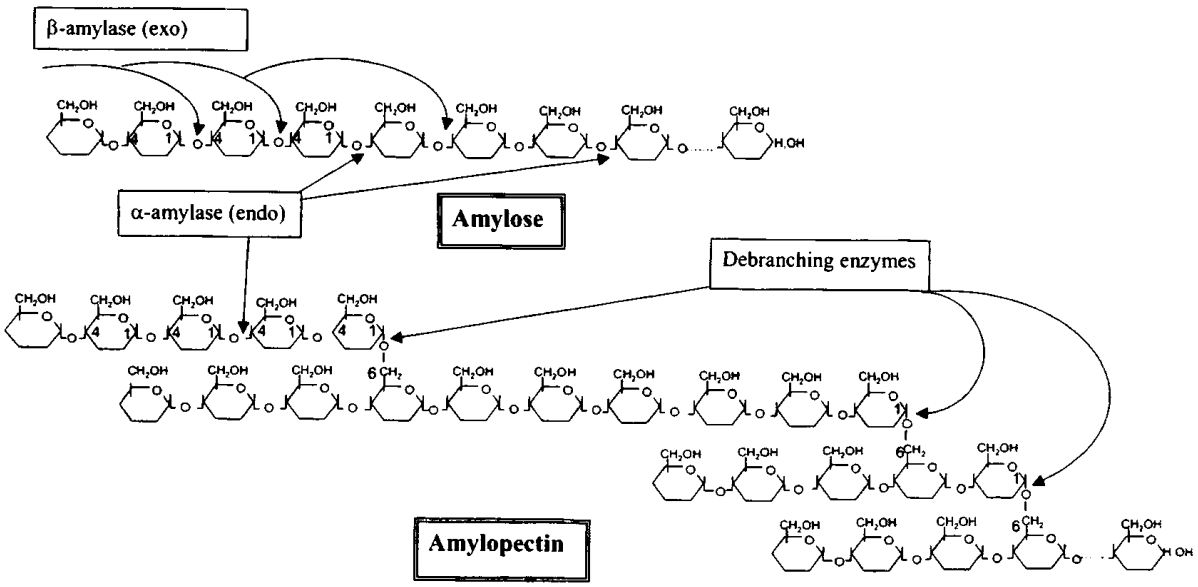


Figure 2.4. Action pattern of hydrolytic enzymes on amylose and amylopectin (Tester *et. al.*, 2004)

2.5 Phenolic compounds

2.5.1 Phenolic compounds in plants and their importance

Phenolic compounds are a diversified group of secondary plant metabolites universally present in all plant species. Their main purpose is to protect the plant against stress conditions, wounding, ultraviolet radiation and infections. They act as phytoalexins, antifeedants and attractants for pollinators and antioxidants in plants. In food applications they provide colour and flavour and are also responsible for enzymatic browning reactions which may affect food quality (Reviewed by Naczk & Shahidi, 2004). The phenolic content of plants can vary even between cultivars of the same species due to genetic and environmental conditions (Reviewed by Bravo, 1998). Processing and storage conditions can also affect the levels of phenolic compounds in plants (Reviewed by Naczk & Shahidi, 2006).

Phenolic compounds in plant tissue are not uniformly distributed (Reviewed by Naczk & Shahidi, 2006). Insoluble phenolic compounds are mostly found in cell walls and the more soluble forms are mostly present inside the plant cell vacuoles. Higher levels of phenolic compounds are present in the outer layers of plants than in the inner plant parts (Reviewed by Naczk & Shahidi, 2006).

Phenolic compounds in plants are derivatives of the amino-acids phenylalanine and tyrosine and produced mainly from two metabolic pathways: the shikimate pathway and the acetate pathway. Phenolic compounds can be categorized into one of the following groups: simple phenolic acids which are derived from benzoic and cinnamic acid, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans and lignins (Reviewed by Bravo, 1998). This classification is based upon the number of carbons present in the side chain (C_n) attached to the phenolic, aromatic (C_6) nucleus

for example: C_6C_n where *n* can be between 0 and 3 or even more in higher plants (Waterman & Mole, 1994a). Sometimes phenolic compounds may be bound or conjugated to one or more sugar units, mostly glucose, due to linkage of the hydroxyl groups of the polyphenol to the sugar unit(s). Polyphenols also have the ability to associate with compounds like carboxylic and organic acids, amines, lipids and other phenols (Reviewed by Bravo, 1998). Some phenolic acids like ferulic and *p*-coumaric acid, mainly found in cell walls, may be esterified to cell wall components like pectin and arabinoxylans or cross-linked to cell wall polysaccharides (Reviewed by Naczk & Shahidi, 2006).

Flavonoids are the most widely distributed and common plant phenols and share a common chemical structure (C_6-C_3-C_6) consisting of 15 carbon atoms arranged in three ring structures as indicated in (Figure 2.5): two aromatic rings (A and B) linked by three carbons which in turn forms an oxygenated heterocycle (C). The position of each carbon atom in the chemical structure is indicated by a number (Reviewed by Bravo, 1998; Reviewed by Naczk & Shahidi, 2006).

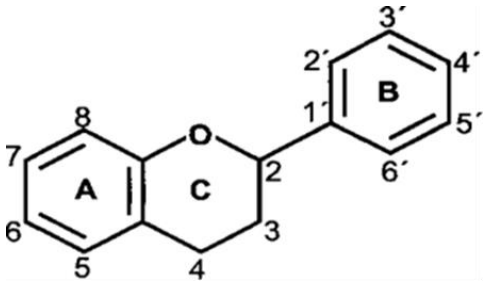
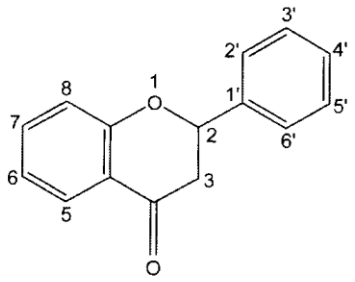


Figure 2.5. The basic structure and numbering system of flavonoids (Reviewed by Bravo, 1998)

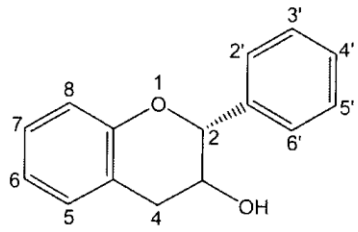
The flavonoid group can be subdivided into 13 sub-classes depending upon the degree of oxidation of the central pyran or C- ring (Figure 2.6). These sub-classes include chalcones, dihydrochalcones, aurones, flavones, flavonols, dihydroflavonols, flavanones, flavanonols, flavandiols or leucoanthocyanidins, anthocyanidins, isoflavonoids, biflavonoids and proanthocyanidins or condensed tannins (Reviewed by Bravo, 1998; Škerget, Kotnik, Hadolin, Hraš, Simonič & Knez, 2005).

The A-ring is synthesized via the acetate pathway and the B-ring via the shikimate pathway (Reviewed by Aherne & O'Brien, 2002; Reviewed by Bravo, 1998). Flavonoids can occur in nature as glycosides (glucose units attached), aglycones (no attached glucose units) and methylated derivatives (Reviewed by Bravo, 1998; Reviewed by Tapas, Sakarkar & Kakde, 2008).



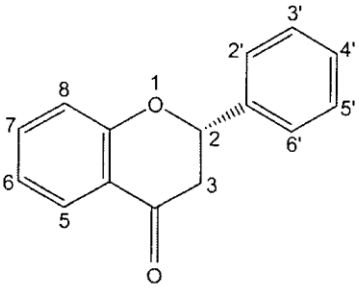
Flavones

Position Compound	5	7	3′	4′
Apigenin	OH	OH	-	OH
Luteolin	OH	OH	OH	OH
Chrysin	OH	OH	–	–



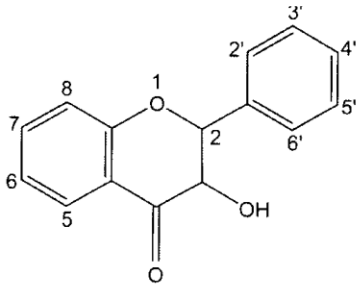
Flavan-3-ols

Position Compound	3	5	7	3′	4′	5′
(+)-Catechin	βOH	OH	OH	OH	OH	–
(−)-Epicatechin	αOH	OH	OH	OH	OH	-
(−)-Epigallocatechin	αOH	OH	OH	OH	OH	OH



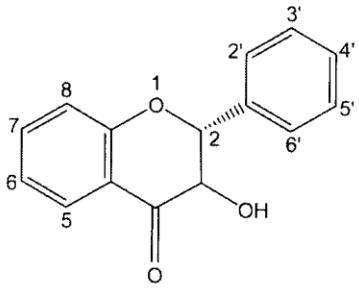
Flavanones

Position Compound	5	7	3′	4′
Naringenin	OH	OH	-	OH
Naringin	OH	*O*-Rha-Glu	–	OH
Hesperetin	OH	OH	OH	OCH_3
Hesperidin	OH	*O*-Rha-Glu	OH	OCH_3



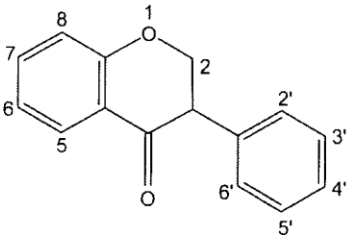
Flavonols

Position Compound	5	7	3′	4′	5′
Quercetin	OH	OH	OH	OH	–
Kaempferol	OH	OH	–	OH	–
Galangin	OH	OH	–	–	–
Fisetin	–	OH	OH	OH	–
Myricetin	OH	OH	OH	OH	OH



Flavanonol

Position Compound	5	7	3′	4′
Taxifolin	OH	OH	OH	OH



Isoflavones

Position Compound	5	7	4′
Genistein	OH	OH	OH
Genistin	OH	*O*-Glu	OH
Daidzein	–	OH	OH
Daidzin	–	*O*-Glu	OH
Ononin	OH	*O*-Glu	CH_3

Figure 2.6. Structures of six of the flavonoid sub-classes (Reviewed by Stalikas, 2007)

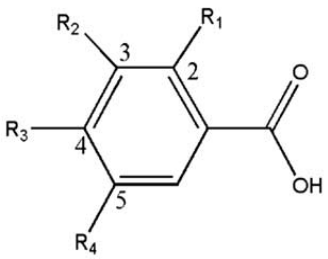
2.5.2 Phenolic compounds in sorghum and maize

The major groups of phenolic compounds found in sorghum are phenolic acids, flavonoids and condensed tannins (Reviewed by Dykes & Rooney, 2006). Phenolic acids and flavonoids are also found in maize, but no condensed tannins have been reported in maize (Taylor & Dewar, 2001). Phenolic acids are present in all sorghums and most cultivars contain flavonoids, but condensed tannins (proanthocyanidins) are only present in sorghum types with a pigmented testa due to the presence of spreader genes (Serna-Saldivar & Rooney, 1995).

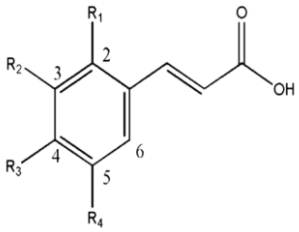
2.5.2.1 Phenolic acids

Phenolic acids in sorghum and maize can occur in both free and bound form (Dykes & Rooney, 2007). Phenolic acids are present as free phenolic acids in the outer layers of the pericarp, testa and aleurone, but mostly as bound phenolic acids associated with the cell walls. Ferulic acid is the main bound phenolic acid, gallic acid is found only in bound form and cinnamic acid only in free form in sorghum. Phenolic acids consist of two classes: hydroxybenzoic and hydroxycinnamic acids as indicated in Table 2.2 (Reviewed by Dykes & Rooney, 2006). Phenolic acids of both these two classes are well represented in sorghum (Table 2.2). Ferulic acid, p-coumaric acid, syringic acid, vanillic acid, p-hydroxybenzoic acid (Guenzi & McCalla, 1966; Mattila, Pihlava & Hellstrom, 2005) and sinapic acid (Chiremba *et al.*, 2012) were the only phenolic acids detected in maize (Table 2.2). The phenolic acids p-coumaric, ferulic and sinapic acid are found in the bound form in maize (Chiremba *et al.*, 2012).

Table 2.2. Some phenolic acid monomers identified in sorghum and maize (Reviewed by Awika & Rooney, 2004; Guenzi & McCalla, 1966; Mattila *et al.*, 2005; Chiremba *et al.*, 2012)



Hydroxybenzoic acid



Hydroxycinnamic acid

	R_1	R_2	R_3	R_4		R_1	R_2	R_3	R_4
Gallic acid	H	OH	OH	OH	**Caffeic acid**	H	OH	OH	H
Gentisic acid	OH	H	H	OH	***Ferulic acid**	H	OCH_3	OH	H
Salicylic acid	OH	H	H	H	***o*-Coumaric acid**	OH	H	H	H
***p*-Hydroxybenzoic acid**	H	H	H	OH	****p*-Coumaric acid**	H	H	OH	H
***Syringic acid**	H	OCH_3	OH	OCH_3	***Sinapic acid**	H	OCH_3	OH	OCH_3
Protocatechuic acid	H	OH	OH	H					
***Vanillic acid**	H	H	OH	OCH_3					

***Phenolic acids reported in maize**

2.5.2.2 Flavonoids

Anthocyanins are the major class of flavonoids detected in sorghum (Table 2.4) and are mostly located in the pericarp (Awika, 2011; Reviewed by Dykes & Rooney, 2006). Sorghum contains overall, higher levels of flavonoids than most other cereals or even some fruit and vegetables (Awika, Rooney & Waniska, 2004). The flavonoid content in sorghum may vary considerably between different varieties and are genetically controlled (Awika, 2011). According to Awika (2011), pigmented sorghum varieties with tan secondary colours contain higher levels of flavones (60 – 386 µg/g) than pigmented sorghums (3.5-47.1 µg/g) with red\purple secondary colours. In tan plant sorghum varieties with a white pericarp colour, no flavanones were detected, but levels of up to 19.4 µg/g flavones were reported (Awika, 2011). The major flavonoids found in maize (Table 2.4) are mainly from the anthocyanin and phlobaphene groups. The anthocyanins can be synthesised by any tissue in the maize plant, but phlobaphenes are mainly found in the cob and pericarp (Styles & Ceska, 1977). Žilić *et al.* (2012) found no anthocyanins in white maize varieties, but still detected flavonoid concentrations of

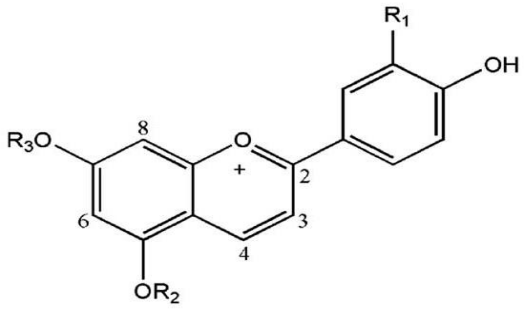
about 248.6 mg catechin equivalents (CE)/ kg. In red maize varieties the flavonoid concentrations were between 267.6 and 270.5 mg CE/ kg with anthocyanin concentrations between 15.4 and 547.5 mg CE/kg. In some yellow and orange coloured maize varieties no anthocyanins were detected, but flavonoid concentrations in these varieties still varied between 280.4 and 268.4 mg CE/kg (Žilić *et al.*, 2012).

Sorghum anthocyanidins are unique in the sense that they do not contain the hydroxyl group in the 3-position of the C-ring like the anthocyanins in fruit, flowers and vegetables. The sorghum anthocyanins are called 3-deoxyanthocyanidins (Table 2.3) and are responsible for most of the red to black pigmentation in sorghum (Awika, 2011). They serve as phytoalexins protecting the sorghum against mold invasion and stresses (Reviewed by Dykes & Rooney, 2006). In contrast to most naturally occurring anthocyanins, the 3-deoxyanthocyanidins in sorghum mostly exist in the aglycone form. Chemically the 3-deoxyanthocyanidins are regarded as very stable due to the absence of the OH-group at the highly reactive C-3 position (Awika, 2011). This stability may also explain why lower levels of glycosylation at position 5 and 7 occur amongst these compounds. With regard to colour properties, the 3-deoxyanthocyanidins, like other anthocyanins also exist as orange – red flavylium cations (AH^+), red-blue quinodial bases, colourless carbinol pseudobases and chalcone species. However, they are found to be more colour-stable at a lower pH than other plant anthocyanins due to their resistance to a drop in molar absorptivity (ability to absorb light). The resistance to change in pH and hydrophilic attacks of the 3-deoxyanthocynidins is believed to contribute to the hydrophobic nature of the heterocyclic ring. These chemical characteristics explain why the 3-deoxyanthocyanins are less soluble in aqueous solvents. They have a tendency to deprotonate into the coloured quinoidal bases with increase in pH rather than into the colourless carbinol bases like most other anthocyanins (Awika, 2011). Extraction of these pigments in aqueous solvents is extremely difficult under atmospheric conditions. Although better extraction yields (90% more) are obtained from acidified organic solvents, there is still indications of under-estimation of the flavonoid content of sorghum bran (Awika, 2011).

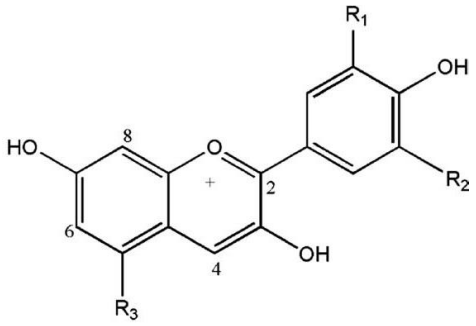
The two common sorghum 3-deoxyanthocyanidins are apigeninidin (yellow) and luteolinidin (orange). Black sorghums have the highest levels of 3-deoxyanthocyanidins concentrated in the bran and luteolinidin and apigeninidin contributes 36 to 50% of 3-deoxyanthocyanidins in black and brown sorghum brans. Reported amounts of 3-deoxyanthocyanins in black sorghum vary between 1.0 µg/g and 2.8 µg/g and levels in the black sorghum bran vary between 4.7 µg/g and 16.0 µg/g (Awika, 2011). In red sorghum, apigeninidin accounts for 19% of the total anthocyanins and amounts between 14 µg/g and 680 µg/g were reported (Awika, 2011; Reviewed by Dykes & Rooney, 2006). Red sorghum contains luteoforol and apiforol, flavan-4-ol compounds, produced from flavanones (naringenin and eriodictyol) which are precursors for sorghum 3-deoxyanthocyanins. The flavan-4-ol compounds also

provide mold resistance (Reviewed by Dykes & Rooney, 2006). In lemon yellow sorghum varieties levels of 3-deoxyanthocyanins between 8 µg/g and 108 µ/g were reported (Awika, 2011). The flavonoids and proanthocyanidins in sorghum and maize are listed in Table 2.4.

Table 2.3. Structures of the 3-deoxyanthocyanidins and their derivatives reported in sorghum compared to the six anthocyanidins found in fruit (Awika *et al.*, 2004)



3-Deoxyanthocyanidins in Sorghum



Anthocyanidins in Fruit

	R1	**R2**	**R3**
Apigeninidin	H	H	H
Apigeninidin-5-glucoside	H	Glc	H
Luteolinidin	OH	H	H
Luteolinidin-5-glucoside	OH	Glc	H
***O*-methyl apigeninidin**	H	H	CH_3

	R1	**R2**	**R3**
Cyanidin	OH	H	OH
Pelargonidin	H	H	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH
Delphinidin	OH	OH	OH
Petunidin	OCH_3	OH	OH

Glc – Glucose unit

Table 2.4. Some flavonoids and proanthocyanidins detected in sorghum and maize (Adapted from Dykes & Rooney, 2007)

Compound	Sorghum	Maize
Anthocyanins:		
Apigeninidin	√	-
Apigeninidin 5-glucoside	√	-
Luteolinidin	√	-
5-Methoxyluteolinidin	√	-
5-Methoxyluteolinidin 7-glucoside	√	-
7-Methoxyapigeninidin	√	-
7-Methoxyapigeninidin 5-glucoside	√	-
Luteolinidin 5-glucoside	√	-
Cyanidin 3-galactoside	-	√
Cyanidin 3-glucoside	-	√
Cyanidin 3-rutinoside	-	√
Pelargonidin 3-glucoside	-	√
Pelargonidin glycoside	-	√
Peonidin 3-glucoside	-	√
Flavan-4-ols:		
Luteoforol	√	-
Apiforol	√	-
Flavones:		
Apigenin	√	-
Luteolin	√	-
Flavanones:		
Eriodictyol	√	-
Eriodictyol 5-glucoside	√	-
Naringenin	√	-

Compound	Sorghum	Maize
Flavonols		
Kaempferol	-	√
Quercetin	-	√
Kaempferol 3-rutinoside-7-glucuronide	√	-
Dihydro-flavonols:	√	-
Taxifolin	√	-
Taxifolin 7-glucoside	√	-
Proanthocyanidin (flavanols) monomers/dimers:	√	-
Catechin	√	-
Procyanidin B-1	√	-
Leucopelargonidin	-	√
Proanthocyanidin polymers:		
Epicatechin-(epicatechin)n-catechin	√	-
Prodelphinidin	√	-
Proapigeninidin	√	-
Proluteolinidin	√	-

√ = Detected; - = Not Detected

2.5.2.3 Tannins

Only sorghum varieties with the $B_1\_B_2\_$ gene contain tannins. Tannins provide some protection against molds and deterioration of the sorghum. Type II and III sorghums have a tannin content of 0.02-0.19 mg/100 g and 0.4-3.5 mg/100 g catechin equivalents respectively (Reviewed by Dykes & Rooney, 2006). Condensed tannins or proanthocyanidins consist of polymerized flavan- 3-ol and/or flavan-3, 4-ol units linked by C4→C8 interflavan bonds (Figure 2.7). They are thus classified as B-type proanthocyanidins. The B-type proanthocyanidins contain (-) epicatechin units as extension units and catechin as terminal units. Condensed tannins like prodelphinidin and heteropolyflavan-3-ols with both A and B interflavan linkages containing procyanidin or prodelphinidin as extension and terminal units have also been reported in sorghum (Reviewed by Dykes & Rooney, 2006). According to Krueger, Vestling and Reed (2002) glucosylated heteropolyflavans containing proluteolinidin or proapigeninidin as extension and terminal units were also found in sorghum. Gupta and Haslam (1978)

also found other flavan-3-ols including catechin and procyanidin B-1 in sorghum. No condensed tannins have been reported in maize (Taylor & Dewar, 2001).

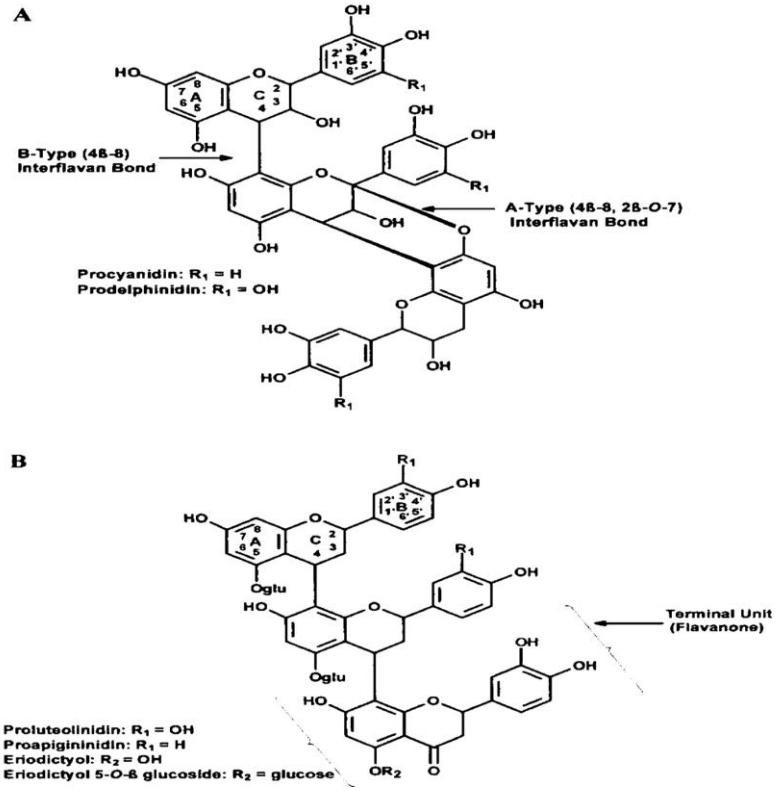


Figure 2.7. (A) Heteropolyflavan-3-ols from Ruby Red Sorghum [Sorghum bicolor (L.) Moench]. (B) Glucosylated heteropolyflavans with a flavanone, eriodictyol or eriodictyol-5-O-β-glucoside as the terminal unit from Ruby Red Sorghum [Sorghum bicolor (L.) Moench] (Krueger *et al.*, 2002)

2.5.3 Extractability of phenolic compounds

In general, the first step in preparation of dietary supplements, nutraceuticals, food ingredients, pharmaceuticals and cosmetic products that are composed predominantly of phenolic compounds is to extract these bioactive compounds from the plant matrix. The most commonly used procedures involve solvent extraction followed by appropriate procedures to store the samples until further use, e.g. freeze-drying (Reviewed by Dai & Mumper, 2010).

The solubility of the phenolic compounds is ruled by their chemical nature, and yield is affected by factors such as polarity of solvents, extraction time, temperature, sample-to-solvent ratio and chemical and physical characteristics of the samples (Reviewed by Dai & Mumper, 2010). Complexity of phenolic compounds may vary in plant material from simple phenolics such as phenolic acids, to highly polymerized compounds such as tannins. In addition, the phenolics may also be associated with other plant components such as carbohydrates and proteins. This may result in the extracts containing non-phenolic compounds including sugars, organic acids and fat. It may therefore be necessary to add additional steps to the preparation process to remove any unwanted substances from the extracts

(Reviewed by Dai & Mumper, 2010). Due to these factors, it is extremely difficult to find a universal procedure suitable for extraction of all plant phenolics (Reviewed by Dai & Mumper, 2010).

Methanol, ethanol, acetone and ethyl acetate are amongst the solvents used in extraction of phenolic compounds and may be used in combination with different concentrations of water to increase polarity. Ethanol is safe to use in preparations suitable for human consumption. Methanol was found to be more effective in extractions of lower molecular weight polyphenols while aqueous acetone has been more effective in extractions of polyphenols with higher molecular weights (Reviewed by Dai & Mumper, 2010). When the objective is mainly to extract anthocyanins, acidified methanol or ethanol would be the most effective solvent of choice. The acidic conditions denature the cell membranes, leading to increased extractability and simultaneously dissolve and stabilise the anthocyanins. Weak organic acids including formic, acetic, citric, tartaric and phosphoric acid and low concentrations (< 1.0%) of stronger mineral acids such as hydrochloric acid are used in combination with organic solvents to obtain good yield of anthocyanins (Reviewed by Dai & Mumper, 2010).

2.6 Flavonoids as inhibitors of α-amylase

Various studies have indicated that phenolic compounds in extracts from food sources including fruits (McDougall *et al.,* 2005), herbs (McCue & Shetty, 2004; McCue, Vattem & Shetty, 2004), tea and wine (Kwon, Apostolidis & Shetty, 2008) inhibit α-amylase activity as indicated in Table 2.5. However, very little research had been done on the inhibitory activity of phenolic extracts from cereal plant sources like non-tannin sorghum on amylase enzymes. The results of a study by Hargrove, Greenspan, Hartle and Dowd (2011) showed that other phenolic compounds apart from tannins, such as flavonoids, in the non-tannin sorghum extracts may also have the ability to inhibit α-amylase. Similar findings were obtained by Lemlioglu-Austin, Turner, McDonough and Rooney (2012) who found that although white corn or maize endosperm flour porridges treated with tannin sorghum bran showed highly reduced starch digestibility, the porridges also showed reduced starch digestibility when treated with non-tannin sorghum bran. These findings were attributed to the phenolic content of the brans. Ju-Sung Kim, Hyun and Kim (2011) compared the inhibitory activity of sorghum, proso and foxtail millet extracts on the activity of α-glucosidase and α-amylase. They found that sorghum extracts showed the highest inhibitory activity against both these enzymes which could be attributed to the higher phenolic content of the sorghum extracts. However, they did not characterize and quantify the phenolic compounds in the extracts. Results of inhibition studies where pure flavonoid compounds were used as inhibitors on starch hydrolysing enzymes like α-amylase, indicated that the inhibitory action of flavonoids like quercetin and luteolin, both found in sorghum (Awika & Rooney, 2004), is related to the chemical structure of the flavonoids (Tadera *et al.*, 2006; Lo Piparo Scheib, Frei, Williamson, Grigorov & Chou, 2008).

Table 2.5. Some studies done on the inhibitory effect of phenolic extracts and pure phenolic compounds against starch hydrolysing enzymes

Inhibitor: Extract/ compound used	Enzyme inhibited	Reference
Acidified methanol extracts from millet seed coats	Pancreatic α-amylase and α-glucosidase	(Shobana, Sreerama & Malleshi, 2009)
Acidified methanol extract of finger millet flour	Malted millet amylase	(Chethan, Sreerama & Malleshi, 2008)
Aqueous ethanol-chloroform extracts from grape seeds	Pancreatic α-amylase	(Gonçalves *et al.*, 2011)
Methanol extracts from tannin and non-tannin sorghum bran	Pancreatic α-amylase activity inhibited by both extracts	(Hargrove *et al.*, 2011)
Commercial tannic acid	Human salivary α-amylase	(Kandra *et al.*, 2004)
Synthetic gallo -tannins	Sweet almond β-glucosidase	(Haslam, 1974)
Ethanol extracts from sorghum, proso and foxtail millets	Porcine and human salivary α-amylase and α-glucosidase strongly inhibited by sorghum extracts	(Ju-Sung Kim *et al.*, 2011)
Flavonoid extracts from various plant sources	Pancreatic α-amylase and yeast α-glucosidase were inhibited more strongly by daidzein, genistein and luteolin; Inhibition was related to the flavonoid structure	(Jong-Sang Kim, Kwon & Son, 2000)
Red wine, white wine; water extracts from oolong tea, green tea, white tea and black tea	α-amylase and α-glucosidase – mostly low or no α-amylase inhibition	(Kwon *et al.*, 2008)
Pure natural flavonoids found in plants	Human salivary α-amylase - the inhibitory activity of the different flavonoids was related to the flavonoid structure	(Lo Piparo *et al.*, 2008)
Commercial oregano and lemon balm extracts	Porcine pancreatic α-amylase	(McCue & Shetty, 2004)
Commercially obtained flavonoid compounds in DMSO	Porcine pancreatic α-amylase; rat and yeast α-glucosidase – Inhibitory activity of different flavonoids on all enzyme activity related to the flavonoid structure	(Tadera *et al.*, 2006)
Water extracts from white and pigmented beans and pea varieties	Beef pancreatic trypsin; porcine pancreatic α-amylase Extracts from pigmented varieties had significantly higher inhibitory activity on the enzymes then white varieties	(Griffiths, 1981)
Extracts from strawberries, blueberries, blackcurrants, red cabbage, red grape juice, red wine and green tea	Salivary, pancreatic α-amylase and α-glucosidase. Degree of inhibition on different enzymes differed depending on phenolic content of each extract	(McDougall *et al.*, 2005)
Proanthochyanins in bark extract of *Acacia mearnsii*	Strongly inhibited α-amylase activity	(Kusano, Ogawa, Matsuo, Tanaka, Yazaki & Kouno, 2010)

Lo Piparo *et al.* (2008) sugested a docking mechanism by which the flavonoid "docks" onto the binding site of the enzyme. This "docking" occurs as a result of hydrogen bonding and covalent interactions between the flavonoid and the amino-acid residues in the binding site of the enzyme. In the study by Lo Piparo *et al.* (2008), a computational ligand docking model was used to indicate that the structure-activity relationship of flavones and flavonols as enzyme inhibitors are dependant on the following structural characteristics of the flavonoid: "i) hydrogen bonds between the hydroxyl groups of the

polyphenol ligands and the catalytic residues of the binding site and (ii) formation of a conjugated π-system that stabilizes the interaction with the active site."

Due to the fact that flavonoid sub-classes like anthocyanins, flavan-4-ols, flavones, and dihydroflavonols are well represented in various sorghum types, including red sorghum (Reviewed by Dykes & Rooney, 2006), it can be expected that these sorghum types might be good inhibitors of porcine pancreatic α-amylase. In contrast to red sorghum, Awika, McDonough and Rooney (2005) found no detectable 3-deoxyanthocyanins in the bran of white sorghum and Chiremba *et al.* (2012) detected lower concentrations of total phenolic compounds in the bran of maize varieties than in the bran of sorghum varieties. These cereal types might therefore be considered to show lower inhibition activity on porcine pancreatic α-amylase.

In conclusion: phenolic compounds in cereals like sorghum, more specifically, the flavonoid group, have the ability to inhibit glycosyl hydrolases like α-amylase (Hargrove *et al.*, 2011); the inhibitory activity of flavonoids on glycosyl hydrolases like α-amylase is structure related (Lo Piparo *et al.*, 2008; Tadera *et al.*, 2006) and different sorghum varieties and cereals like maize differ with regard to phenolic content (Awika *et al.*, 2005; Reviewed by Awika & Rooney, 2004; Chiremba *et al.*, 2012) and therefore could be expected to have different levels of inhibitory activity against α-amylase.

CHAPTER 3

3 Hypotheses and Objectives

3.1 Hypotheses

Hypothesis 1

The bran of red non-tannin sorghum will have higher total phenolic content and total flavonoids than that of white maize and white non-tannin sorghum. Pigmented sorghums generally have higher total phenolic content than non-pigmented types and other non-pigmented cereals such as white maize (Awika *et al.*, 2005). This is because pigmented sorghums contain a wider range of phenolics including anthocyanins and anthocyanidins (Taylor, 2005) which are not present in non-pigmented sorghums (Awika *et al.*, 2005; Dykes, Seitz, Rooney & Rooney, 2009) and white maize (Žilić *et al.*, 2012).

Hypothesis 2

The bran and bran extracts of red non-tannin sorghum will have higher inhibitory activity against porcine pancreatic α-amylase compared to that of white maize and white non-tannin sorghum and this inhibitory effect will be related to the flavonoid content of the bran and bran extracts of the grain.

Sorghum contains overall, higher levels of various flavonoids than most other cereals (Awika *et al.*, 2004; Dykes & Rooney, 2007). Red non-tannin sorghum would generally contain a wider variety of various flavonoids compounds such as anthocyanins (apigeninidin and luteolinidin), flavan-3-ols (catechin and epicatechin), flavan-4-ols (luteoforol and apiforol), flavones (luteolin and apigenin), flavanones (eriodictyol and naringenin) and dihydro-flavonols (taxifolin) compared to white maize and white non-tannin sorghum (Dykes & Rooney, 2007). Some of these flavonoids such as luteolin, apigenin and naringenin are known to be powerful inhibitors of porcine α-amylase (Tadera *et al.*, 2006; Lo Piparo *et al.*, 2008) due to their ability to interact with the enzyme via various mechanisms such as hydrogen bonding (Lo Piparo *et al.*, 2008), ionic bonding and hydrophobic interactions (Papadopoulou, Green & Frazier, 2004).

3.2 Objectives

To characterize the bran of white maize and white and red non-tannin sorghum in terms of their total phenolic and flavonoid contents.

To determine the effect of bran and bran extracts (prepared with various methanolic, acetone and water solvents) of white maize and white and red non-tannin sorghum on the activity of porcine pancreatic α-amylase.

CHAPTER 4

4 Experimental Design and Research

4.1 Experimental Design

The experimental design used in this research is illustrated in Figure 4.1 below. The independent variables were grain type and solvent type while the dependent variables were total phenolic content, flavonoid content and enzyme inhibitory activity.

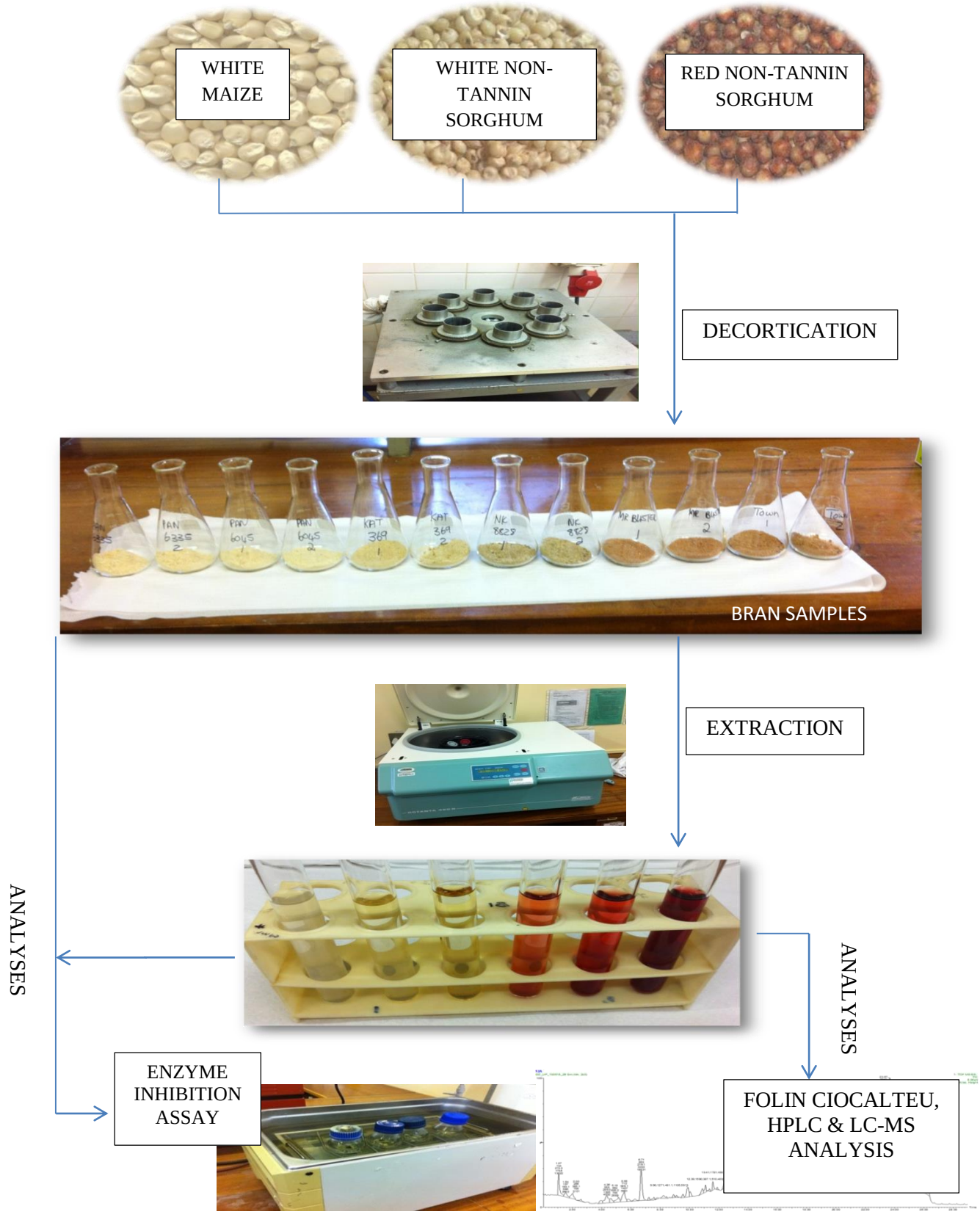


Figure 4.1. Schematic illustration of experimental design

4.2 Characterization of flavonoids and total phenolic content of bran extracts of white maize and white and red non-tannin sorghum

Abstract

Sorghum and maize contain phenolic compounds most of which are flavonoids. Some of these flavonoids are known to be powerful inhibitors of the starch hydrolysing enzyme α-amylase. In order to provide a basis for an understanding of the amylase enzyme inhibitory properties of sorghum and maize bran in relation to their phenolic content and therefore, their potential anti-diabetic properties, total phenolic content (Folin Ciocalteu assay) and flavonoid composition (using High Performance Liquid Chromatography) of white maize and red and white non-tannin sorghum bran extracts were determined. The extracts were prepared using organic (methanol and acetone), aqueous organic (70% methanol and acetone), acidified organic (1% acidified methanol) and water solvents. The bran of the red non-tannin sorghum varieties had higher levels of total phenolics (3.35 – 4.13 g CE/100 g) than that of the white maize (1.07-1.20 g CE/ 100 g) and white non-tannin sorghum varieties (0.99-1.15 g CE/ 100 g) at the 95% significance level. The level of total flavonoids was higher in extracts from red sorghum varieties (166.8-269.8 mg/100 g) than in extracts from white maize (18.7-24.8 mg/ 100 g) and white non-tannin sorghum (64.9-69.9 mg/100 g). Hesperidin and naringenin were present in all the extracts. No catechin, fisetin and quercetin were present in the extracts from the white maize varieties. These observations indicate that non-tannin sorghum bran (particularly of the red variety) is a good source of phenolic compounds such as flavonoids with the potential to exhibit anti-diabetic properties by inhibiting starch hydrolysing enzymes.

4.2.1 Introduction

Diabetes mellitus is becoming an illness of great concern, not only in developed countries, but also in developing regions of the world, like sub-Saharan Africa, (Wagman & Nuss, 2001). In these regions, the problem is exacerbated as a result of ineffective and under-developed health care systems, inadequate resources and limited choice of medication (Bannon, 2011).

The main problem for people suffering from diabetes is to control their postprandial plasma glucose levels. One way of addressing the problem effectively is by inhibition of digestive enzymes like α-amylase, responsible for hydrolysis of carbohydrates into glucose after intake. The impaired glucose digestion due to enzyme inhibition may then prevent a spike in post prandial plasma glucose levels. (Brody, 1999). Studies indicate that flavonoids such as quercetin and luteolin are powerful inhibitors of porcine α-amylase (Tadera *et al.*, 2006). Hargrove *et al.* (2011) also found evidence that simple flavonoids and phenolic acids in sorghum inhibit α-amylase activity. The inhibiting potential of the flavonoids is related to their chemical structure (Tadera *et al.*, 2006).

Sorghum and maize are important food cereals in various regions of the world. Sorghum is a drought-resistant cereal grown extensively in semi-arid developing regions like Africa and Asia (Leder, 2004) while maize is amongst the three crops with the greatest production world-wide. (Fahrnham, Benson & Pearce, 2003). They both contain phenolic compounds consisting mainly of flavonoids, and phenolic acids (Awika, 2011). It has been suggested that in comparison with other cereals, sorghum contains an abundance of flavonoids present in various sorghum varieties (Awika, 2011). Their content of flavonoids suggests that these two cereals (sorghum and maize) could exhibit potential anti-diabetic properties as a result of potential ability to inhibit starch hydrolysing enzymes.

Anthocyanins are the major class of flavonoids detected in pigmented sorghum, mostly located in the pericarp (Awika, 2011; Reviewed by Dykes & Rooney, 2006). In pigmented maize varieties, the major classes of flavonoids present are anthocyanins and phlobaphenes (Styles & Ceska, 1977) . According to Dykes and Rooney (2007) the anthocyanins in maize mainly include cyanidin , peonidin and pelargonidin and their derivatives. However, sorghum contains overall, higher levels of flavonoids than other cereals and even some fruit and vegetables (Awika *et al.*, 2004). The flavonoid content in sorghum may vary considerably between different varieties and are genetically controlled (Awika, 2011). Sorghum contains a unique group of anthocyanins known as 3-deoxyanthocyanidins (red to black pigmentation). The 3-deoxyanthocyanidins in sorghum mostly exist in the aglycone form. The two common sorghum 3-deoxyanthocyanidins are apigeninidin (yellow) and luteolinidin (orange). In red sorghum, apigeninidin accounts for 19% of the total anthocyanins (Awika, 2011; Reviewed by Dykes & Rooney, 2006). Red sorghum contains luteoforol and apiforol, (flavan-4-ol compounds),

produced from flavanones (naringenin and eriodictyol) which are precursors for sorghum 3-deoxyanthocyanins (Reviewed by Dykes & Rooney, 2006). Awika *et al.* (2005) found no detectable 3-deoxyanthocyanins in the bran of white sorghum. White sorghum varieties contain low levels of flavonoids (Reviewed by Dykes & Rooney, 2006). Similarly, Žilić *et al.* (2012) found no anthocyanins in white maize, but considerable amounts of anthocyanins are present in coloured maize varieties.

The objective of this study was to characterize the bran of white maize and white and red non-tannin sorghum in terms of their total phenolic and flavonoid contents. This information will provide a basis for an understanding of the potential amylase enzyme inhibitory properties of the brans in relation to their phenolic content and therefore, their potential anti-diabetic properties.

4.2.2 Materials and Methods

Samples: Two white maize varieties (PAN 6045 and PAN 6335) of similar hardness, two white non-tannin sorghums (KAT 369 and NK8820) and two red non-tannin sorghums (Town and MR Buster) of different hardness were used in this research. The white maize varieties were used as a control. The PAN 6045 and PAN 6335 maize varieties and NK8820 sorghum were from South Africa. KAT 369 sorghum was obtained from Kenya Industrial Research and Development Institute (KIRDI), Kenya. The Town and MR BUSTER sorghum varieties were from Botswana.

Chemicals and standard phenolic compounds: Methanol, concentrated HCl (32%), Folin Ciocalteu reagent, sodium carbonate, HPLC grade acetic acid and acetonitrile (gradient grade for liquid chromatography) were purchased from Merck. (+) Catechin, rutin, hesperidin, naringin, fisetin, quercetin, naringenin and hesperitin were purchased from Sigma-Aldrich.

Decortication of maize and sorghum grains to produce bran: The maize and sorghum grains were decorticated using a Tangential Abrasive Dehulling Device (TADD) fitted with a R284 Norton type metalite sandpaper disc (Norton Abrasives, Worcester, USA), grit size 50. A 100 g sample of each of the maize and sorghum grains was cleaned by removing visible dirt and damaged kernels by hand and by sifting, and placed into the cups of the TADD. The maize samples were decorticated for 3.5 min and the sorghum samples for 1 min to remove an amount of not more than 10% of the kernels from each grain. The bran was then sifted through a 710 µm Madison test sieve to ensure uniform particle size. The TADD was cleaned thoroughly between the decortication of each grain variety. The bran samples of each grain were placed in zip-lock bags, sealed and stored in a dark room at -10° C until needed for analyses.

Preparation of acidified methanol extracts: Extracts from the maize and sorghum bran samples were prepared using acidified methanol (1% conc. HCl in methanol) according to the method described by

Awika *et al.* (2004) with modification. An amount of 10 g bran was weighed out in duplicate into 250 ml Erlenmeyer flasks and 150 ml solvent was added. The flasks were covered entirely with aluminium foil and shaken on a Grand-Bio shaker model Pos 300 (Grant Instruments, Cambridge, UK) at a low speed (200 rpm) overnight at room temperature to allow for maximum diffusion of phenolics from the cellular matrix. The samples were transferred into plastic centrifuge bottles and centrifuged in a Rotanta model 460 R centrifuge (Labotec, Alberton, South Africa) at 3150 x *g* for 10 min. The supernatants were decanted into glass screw-top bottles covered with aluminium foil and the residues were rinsed twice with 50 ml solvent each time, collected into the glass bottles and mixed. The extracts were stored at -20˚ C until needed for analysis.

Determination of Total phenolic content: The total phenolic content of the extracts from the maize and sorghum bran samples was determined using the Folin Ciocalteu method (Waterman & Mole, 1994b). The principle of the method involves the reaction of phenolic hydroxyl groups with the Folin Ciocalteu reagent under alkaline conditions forming chromogens that can be detected spectrophotometrically.

A 0.5 ml aliquot of each extract or (+) catechin standard was added to a 50 ml volumetric flask containing 10 ml distilled water. Then 2.5 ml Folin-Ciocalteu's phenol reagent was added and mixed with the sample. After 2 min, 7.5 ml of sodium carbonate (Na_2CO_3) solution (20 g/ 100 ml) was added. The contents of the volumetric flasks were mixed, made up to volume with deionised water, stoppered and mixed thoroughly. The flasks were allowed to stand for 2 h from the addition of sodium carbonate after which the absorbance was measured at 760 nm, using a UV/VIS Spectrophotometer Model T80$^+$ (PG Instruments, Leicestershire, UK). A standard catechin calibration curve was obtained by using concentrations of 0.2, 0.4, 0.6 and 0.8 mg/ml in acidified methanol. Results were expressed as g catechin equivalents per 100 g sample on a dry basis.

Identification and quantification of flavonoids in bran extracts by HPLC: Reverse phase HPLC (M.J. Kim, Hyun, Kim, Park, Kim, Kim, Lee, Chun & Chung, 2007) was used to characterize and quantify flavonoids in the bran extracts. The HPLC system consisted of a Waters 1525 binary HPLC pump and a Waters 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA). A Sunfire C18 column (150 mm x 4.6 mm i.d., 3.5 µm particle size) (Waters, Milford, MA, USA) was used for the separation process. The analysis was monitored with BreezeTM software (Waters, Milford, MA, USA).

Each bran extract was filtered through a 0.2 µm PTFE syringe filter and a 20 µL aliquot of each sample was then injected into the HPLC system. The analysis was carried out at a flow rate of 1.0 ml/min and monitored at 280 nm using a linear gradient of solvent A (0.1% acetic acid in water) and solvent B

(0.1% acetic acid in acetonitrile). The linear gradient used is shown in Table 4.1 below. The column temperature was maintained at 25˚C for the running time of 30 min.

Table 4.1. Linear gradient parameters used for HPLC

TIME (MIN)	% SOLVENT A	% SOLVENT B
0.00	92.0	8.0
1.00	90.0	10.0
11.00	80.0	20.0
20.00	10.0	90.0
21.00	0.0	100.0
25.00	0.0	100.0
26.00	92.0	8.0

For the HPLC standard calibration process, flavonoid standards (rutin, hesperidin, naringin, fisetin, quercetin, naringenin, hesperitin and catechin) were prepared in dimethyl sulphoxide at concentrations of 200, 150, 100, 50, 25 and 10 ppm. The standards were chromatographed singly and as mixtures. Standard curves of peak area (y-axis) against concentration (x-axis) were plotted for each standard. Regression equations were obtained from the standard curve of each flavonoid compound. Flavonoid compounds in the extracts were identified by comparing the retention time of the unknown with those of the standard flavonoid compound. The regression equations were used to quantify the flavonoid compounds and the concentrations were expressed as mg/100 g of bran on a dry basis.

4.2.3 Statistical Analysis

The analysis for total phenolic content was done in duplicate. HPLC analysis of the bran extract samples were done in triplicate. The results were subjected to one-way analysis of variance (ANOVA) and significant differences between the sample means were determined using Fisher's least significance difference (LSD) test at the 95% significance level (STATISTICA version 11 StatSoft, Tulsa, OK, USA).

4.2.4 Results

4.2.4.1 Total phenolic content

Table 4.2 below shows that the extracts from the bran of the red non-tannin sorghum varieties had higher levels of total phenolics (3.35 – 4.13 g CE/100 g) than that of the white maize (1.07-1.20 g CE/ 100 g) and white non-tannin sorghum varieties (0.99-1.15 g CE/ 100 g). There was no significant difference in total phenolic content of the extracts from the white maize and white non-tannin sorghum brans.

Table 4.2 Total phenolic content (g CE/100 g) of methanolic extracts from white maize, white and red non-tannin sorghum bran

Grain Type	Variety	Total phenolic content
White maize	PAN 6335	1.20^{ab}(0.05)
	PAN 6045	1.07^{a}(0.04)
White non-tannin sorghum	NK 8828	0.99^{a}(0.04)
	KAT 369	1.15^{a}(0.09)
Red non-tannin sorghum	MR BUSTER	4.13^{d}(0.47)
	TOWN	3.35^{c}(0.37)

abcd – Mean values with the same superscript letters do not differ significantly ($p < 0.05$)

Figures in parentheses are standard deviations

4.2.4.2 Identification and quantification of flavonoids in bran extracts

No hesperitin was detected in any of the samples (Figure 4.2 and Table 4.3). Hesperidin and naringenin were present in all the extracts (Figure 4.2 and Table 4.3). No catechin, fisetin and quercetin were present in the extracts from the white maize varieties (Figure 4.2 and Table 4.3). The level of total flavonoids was higher in extracts from red sorghum varieties (166.8-269.8 mg/100 g) than in extracts from white maize (18.7-24.8 mg/ 100 g) and white non-tannin sorghum (64.9-69.9 mg/100 g) (Table 4.3). This trend is in agreement with the trends in total phenolic content of the bran extracts (Table 4.3).

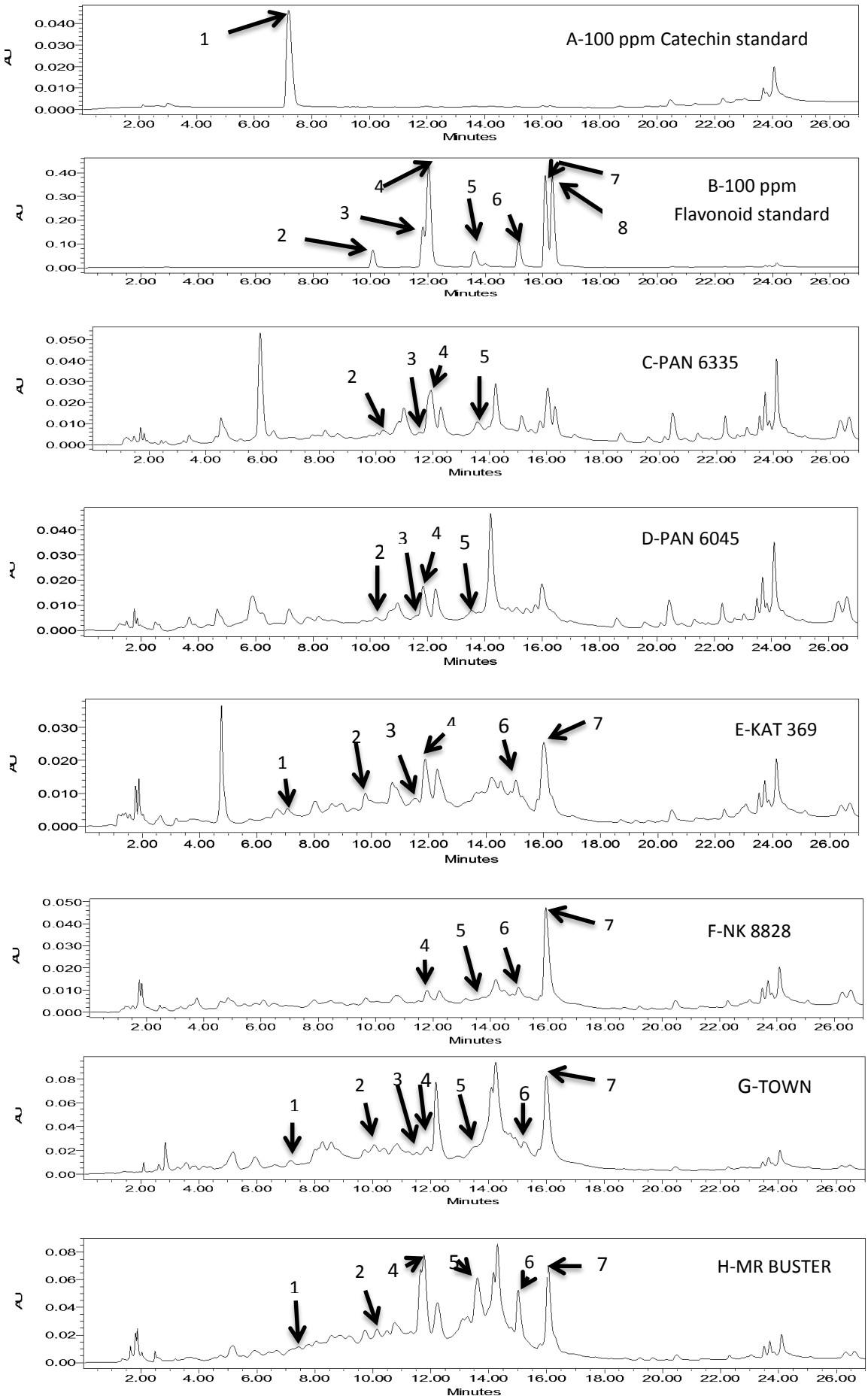


Figure 4.2. HPLC chromatograms of methanolic extracts from bran of white maize and white and red non-tannin sorghum showing flavonoid peaks:

1-catechin
2-rutin
3-naringin
4-hesperidin
5-fisetin
6-quercetin
7-naringenin
8-hesperitin
7-naringenin
8-hesperitin

Table 4.3 Flavonoid content (mg/100 g) of methanolic extracts from bran of white maize and red and white non-tannin sorghum

	White Maize		White non-tannin sorghum		Red non-tannin sorghum	
Flavonoid	**PAN 6335**	**PAN 6045**	**KAT 369**	**NK 8828**	**MR BUSTER**	**TOWN**
Catechin	ND	ND	6.78^{b} (0.71)	ND	4.58^{a} (0.56)	21.53^{c} (1.23)
Rutin	1.75^{a} (0.04)	3.55^{a} (0.21)	16.93^{b} (0.83)	ND	21.74^{c} (2.60)	29.71^{d} (2.77)
Naringin	0.67^{ab} (0.07)	1.30^{bc} (0.33)	2.37^{d} (0.10)	ND	ND	1.74^{cd} (0.83)
Hesperidin	6.66^{d} (2.30)	10.15^{b} (0.10)	10.92^{bc} (0.83)	2.21^{a} (0.23)	13.79^{c} (2.02)	2.20^{a} (0.20)
Fisetin	ND	ND	ND	0.21^{a} (0.03)	115.84^{c} (11.21)	17.38^{b} (0.16)
Quercetin	ND	ND	9.57^{a} (0.56)	10.50^{a} (0.16)	64.90^{c} (2.69)	15.14^{b} (0.89)
Naringenin	9.66^{a} (1.68)	9.77^{a} (0.01)	18.33^{c} (0.35)	56.39^{b} (5.44)	48.99^{b} (5.66)	79.14^{d} (0.20)
Hesperitin	ND	ND	ND	ND	ND	ND
TOTAL	18.74	24.77	64.90	69.85	269.84	166.84

abcd – Mean values with the same superscript letter in the same row do not differ significantly ($p < 0.05$); ND – Not Detected
Values in parentheses are standard deviations

4.2.5 Discussion

The lower total phenolic content of the maize bran extracts compared to that of the red non-tannin sorghum in this study is in agreement with the findings of a study by Chiremba *et al.* (2012) who reported lower total phenolic content of bran of maize varieties than bran of sorghum varieties. Dykes, Rooney, Waniska and Rooney (2005) also indicated that lower levels of total phenolics were detected in white sorghum than in red sorghum varieties.

According to Taylor (2005), red non-tannin sorghum contains higher amounts of pigments than most other cereals in the form of anthocyanins and anthocyanidins, mostly concentrated in the bran. This explains the observation in this study that higher levels of flavonoids were detected in the extracts from bran of red non-tannin sorghum than in that of the white maize and white non-tannin sorghum. Dykes *et al.* (2005) found higher levels of various flavonoids in the bran of red sorghum genotypes than in white sorghum grains, and the red sorghums also contained various anthocyanidins absent in the white sorghum. This contributes to higher flavonoid levels in red sorghum than in white sorghum grains. Awika *et al.* (2004) indicated that sorghum contains overall, higher levels of flavonoids than any other cereals or even some fruit and vegetables. Awika *et al.* (2005) found no detectable 3-deoxyanthocyanins in the bran of white sorghum.

As shown in Table 4.3, the presence of flavonoids such as catechin and naringenin has been reported in sorghum by Gujer, Magnolato and Self (1986). However there does not seem to be any reports in the literature concerning the presence of other flavonoids reported in Table 4.3 (rutin, naringin, hesperidin, fisetin and quercetin) in sorghum or maize. The flavonol quercetin is a well-known component of legumes such as cowpeas (Nderitu, Dykes, Awika, Minnaar & Duodu, 2013) but not necessarily of cereals. Although it must be mentioned that a study by Larson (1971) indicated the presence of quercetin in maize kernels. According to Dykes and Rooney (2007), it is not uncommon to find flavonoids normally present in fruits (e.g. berries and citrus fruit) and vegetables (e.g. parsley and celery), also present in cereals such as sorghum. It must also be borne in mind that identification of flavonoids in this study was done by comparing retention times in chromatograms of the samples to those of standards run under the same conditions. A limitation of this method is that in the absence of the required standards, it is not possible to distinguish between glycoside and aglycone forms of flavonoids (e.g. catechin and its glucosides) or flavonoids that are structurally closely related which may possibly co-elute. A technique such as liquid chromatography coupled with mass spectrometry (LC-MS) which allows for identification of compounds based on their masses and fragmentation patterns will be expected to be more diagnostic in identifying the flavonoids in the bran extracts.

4.2.6 Conclusion

The bran extracts of red non-tannin sorghum contain significantly higher levels of total phenolics compounds and flavonoids than that of white maize and white non-tannin sorghum. While all the sorghum and maize bran extracts appear to contain hesperidin and naringenin, none of the extracts appears to contain any hesperitin. While only rutin, naringin, hesperidin and naringenin are present in the white maize bran extracts, a wider variety of flavonoids namely, catechin, rutin, hesperidin, fisetin, quercetin and naringenin are present in the red non–tannin sorghum bran extracts. The presence of a larger variety of flavonoids and total flavonoids in extracts from red non-tannin sorghum bran compared to extracts from white maize and white non-tannin sorghum bran suggests that the red non-tannin sorghum bran extracts may have a superior ability to inhibit starch hydrolysing enzymes such as α-amylase. Therefore, inclusion of red sorghum bran in the diet may have the potential to control postprandial blood glucose levels in people suffering from diabetes.

4.2.7 References

AWIKA, J. M. 2011. Sorghum flavonoids: Unusual compounds with promising implications for health. *In:* AWIKA, J. M., PIIRONEN, V. & SCOTT, B. (eds.) *Advances in Cereal Science: Implications to Food Processing and Health Promotion* 1st ed. USA: American Chemical Society, Pages 107-200.

AWIKA, J. M., MCDONOUGH, C. M. & ROONEY, L. W. 2005. Decorticating sorghum to concentrate healthy phytochemicals. *Journal of Agricultural and Food Chemistry,* 53**,** 6230-6234.

AWIKA, J. M., ROONEY, L. W. & WANISKA, R. D. 2004. Properties of 3-deoxyanthocyanins from sorghum. *Journal of Agricultural and Food Chemistry,* 52**,** 4388-4394.

BANNON, M. 2011. Challenges for diabetic care: rural Africa & type 1 brittle diabetes. *QJM,* 104**,** 559-560.

BRODY, T. 1999. Digestion and absorption. *Nutritional Biochemistry*. 2nd Edition. Academic Press. London, Pages 103-180.

CHIREMBA, C., TAYLOR, J. R. N., ROONEY, L. W. & BETA, T. 2012. Phenolic acid content of sorghum and maize cultivars varying in hardness. *Food Chemistry,* 134**,** 81-88.

DYKES, L. & ROONEY, L. W. 2006. Sorghum and millet phenols and antioxidants. *Journal of Cereal Science,* 44**,** 236-251.

DYKES, L. & ROONEY, L. W. 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World,* 52**,** 105-111.

DYKES, L., ROONEY, L. W., WANISKA, R. D. & ROONEY, W. L. 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. *Journal of Agricultural and Food Chemistry,* 53**,** 6813-6818.

FAO. 1997. White maize: A traditional food grain in developing countries [Online]. Available: http://www.fao.org/docrep/w2698e/w2698e00.htm#Contents [Accessed 18 February 2014].

FAOSTAT. 2012. Available: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567 [Accessed 17 April 2012].

FAHRNHAM, D. E., BENSON, G. O. & PEARCE, R. B. 2003. Corn perspective and culture. In: WHITE, P. J. & JOHNSON, L. A. (eds.) Corn Chemistry and Technology. Minnesota, USA: American Association of Cereal Chemists, Inc., Pages 1 - 33.

GUJER, R., MAGNOLATO, D. & SELF, R. 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry,* 25**,** 1431-1436.

HARGROVE, J. L., GREENSPAN, P., HARTLE, D. K. & DOWD, C. 2011. Inhibition of aromatase and α-amylase by flavonoids and proanthocyanidins from Sorghum bicolor bran extracts. *Journal of Medicinal Food,* 14**,** 799-807.

KIM, M. J., HYUN, J. N., KIM, J. A., PARK, J. C., KIM, M. Y., KIM, T. G., LEE, S. J., CHUN, S. C. & CHUNG, I. M. 2007. Relationship between phenolic compounds, anthocyanins content antioxidant activity in colored barley germplasm. *Journal of Agricultural and Food Chemistry,* 55**,** 4802-4809.

LARSON, R. L. 1971. Glucosylation of quercetin by a maize pollen enzyme. *Phytochemistry,* 10**,** 3073-3076.

LEDER, I. 2004. *Sorghum and millets. Cultivated plants, primarily as food sources.* [Online]. Available:http://www.eolss.net/ebooks/Sample%20Chapters/C10/E5-02-01-04.pdf [Accessed 1 March 2012].

NDERITU, A. M., DYKES, L., AWIKA, J. M., MINNAAR, A. & DUODU, K. G. 2013. Phenolic composition and inhibitory effect against oxidative DNA damage of cooked cowpeas as affected by simulated in vitro gastrointestinal digestion. *Food Chemistry*, 141, 1763-1771.

STYLES, E. D. & CESKA, O. 1977. ABSTRACT. The genetic control of flavonoid synthesis in maize. *Canadian Journal of Genetics and Cytology*, 19, 289-302.

TADERA, K., MINAMI, Y., TAKAMATSI, K. & MATSUOKA, T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology*, 52**,** 149-153.

TAYLOR, J. R. N. & DEWAR, J. 2001. Developments in sorghum food technologies. *Advances in Food and Nutrition Research*, 43, 217-264.

TAYLOR, J. R. N. 2005. Non-starch polysaccharides, protein and starch: Form function and feed - Highlight on sorghum. *Australian Poultry Science Symposium,* 17**,** 9-16.

WAGMAN, A. S. & NUSS, J. M. 2001. Current therapies and emerging targets for the treatment of diabetes. *Current Pharmaceutical Design*, 7, 417-450

WATERMAN, P. G. & MOLE, S. 1994. *Analysis of Phenolic Plant Metabolites.* Blackwell Scientific Publications. London. Pages 66-103.

ŽILIĆ, S., SERPEN, A., AKıLLıOĞLU, G., GÖKMEN, V. & VANČETOVIĆ, J. 2012. Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. *Journal of Agricultural and Food Chemistry*, 60, 1224-1231.

4.3 Characterization and quantification of flavonoids in bran of white maize and white and red non-tannin sorghum and their inhibitory effects against porcine pancreatic α-amylase activity

Abstract

Foods with high starch digestibility are not suitable for diabetic patients due to their inability to metabolise glucose properly. Therefore, a potential way of preventing diabetes would be to limit starch digestibility of food through inhibition of starch-hydrolysing enzymes in order to control blood glucose levels. Flavonoids, which are also found in cereals such as sorghum and maize, are known to exert inhibitory effects against starch-hydrolysing enzymes such as α-amylase. In this study, the bran of white maize and white and red non-tannin sorghum were characterized for their flavonoid contents and the effect of the bran and extracts from the bran of these grains on the activity of porcine pancreatic α-amylase was determined. Total phenolic content was determined using the Folin-Ciocalteu assay, α-amylase inhibitory activity was determined using the Megazyme Ceralpha α-amylase assay kit and flavonoid content was determined using liquid chromatography mass spectrometry (LC-MS). The bran and bran extracts of red non-tannin sorghum had the highest amount of total phenolics, total flavonoids (flavones and flavanones) and inhibitory activity on porcine pancreatic α-amylase. Acidified organic bran extracts had higher total phenolic and flavonoid contents than non-acidified organic and water extracts. Unextracted bran samples of the grains showed high inhibition of α-amylase. Nutraceutical-type preparations from red non-tannin sorghum bran in particular could have anti-diabetic properties by inhibiting α-amylase activity and thus control postprandial glucose levels in people suffering from diabetes.

4.3.1 Introduction

Diabetes mellitus is a chronic metabolic disease caused by insufficient insulin production by the pancreas and/or when the body loses its ability to utilise insulin effectively (Brody, 1999). After consumption of food with a high starch digestibility, the level of blood plasma glucose rise rapidly in people with diabetes and remains above normal levels due the body's inability to metabolise glucose effectively (Whitney & Rolfes, 2011). A potential way of preventing diabetes is to limit starch digestibility of food through inhibition of starch hydrolysing enzymes (Wagman & Nuss, 2001) in order to control blood glucose levels. According to Uchida, Nasu, Tokutake, Kasai, Tobe and Yamaji (1999), inhibitors of α-amylase are effective suppressors of post prandial glucose levels in diabetes patients. The astronomic cost of diabetic health care world-wide (IDF, 2011), as well as inadequate health care in remote and rural areas (Bannon, 2011), creates a need for research in finding more cost-effective ways e.g. a food source, to control the escalating incidence of diabetes mellitus.

Lo Piparo *et al.* (2008) specified that flavones and flavonols are the two groups exhibiting the most powerful inhibitory activity on human salivary α-amylase amongst the flavonoids. Sorghum contains overall, higher levels of flavonoids than other cereals (Awika *et al.*, 2004) and these flavonoids are mostly concentrated in the bran (Chiremba *et al.*, 2012). Flavonoids such as quercetin and luteolin in sorghum extracts (Awika & Rooney, 2004), are powerful inhibitors of porcine-α-amylase (Tadera *et al.*, 2006). Hargrove *et al.* (2011) also found that α-amylase activity was inhibited by simple flavonoids in sorghum. This inhibitory effect is related to the chemical structure of the flavonoids (Tadera *et al.*, 2006).

The inhibition of digestive enzymes such as α-amylase by flavonoids (Tadera *et al.*, 2006) in cereals such as sorghum may suggest that the inclusion of non-tannin sorghum varieties in the diet could contribute to controlling blood glucose levels and thereby possibly prevent the development of Type 2 diabetes without using expensive drugs. Flavonoid-rich extracts from cereals such as sorghum and maize may also have potential uses as nutraceuticals in anti-diabetic applications.

The objective of this study was to characterize the bran of white maize and white and red non-tannin sorghum in terms of their flavonoid contents and to determine the effect of bran and bran extracts of white maize and white and red non-tannin sorghum on the activity of porcine pancreatic α-amylase.

4.3.2 Materials and Methods

Samples: Two white maize varieties (PAN 6045 and PAN 6335) of similar hardness, two white non-tannin sorghums (KAT 369 and NK8820) and two red non-tannin sorghums (Town and MR Buster)

of different hardness were used in this research. The white maize varieties were used as a control. The origins of the samples are described in Chapter 4.2.

Chemicals and standard phenolic compounds: Methanol, acetone, concentrated HCl (32%), acetic acid, Folin Ciocalteu Reagent, sodium carbonate and tri-sodium phosphate were purchased from Merck. Kaempferol 3-β-D-glucopyranose, kaempferol, eriodictyol, eriodictyol-7-O-β-D-glucopyranoside, luteolin, luteolin-7-glucoside were purchased from Sigma-Aldrich as well as the HEPES-buffer (1 M), p-nitrophenol and porcine pancreatic α-amylase Type I-A, saline suspension (±1000 units/mg protein). Amylase HR Assay reagent was purchased from Megazyme, Ireland.

Decortication of maize and sorghum grains to produce bran: The bran samples from the sorghum and maize grains were prepared as described in Chapter 4.2.

Preparation of acidified (1% HCl and 1% acetic acid) methanol extracts: Bran (10 g) was extracted with 150 ml solvent by shaking on a Grand-Bio shaker model Pos 300 (Grant Instruments, Cambridge, UK) at a speed of 200 rpm overnight at room temperature to allow for maximum diffusion of phenolics from the cellular matrix. The samples were centrifuged in a Rotanta model 460 R centrifuge (Labotec, Alberton, South Africa) at 3150 x *g* for 10 min. The supernatants were decanted into glass screw-top bottles covered with aluminium foil and the residues were rinsed twice with 50 ml solvent each time, collected into the glass bottles and pooled. The extracts were stored at -20˚ C until needed for analysis.

Preparation of 70% aqueous acetone, 100% acetone, 70% aqueous methanol and 100% methanol extracts: Bran (3 g) was extracted with 30 ml solvent by shaking overnight at 200 rpm on a Grand-Bio shaker model Pos 300 (Grant Instruments, Cambridge, UK). The samples were centrifuged at 3150 x *g* for 10 min in a Rotanta model 460 R centrifuge (Labotec, Alberton, South Africa) and supernatants were collected in 50 ml glass beakers. The 70% aqueous acetone and 70% aqueous methanol extracts were evaporated off to a volume of 3 ml and the 100% acetone and 100% methanol extracts were taken to dryness at room temperature in a fume cupboard. After evaporation, 27 ml of distilled water was added to the 70% aqueous acetone and 70% aqueous methanol residues and 30 ml of sterilized, distilled water was added to the residues of the 100% acetone and 100% methanol extracts. These were mixed well by stirring with a glass rod and transferred into screw-top glass bottles and stored at -20˚ C until analysed.

Preparation of water extracts: Bran (3 g) was extracted with 30 ml distilled water by magnetic stirring at room temperature for 30 min. This was followed by further extraction for 2 h in a Grant shaking water bath, model OLS 200 (Grant Instruments, Cambridgeshire, UK), at 37˚ C at a shaker

speed of 80 shakes per minute. The samples were then transferred into plastic centrifuge tubes and centrifuged at 3150 x *g* for 15 min in a Rotanta model 460 R centrifuge (Labotec, Alberton, South Africa) at ambient temperature. The supernatants were decanted into glass screw-top bottles and stored at -20˚ C until analysed.

Preparation of bran extracts for LC-MS: Acidified (1% HCl) methanol, 70% aqueous methanol, 70% aqueous acetone and water extracts from white maize (PAN 6334) and white (KAT 369) and red (MR BUSTER) non-tannin sorghum bran were selected for LC-MS analysis. Extracts were prepared as described above and filtered through Whatman No 4 filter paper in glass funnels into 50 ml glass beakers. This was followed by filtration through 0.45 µm GHP membranes (PAL) into 1.5 ml amber glass vials in preparation for LC-MS analysis.

Determination of Total phenolic content: The total phenolic content of the extracts was determined using the Folin-Ciocalteu assay as described in Chapter 4.2.

Determination of the effect of maize and sorghum flavonoids on the activity of porcine α-amylase: The determination of the effect of the bran from sorghum and maize and their extracts on the activity of α-amylase was conducted using the Megazyme Ceralpha α-amylase assay kit (Megazyme International, Ireland) with some modification. The procedure involves the use of the oligosaccharide, non-reducing end-blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) as substrate, in the presence of α-glucosidase. The α-glucosidase does not act on the native substrate due to the presence of the blocking-group. The BPNPG7 is hydrolysed by the endo-acting α-amylase into a blocked maltosaccharide and *p*-nitrophenyl maltosaccharide, which is then further hydrolysed to glucose and free *p*-nitrophenol by excess quantities of α-glucosidase (Figure 4.3). The amount of released *p*-nitrophenol is measured at an absorbance of 400 nm and is in direct relation to the activity of the α-amylase enzyme.

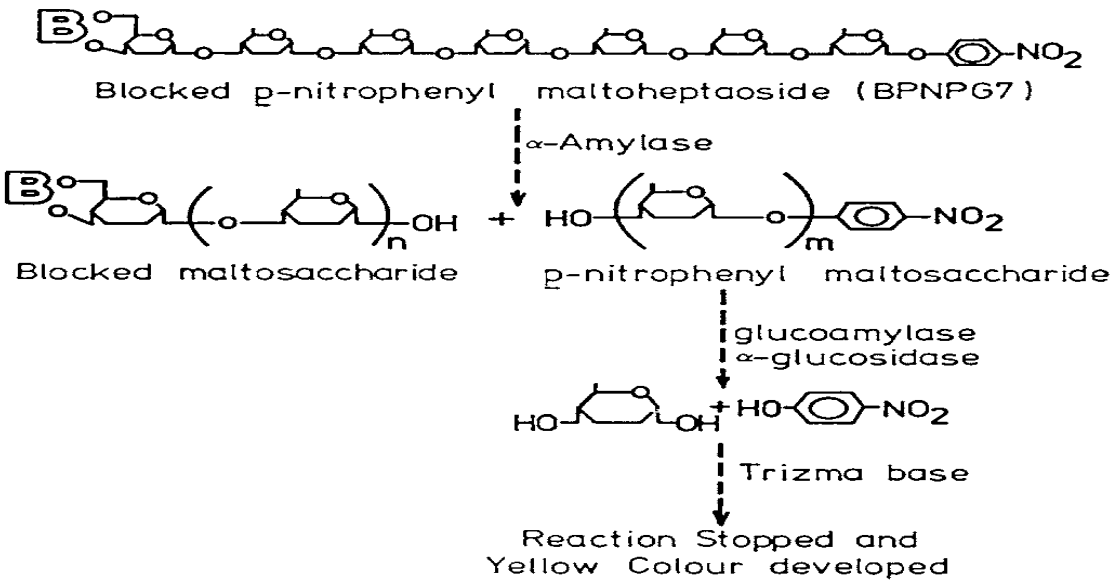


Figure 4.2 Schematic illustration of the hydrolysis of the BPNPG7 to glucose and free p-nitrophenol (Megazyme, 2012)

For the acidified extracts an aliquot of 1 ml extract was diluted in 10 ml HEPES buffer (pH 6.9) to obtain a suitable pH of 5.9-6.0 for optimum enzyme activity. An aliquot of 200 µl of Amylase HR Reagent (substrate) was transferred into clean test tubes in duplicate. Then a 100 µl (equivalent to 10 mg bran) aliquot of each of the different extracts was added. Due to the extra dilution to adjust the pH, a 1000 µl (equivalent to 10 mg bran) aliquot was added for the acidified extracts. For the un-extracted bran, 10 mg of bran was added. These served as reaction mixtures. A reaction blank was included in duplicate, containing 200 µl of substrate, but no extract or bran. A single reagent blank (reaction stopped in advance) for each sample, containing extracts, stopping reagent and enzyme was also included. The tubes with contents were pre-incubated at 37 °C for 5 min before addition of the enzyme. Then 200 µl of porcine pancreatic α-amylase (10 µl of enzyme into 500 ml 0.1 M HEPES buffer with pH 6.9 to give 5 units) was added to the reaction mixture in the test tube, directly to the bottom of the tubes and vortexed to ensure that the substrate and enzyme were in proper contact with each other. This was done at exact time intervals (10 s) to ensure that each sample was incubated for exactly 20 min at 37° C. At the end of the 20 min incubation period, exactly 3 ml of 1% tri-sodium phosphate (pH 11.0) was added to stop the reaction and the tubes were vortexed again. The samples were then transferred into Eppendorf tubes and centrifuged at 1100 g for 5 min. The absorbance of the solutions and the blanks were read at 400 nm against distilled water on a spectrophotometer. A p-nitrophenol standard in 1% tri-sodium phosphate was prepared by diluting 1 ml of 10 mM p-nitrophenol solution to 200 ml with 1% tri-sodium phosphate and used to standardise the spectrophotometer. This gave an absorbance of approximately 0.905 at 400 nm. If the absorbance value for a specific sample was more than 1.20, the extract was diluted and re-assayed.

The inhibitory effect of the extracts on α-amylase activity was then calculated as follows:

$A - (B - C)/A \times 100$, where A is the absorbance of p-nitrophenol in the absence of the extract, B is the absorbance of p-nitrophenol in the presence of the extract and C is the absorbance of the reagent blank.

Liquid chromatography mass spectrometry (LC-MS): LC-MS analysis was done using a Waters UPLC model Synapt G2 equipped with a Waters BEH C18 column (2.1 x 100 mm) (Waters, Milford, MA, USA). A solvent gradient (Table 4.4) using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 0.30 ml/min was employed. The data was obtained in the negative mode $(M-H)^-$. An electrospray positive source was used with capillary voltage of 3 kV and cone voltage of 15 V. Leucine enkaphelin was used as lock mass compound and the analysis was monitored using ACQUITY Binary Solvent Manager Software.

Table 4.4 Linear gradient parameters used for LC-MS analysis

TIME (MIN)	FLOW (ml/min)	% SOLVENT A	% SOLVENT B
0.00	0.3	100.0	0.0
1.00	0.3	100.0	0.0
22.00	0.3	72.0	28.0
22.50	0.3	60.0	40.0
23.00	0.3	0.0	100.0
24.00	0.3	0.0	100.0

For the LC-MS standard calibration process, flavonoid standard mixtures (rutin, hesperidin, naringin, fisetin, quercetin, naringenin, catechin, eriodictyol and luteolin) were prepared in dimethyl sulphoxide (DMSO) at concentrations of 50, 25, 10 and 5 ppm. Standard curves of peak area (y-axis) against concentration (x-axis) were plotted for each standard. Regression equations were obtained from the standard curve of each flavonoid compound. Flavonoid compounds in the extracts were identified by comparing the retention time and molecular weights of the unknown with those of the standard flavonoid compound. Possible identification of compounds not included in the standard mixture was done by comparing their molecular weights with phenolic compounds listed in the polyphenol data base Phenol Explorer (INRA, 2013) and comparing fragment patterns with that described in literature (Cuyckens & Claeys, 2004; Gujer *et al.*, 1986). The regression equations were used to quantify the flavonoid compounds and the concentrations were expressed as g catechin/eriodictyol/luteolin/naringenin equivalents/100 g of bran on a dry basis.

4.3.3 Statistical Analysis

Statistical analysis for the enzyme inhibition analysis and total phenolic content was done using one-way analysis of variance (ANOVA) and significant differences between the sample means were determined using Fisher's least significance difference (LSD) test at the 95% significance level. Regression analysis was conducted using Statistica version 11 (StatSoft Inc., Tulsa, OK, USA).

4.3.4 Results and Discussion

4.3.4.1 Total phenolic content of bran extracts of white maize and white and red non-tannin sorghum

The total phenolic content (Table 4.5) of all the bran extracts of red non-tannin sorghum (0.19 – 4.13 g CE/100 g bran) was significantly higher than that of white maize (0.03-1.20 g CE/100 g bran) and white non-tannin sorghum (0.03 – 1.15 g CE/ g bran). These findings are in agreement with Awika *et al.* (2005) and Taylor (2005) who stated that pigmented grains e.g. red non-tannin sorghum contain higher amounts of phenolic compounds than non-pigmented grains e.g. white maize and white non-tannin sorghum. Chiremba *et al.* (2012), also reported higher amounts of total phenolics in the bran of various red non-tannin sorghum varieties than in that of white maize.

The 70% aqueous organic solvent extracts had higher total phenolic contents (0.14 – 1.28 g CE/100 g bran) than the 100% organic solvent extracts (0.11 – 0.86 g CE/100 g bran). This may be attributed to the differing polarities of the two solvent systems (aqueous organic vs. 100% organic solvents). Garcia-Salas, Morales-Soto, Segura-Carretero and Fernández-Gutiérrez (2010), Xu and Chang (2007) and Naczk and Shahidi (2006) reported that solvents with different polarity may yield different phenolic content, because the solubility of phenolic compounds depends on their chemical nature which in turn determines their polarity.

An appreciable amount of total phenolics was extractable in water (0.29 – 0.78 g CE/100 g bran), and this was higher than total phenolics from organic solvent extracts in some instances. Specifically, water extracts from white non-tannin sorghum and white maize had higher total phenolics than corresponding 70% and 100% methanol or acetone extracts. This observation may be explained by the fact that the Folin-Ciocalteu method used for this analysis is not specific for phenolic compounds. It is therefore possible that other components extractable in water e.g. reducing sugars may be a source of interference and lead to an overestimation of the total phenolic content of the water extracts (Everette, Bryant, Green, Abbey, Wangila & Walker, 2010).

For all the sorghum and maize bran samples, acidified (1% HCl) methanol extracts had the highest total phenolic content. The acidified organic extracts of the brans had higher total phenolic contents

than non-acidified organic extracts. Xu and Chang (2007) reported that acidic conditions in extracts can release bound phenolic compounds and make them more extractable.

Table 4.5 Total phenolic content (g Catechin Equivalents/100 g bran) of extracts from the bran of white maize and white and red non-tannin sorghum prepared with different solvents

Grain Type	Variety	Acidified (1% HCL) methanol	Acidified (1% acetic acid) methanol	70% aqueous methanol	70% aqueous acetone	100% methanol	100% acetone	Water
White maize	PAN 6335	1.20^{ab}(0.05)	0.43^{a}(0.01)	0.18^{b}(0.00)	0.18^{b}(0.00)	0.11^{a}(0.00)	0.03^{a}(0.00)	0.31^{a}(0.02)
	PAN 6045	1.07^{a}(0.04)	0.37^{a}(0.01)	0.14^{a}(0.00)	0.16^{a}(0.02)	0.12^{ab}(0.00)	0.05^{b}(0.01)	0.29^{a}(0.01)
White non-tannin sorghum	KAT 369	1.15^{a}(0.09)	0.37^{a}(0.01)	0.23^{d}(0.01)	0.26^{c}(0.00)	0.16^{c}(0.00)	0.03^{a}(0.00)	0.34^{ab}(0.00)
	NK 8828	0.99^{a}(0.04)	0.38^{a}(0.02)	0.20^{c}(0.00)	0.24^{c}(0.01)	0.13^{b}(0.00)	0.04^{ab}(0.00)	0.38^{b}(0.00)
Red non-tannin sorghum	TOWN	3.35^{c}(0.47)	1.88^{b}(0.05)	0.93^{e}(0.00)	0.94^{d}(0.00)	0.76^{d}(0.01)	0.19^{c}(0.00)	0.78^{d}(0.01)
	MR BUSTER	4.13^{d}(0.37)	2.50^{c}(0.07)	1.00^{f}(0.01)	1.28^{e}(0.01)	0.86^{e}(0.00)	0.32^{d}(0.00)	0.71^{c}(0.03)

Total phenolic content with different superscripts in the same column differ significantly
Values in parentheses are standard deviations from the means

4.3.4.2 The effect of maize and sorghum bran and bran extracts on the activity of porcine pancreatic α-amylase

For the unextracted bran samples, the two red non-tannin sorghum brans had higher inhibition of porcine α-amylase (59.4 – 71.4%) than the white maize and white non-tannin sorghum bran samples (16.2 – 24.6%) (Table 4.6). All organic solvent extracts from red non-tannin sorghum bran had higher inhibition of porcine α-amylase than corresponding organic solvent extracts from white maize and white non-tannin sorghum bran. These trends in α-amylase inhibitory capacity of the extracts are similar to the trends in total phenolic content (Table 4.5) which showed that organic solvent extracts from the red non-tannin sorghum bran had higher total phenolic content than extracts from white maize and white non-tannin sorghum bran.

Table 4.6 Inhibitory capacity (%) against porcine pancreatic α-amylase of bran and bran extracts of white maize and red and white and red non-tannin sorghum

Grain Type	Variety	Bran	Extracts					
			Acidified (1% acetic acid) methanol	70% methanol	70% acetone	100% methanol	100% acetone	Water
White maize	PAN 6335	21.5^{b}(0.1)	22.8^{a}(2.4)	NI	NI	NI	12.1^{a}(0.0)	NI
	PAN 6045	24.6^{c}(0.2)	21.1^{a}(2.5)	NI	NI	NI	17.7^{ab}(3.0)	NI
White non-tannin sorghum	KAT 369	16.2^{a}(0.8)	12.1^{a}(12.1)	19.7^{b}(0.2)	19.7^{a}(1.2)	3.2^{a}(3.1)	19.8^{b}(2.4)	NI
	NK 8828	21.0^{b}(0.7)	27.3^{a}(3.1)	0.2^{a}(0.2)	26.1^{b}(2.2)	15.3^{b}(0.2)	11.6^{a}(0.8)	NI
Red non-tannin sorghum	TOWN	59.4^{d}(0.4)	54.6^{b}(3.2)	34.6^{c}(1.5)	62.7^{d}(2.0)	41.0^{c}(1.4)	21.7^{b}(2.4)	NI
	MR BUSTER	71.4^{e}(0.6)	52.9^{b}(0.3)	41.2^{d}(1.3)	53.8^{c}(0.6)	40.2^{c}(0.2)	22.8^{b}(0.2)	5.1^{a}(0.3)

NI-No Inhibition
Percentage inhibition with different superscripts in the same column differ significantly ($p<0.05$)
Values in parentheses indicate standard deviation from means

These findings show that in general, extracts with high total phenolic contents had high α-amylase inhibitory capacity. Shobana *et al.* (2009) also reported that millet seed coat extracts with high polyphenol concentrations had high inhibitory activity against pancreatic α-amylase. This is further demonstrated by the observation that a positive correlation was obtained between total phenolic content of the bran extracts of white maize and white and red non-tannin sorghum and their inhibitory capacity against porcine pancreatic α-amylase (Figure 4.4).

However, the linear correlation coefficient (r) was different for each of the grain types. The correlation between total phenolic content and alpha-amylase inhibitory activity for red non-tannin sorghum extracts was stronger than for white non-tannin sorghum extracts. This may be related to the relatively higher total phenolic content of the red non-tannin sorghum bran extracts compared to the extracts from white non-tannin sorghum bran. Generally, pigmented non-tannin sorghums have higher phenolic content than white non-tannin sorghums. This has been reported by other workers such as Awika *et al.* (2005) who found that red sorghum varieties contain higher amounts of phenolic compounds than white sorghum varieties, and Chiremba *et al*. (2012), who also reported higher amounts of total phenolics in the bran of various red non-tannin sorghum varieties than in that of white maize. The white maize bran extracts showed an unexpected high value for r, which may be attributed to the fact that fewer values were used to determine the r-value, as some of the white maize bran extracts did not show any inhibition against the enzyme activity.

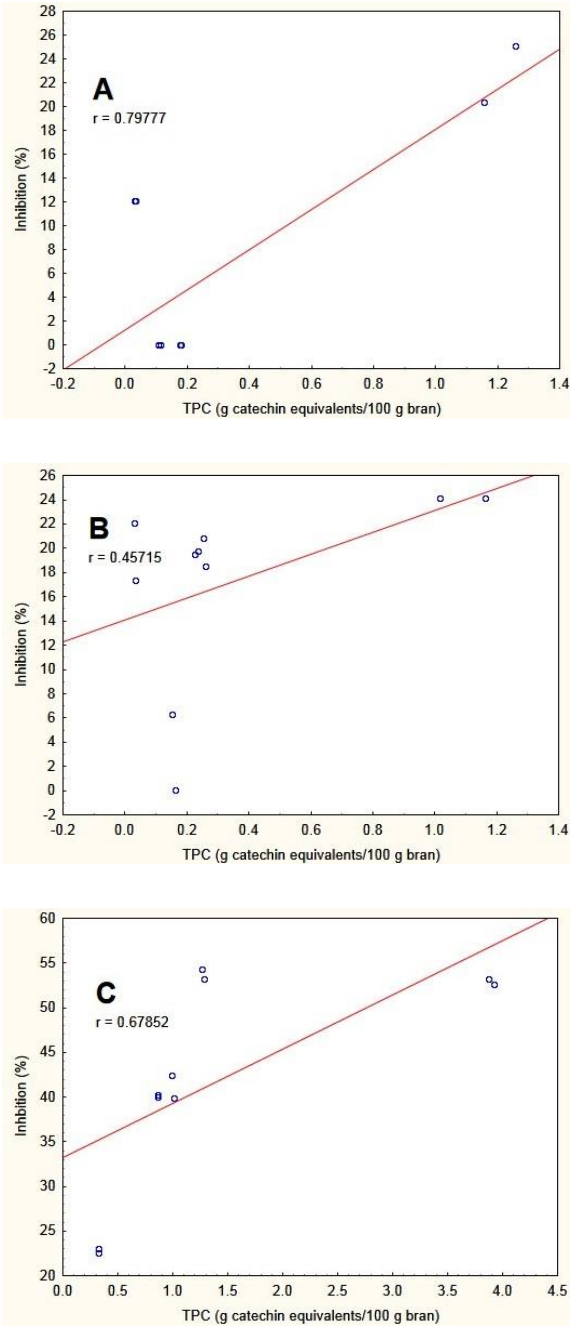


Figure 4.3 Correlation between total phenolic content (TPC) and inhibition of porcine pancreatic α-amylase activity by organic extracts of the bran of white maize (A) at p<0.01, white non-tannin sorghum (B) at p<0.5 and red non-tannin sorghum (C) at p<0.05

With the exception of the extract from MR Buster red non-tannin sorghum, all the water extracts did not show porcine α-amylase inhibition, although they contained total phenolics. This suggests that the inhibitory activity of extracts depends on the type of phenolic compound present. This, as well as the presence of very low concentrations of phenolic compounds, might also be the reason why no inhibition was exhibited by the organic and aqueous organic extracts from the white maize. Some studies have shown that certain flavonoids such as quercetin and luteolin are powerful inhibitors of

porcine-α-amylase compared to catechin and kaempferol which are less powerful. This inhibitory potential is related to the chemical structure of these flavonoids (Tadera *et al*., 2006; Hargrove *et al*., 2011).

The unextracted bran of white maize and white and red non-tannin sorghum exhibited much higher inhibition compared to corresponding extracts. The highest concentration of phenolics can be found in the bran of these cereals (Taylor, 2005). The presence of other components like phytates and fibre present in the bran may also contribute to the enzyme inhibition shown by the bran (Yoon, Thompson & Jenkins, 1983).

4.3.4.3 Identification of phenolic compounds and quantification of flavonoids in bran extracts by LC-MS

Flavanones (eriodictyol and naringenin and their derivatives) and flavones (apigenin and luteolin and their derivatives) were the only two groups of flavonoids identified in the bran extracts (Table 4.7). The flavanones and derivatives were present only in extracts from red non-tannin sorghum bran while the flavones and derivatives were present in extracts from red non-tannin sorghum and white non-tannin sorghum bran. The monomeric flavanones naringenin and eriodictyol and their glycosides and the dimeric derivative of eriodictyol, 5,7,3,4,-tetrahydroxyflavan-5-0-β-glucosyl-4,8-eriodictyol and its glucoside forms have been reported in sorghum (Gujer et al. (1986). Dykes et al. (2009) also identified the flavones apigenin and luteolin in various sorghum grain varieties. Overall, Table 4.7 shows that flavonoids were predominantly detected in the extracts of the red non-tannin sorghum bran. No flavonoids were detected in any of the extracts from white maize bran.

Four phenolic acids were detected only in water extracts. Chiremba et al. (2012) detected ferulic acid, caffeic acid and p-coumaric acid in extracts from both maize and sorghum bran. In this study, these phenolic acids were detected in the water extracts of the white maize and white and red non-tannin sorghum, which is in agreement with the findings of Chiremba et al. (2012). Svensson, Sekwati-Monang, Lutz, Schieber and Gänzle (2010) identified p-hydroxybenzoic acid as one of the most abundant free phenolic acids in red sorghum.

Table 4.7 Phenolic compounds (flavonoids and phenolic acids) identified and flavonoid content in bran extracts of white maize and white and red non-tannin sorghum prepared with different solvents

Compound	Ret time t_R (min)	Parent ion $M\text{-}H^+$ (m/z)	MS-MS fragments (m/z)	Extract	Concentration
Flavonoids					
Flavanones and derivatives					
Eriodictyol galactoside	11.00	449.1	287	Red non-tannin sorghum; 70% methanol	1.9 (0.1)†
				Red non-tannin sorghum; 70% acetone	1.7 (0.1)†
Eriodictyol glucoside	11.56	449.1	287	Red non-tannin sorghum; Acid methanol	3.8 (1.4)†
				Red non-tannin sorghum; 70% methanol	6.3 (0.4)†
				Red non-tannin sorghum; 70% acetone	7.5 (0.4)†
				Red non-tannin sorghum; Water	2.3 (0.4)†
Naringenin glucoside	13.35	433	271	Red non-tannin sorghum; Acid methanol	4.9 (1.5)*
				Red non-tannin sorghum; 70% methanol	10.3 (0.0)*
				Red non-tannin sorghum; 70% acetone	10.7 (0.2)*
				Red non-tannin sorghum; Water	3.5 (0.2)*
5,7,3,4,-tetrahydroxyflavan-5-0-β-glucosyl-4,8-eriodictyol glucoside	15.48	883.2	721	Red non-tannin sorghum; 70% methanol	3.3 (0.0)†
				Red non-tannin sorghum; 70% acetone	3.5 (0.1)†
5,7,3,4,-tetrahydroxyflavan-5-0-β-galactosyl-4,8-eriodictyol	16.43	721	287, 271	Red non-tannin sorghum; Acid methanol	16.0 (1.7)†
5,7,3,4,-tetrahydroxyflavan-5-0-β-glucosyl-4,8-eriodictyol	17.42	721	287, 271	Red non-tannin sorghum; Acid methanol	3.3 (0.0)†
Eriodictyol	18.66	287		Red non-tannin sorghum; Acid methanol	15.7 (0.8)†
				Red non-tannin sorghum; Water	1.7 (0.1)†
Naringenin	21.47	271		Red non-tannin sorghum; Acid methanol	29.9 (0.7)*
				Red non-tannin sorghum; Water	2.8 (0.1)*
Flavones and derivatives					
Apigenin glucoside	10.98	431	269	Red non-tannin sorghum; Acid methanol	NQ
Luteolin-7-O-glucoside	14.28	447	285	Red non-tannin sorghum; 70% methanol	5.7 (0.1)¤
				Red non-tannin sorghum; 70% acetone	4.6 (0.6)¤
Luteolin	19.4	285		White non-tannin sorghum; Acid methanol	10.4 (0.4)
Apigenin	21.93	269		White non-tannin sorghum; Acid methanol	NQ
				Red non-tannin sorghum; Water	NQ
Phenolic acids					
4-Hydroxybenzoic acid 4-O-glucoside	4.61	299.1	137	Red non-tannin sorghum; Water	NQ
Caffeic acid	8.64	179		Red non-tannin sorghum; Water	NQ

Compound	Ret time t_R (min)	Parent ion $M-H^+$ (m/z)	MS-MS fragments (m/z)	Extract	Concentration
p-Coumaric acid	10.91	163		White maize; Water	NQ
Ferulic acid	12.5	193.1		White non-tannin sorghum; Water	NQ
				Red non-tannin sorghum; Water	NQ

NQ-Not Quantified
Values in parentheses are standard deviations from the mean
†-g Eriodictyol equivalents/100 g bran; *-g Naringenin equivalents/100 g bran; ¤-Luteolin equivalents/100 g bran

Table 4.8 shows that acidified methanol extracts of red non-tannin sorghum bran contained the highest amount of total flavonoids of all the extracts, followed by aqueous organic extracts of red non-tannin sorghum bran. Water extracts of red non-tannin sorghum bran (containing only flavanones) and acidified methanol extracts of white non-tannin sorghum (containing only flavones) each had the lowest amounts of total flavonoids.

Table 4.8 Total flavonoids quantified in extracts from bran of red non-tannin sorghum and white non-tannin sorghum

	Red non-tannin sorghum; 70% methanol	Red non-tannin sorghum; 70% acetone	Red non-tannin sorghum; acid methanol	Red non-tannin sorghum; water	White non-tannin sorghum; acid methanol
Total flavanones					
(g /100 g bran)	21.8	23.4	73.6	10.3	ND
Total flavones					
(g /100 g bran)	5.7	4.6	ND	ND	10.4
Total flavonoids					
(g /100 g bran)	27.5	28.0	73.6	10.3	10.4

The findings of studies done by Lo Piparo *et al.* (2008) and Tadera *et al.* (2006) indicated that the flavones luteolin and apigenin, as well as the flavanone naringenin are amongst the most powerful inhibitors of α-amylase. Furthermore, Kusano *et al.* (2010) found that eriodictyol in Acacia bark was a powerful inhibitor of α-amylase. These findings are corroborated by the results obtained in this study showing high inhibitory activity of the bran and red non-tannin sorghum extracts against porcine α-amylase which can be related to their flavonoid content. Flavonoids from both the flavanone and flavone groups were detected in these extracts. The observed 5% enzyme inhibition exhibited by the water extracts of the red non-tannin sorghum bran may be due to the presence of flavones and flavanones which were detected in them.

The lower inhibitory activity of the white non-tannin sorghum bran extracts compared to that of the red non-tannin sorghum may be due to the presence of lower concentrations of total flavonoids. Although no flavonoids were detected in the bran extracts of the white maize, inhibitory activity comparable to that of white non-tannin sorghum was detected by the bran, acidified methanol and 100% acetone extracts of the white maize, following the same trend as the total phenolic content of these samples. McCue *et al.* (2004) found that *p*-coumaric acid detected in oregano extracts contributed to α-amylase inhibition of these extracts. However, *p*-coumaric acid was only detected in the water extracts of the white maize which did not have any inhibitory effect on the activity of α-amylase. However, the presence of flavonoids in white maize has been reported (Žilić *et al.* (2012). Therefore

it might be possible that this inhibitory activity could be due to the presence of flavonoids in the maize extracts which were not detected in this study. Further analysis on the phenolic content of these extracts would be necessary to determine which compounds could have played a role in their inhibitory activity.

4.3.5 Conclusion

All the extracts from bran of red non-tannin sorghum have higher total phenolic contents and higher inhibitory activity against porcine pancreatic α-amylase than corresponding extracts from white non-tannin sorghum and white maize. Unextracted red non-tannin sorghum bran has higher porcine pancreatic α-amylase inhibitory activity than white non-tannin sorghum and white maize bran. The high α-amylase inhibitory activity of the red non-tannin sorghum bran extracts may be related to their content of various flavanones and flavones and their derivatives which are not present in extracts from white non-tannin sorghum and white maize bran. These results show that diets rich in sorghum or maize bran and nutraceutical-type preparations particularly from red non-tannin sorghum bran could have anti-diabetic properties by inhibiting α-amylase activity and thus control postprandial glucose levels in people suffering from diabetes.

4.3.6 References

AWIKA, J. M., MCDONOUGH, C. M. & ROONEY, L. W. 2005. Decorticating sorghum to concentrate healthy phytochemicals. *Journal of Agricultural and Food Chemistry*, 53, 6230-6234.

AWIKA, J. M. & ROONEY, L. W. 2004. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry*, 65, 1199-1221.

AWIKA, J. M., ROONEY, L. W. & WANISKA, R. D. 2004. Properties of 3-deoxyanthocyanins from sorghum. *Journal of Agricultural and Food Chemistry*, 52, 4388-4394.

BANNON, M. 2011. Challenges for diabetic care: rural Africa & type 1 brittle diabetes. *QJM*, 104, 559-560.

BRODY, T. 1999. Nutritional Biochemistry. 2nd Edition. Academic Press. London, Pages 103-180.

CHIREMBA, C., TAYLOR, J. R. N., ROONEY, L. W. & BETA, T. 2012. Phenolic acid content of sorghum and maize cultivars varying in hardness. *Food Chemistry*, 134, 81-88.

CUYCKENS, F. & CLAEYS, M. 2004. Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry*, 39, 1-15.

DYKES, L., SEITZ, L. M., ROONEY, W. L. & ROONEY, L. W. 2009. Flavonoid composition of red sorghum genotypes. *Food Chemistry*, 116, 313-317.

EVERETTE, J. D., BRYANT, Q. M., GREEN, A. M., ABBEY, Y. A., WANGILA, G. W. & WALKER, R. B. 2010. Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. *Journal of Agricultural and Food Chemistry*, 58, 8139-8144.

GARCIA-SALAS, P., MORALES-SOTO, A., SEGURA-CARRETERO, A. & FERNÁNDEZ-GUTIÉRREZ, A. 2010. Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules*, 15, 8813-8826.

GUJER, R., MAGNOLATO, D. & SELF, R. 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry*, 25, 1431-1436.

HARGROVE, J. L., GREENSPAN, P., HARTLE, D. K. & DOWD, C. 2011. Inhibition of aromatase and α-amylase by flavonoids and proanthocyanidins from Sorghum bicolor bran extracts. *Journal of Medicinal Food*, 14, 799-807.

IDF. 2011. *IDF diabetes atlas – Fifth Edition* [Online]. Available: http://www.idf.org/diabetesatlas/5e/the-global-burden [Accessed 15 February 2012].

INRA. 2013. *Phenol-Explorer Version 3.0* [Online]. Available: www.phenol-explorer.eu/compounds [Accessed 17 July 2013].

KUSANO, R., OGAWA, S., MATSUO, Y., TANAKA, T., YAZAKI, Y. & KOUNO, I. 2010. α-Amylase and lipase inhibitory activity and structural characterization of acacia bark proanthocyanidins. *Journal of Natural Products*, 74, 119-128.

LO PIPARO, E., SCHEIB, H., FREI, N., WILLIAMSON, G., GRIGOROV, M. & CHOU, C. J. 2008. Flavonoids for controlling starch digestion: Structural requirements for inhibiting human α-amylase. *Journal of Medicinal Chemistry,* 51, 3555-3561.

MCCUE, P. & SHETTY, K. 2004. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro. *Asia Pacific Journal of Clinicial Nutrition,* 13, 101-106.

MCCUE, P., VATTEM, D. & SHETTY, K. 2004. Inhibitory effect of clonal oregano extracts against porcine pancratic amylase *in vitro. Asia Pacific Journal of Clinical Nutrition,* 13, 401-408.

MEGAZYME. 2012. *Alpha-amylase assay procedure (Ceralpha Method K-CERA 01/12) for the measurement of plant and microbial alpha-amylases.* [Online]. Available: http://management.ebooks6.com/ALPHA-AMYLASE-ASSAY-PROCEDURE---(CERALPHA-METHOD)-pdf-e8287.html [Accessed 1 June 2012].

NACZK, M. & SHAHIDI, F. 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*, 41, 1523-1542.

SHOBANA, S., SREERAMA, Y. N. & MALLESHI, N. G. 2009. Composition and enzyme inhibitory properties of finger millet (Eleusine coracana L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. *Food Chemistry*, 115, 1268-1273.

SVENSSON, L., SEKWATI-MONANG, B., LUTZ, D. L., SCHIEBER, A. & GÄNZLE, M. G. 2010. Phenolic acids and flavonoids in nonfermented and fermented red sorghum (Sorghum bicolor (L.) Moench). *Journal of Agricultural and Food Chemistry,* 58, 9214-9220.

TADERA, K., MINAMI, Y., TAKAMATSI, K. & MATSUOKA, T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology,* 52, 149-153.

TAYLOR, J. R. N. 2005. Non-starch polysaccharides, protein and starch: Form function and feed - Highlight on sorghum. *Australian Poultry Science Symposium,* 17, 9-16.

UCHIDA, R., NASU, A., TOKUTAKE, S., KASAI, K., TOBE, K. & YAMAJI, N. 1999. Synthesis of new N-containing maltooligosaccharides, a-amylase inhibitors and their biological activities. *Chemical and Pharmaceutical Bullitin,* 47, 187-193.

WAGMAN, A. S. & NUSS, J. M. 2001. Current therapies and emerging targets for the treatment of diabetes. *Current Pharmaceutical Design,* 7, 417-450.

WHITNEY, E. & ROLFES, S. R. 2011. *Understanding Nutrition. 12th Edition. Wadsworth. USA. 103-105.* Twelfth ed., 103-105.

XU, B. J. & CHANG, S. K. C. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science,* 72, S159-S166.

ŽILIĆ, S., SERPEN, A., AKıLLıOĞLU, G., GÖKMEN, V. & VANČETOVIĆ, J. 2012. Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. *Journal of Agricultural and Food Chemistry*, 60**,** 1224-1231.

CHAPTER 5

5 General Discussion

This chapter will present a critical review of the main methods used in this study. It will also include a discussion of the observed trends in results and the mechanisms involved in the inhibition of porcine pancreatic α-amylase by the flavonoids detected in the bran of the different grains.

5.1 Critical discussion of experimental design and methodologies

It was necessary to prepare bran samples from the maize and sorghum grains with as little endosperm contamination as possible in order to maximise the chances of obtaining the highest possible concentration of phenolic content from the bran samples during extraction. Decortication of the grains to produce bran was done using a tangential abrasive dehulling device (TADD). According to Awika *et al.* (2005) bran fractions obtained after the first two minutes of dehulling provided the highest concentration of phenolic compounds for most sorghum types and can be regarded as the "optimum" decortication time for preparation of these bran samples. Da Silva and Taylor (2004) reported the highest concentration of phenolic compounds in the first 10% of decorticated sorghum bran. Therefore in this study, a bran yield of not more than 10% was targeted in order to obtain bran samples that were as pure as possible.

The decortication times and bran yields of the maize and sorghum grains are shown in Table 5.1. It was found during decortication trials, and as shown in Table 5.1, that it took relatively longer to decorticate 10% of the maize kernels than the sorghum kernels. This observation may be due to one or a combination of the following reasons: the relatively bigger size of the maize kernels; the irregular shape of the maize kernels and the relatively harder bran layer of the maize compared to that of the sorghum kernels.

Table 5.1 Decortication times and bran yield of white maize and red and white non-tannin sorghum samples used for preparation of extracts

	PAN 6335	PAN 6045	KAT 369	NK 8828	MR BUSTER	TOWN
Initial Sample Weight (g)	1749.37	1818.01	1604.87	1693.89	1714.62	1642.26
Sample weight (g) after decortication	1583.18	1652.47	1484.50	1548.75	1546.59	1523.25
Bran Yield (%)	9.5	9.1	7.5	8.5	9.8	7.3
Decortication time (min)	3.5	3.5	1.0	1.0	1.0	1.0

For the preparation of nutraceutical-type products containing bioactive compounds such as phenolics, their effective extraction from the plant matrix is an important step. The solubility of phenolic compounds is governed by their chemical nature and extract yield is affected by factors such as polarity of solvents, extraction time, temperature, sample-to-solvent ratio and chemical and physical characteristics of the samples e.g. particle size (Reviewed by Dai & Mumper, 2010). Phenolic compounds differ in type and complexity in different types of plant material and this is compounded by the fact that a lot of the phenolics (e.g. phenolic acids and flavonoids) may be associated with other plant components such as carbohydrates, proteins and lipids. Due to these factors, it is difficult to find a universal procedure and solvent suitable for extraction of all plant phenolics (Reviewed by Dai & Mumper, 2010). This was demonstrated in this study by the difference in total phenolic content of the white maize and white and red non-tannin bran extracts prepared with different solvents. The 70% aqueous organic solvent extracts had higher total phenolic contents than the 100% organic solvent extracts possibly due to the differing polarities of the two solvent systems (aqueous organic vs. 100% organic solvents). It has been shown that in general, aqueous organic solvents are more efficient in the extraction of phenolic compounds compared to pure organic solvents (Xu & Chang, 2007; Zhao & Hall, 2008). Results further indicated that for all the sorghum and maize bran samples, acidified (1% HCl) methanol extracts had the highest total phenolic content. This was possibly due to the fact that the acid can hydrolyse ester bonds and release bound phenolic compounds making them more extractable (Reviewed by Robards, 2003; Reviewed by Stalikas, 2007).

The Folin-Ciocalteu assay involves the measurement of the total reducing phenolic hydroxyl groups in the bran extracts, based upon the reduction-oxidation reaction of the Folin Ciocalteu phenol reagent with the phenols, to form chromogens. The reagent mixture contains sodium molybdate and sodium tungstate (Everette *et al.*, 2010). The phenols are oxidised and the phosphomolybdic/phosphotungsten acid complexes in the reagent are reduced, causing the formation of blue chromogens which can be detected spectrophotometrically (Waterman & Mole, 1994a). The method is generally used in food and agricultural research fields in establishing total phenolic content in biological material. However, it is not specific and may be affected by interfering substances present in the biological material like sugars, proteins and ascorbic acid. Phenolic compounds are the most abundant reducing compounds in most plants; therefore the method provides a rough estimate of total phenolic content in most plant extracts (Everette *et al.*, 2010). It could therefore be possible that the higher total phenolic content of the water extracts from white non-tannin sorghum and white maize compared to that of corresponding 70% and 100% methanol or acetone extracts, might be due to an over-estimation of phenolic compounds in these crude extracts due to the non-phenolic interfering substances like reducing sugars, mentioned earlier. Chirinos, Rogez, Campos, Pedreschi and Larondelle (2007) have pointed out that the use of water only, as a solvent, yields an extract with high content of water-soluble substances with

reducing properties (e.g. organic acids, reducing sugars, soluble proteins) which can interfere with phenolic quantification methods such as the Folin Ciocalteu assay.

According to a review by Khoddami, Wilkes and Roberts (2013) HPLC is the desired technique for both separation and quantification of phenolic compounds. However, HPLC analysis can be influenced by factors such as sample purity (Reviewed by Stalikas, 2007). In this study, crude extracts from the bran of the white maize and white and red non-tannin grain varieties were analysed. With initial reversed phase HPLC analysis with UV detection at 280 nm, compounds such as rutin, naringin, hesperidin, fisetin and quercetin were provisionally identified in the sorghum and maize samples. With the exception of quercetin in maize, identification of these compounds in the samples could not be verified from the literature. Limitations with regard to reversed phase HPLC analysis used in this study in identifying flavonoids with closely related structures were described in the first research chapter. According to Hansen, Jensen, Cornett, Bjørnsdottir, Taylor, Wright and Wilson (1999), HPLC with UV detection does not always provide enough data for full structure analysis. LC-MS was found to be more effective in identifying specific flavonoids noted in the literature to be present in sorghum. More compounds could be identified with LC-MS than reversed phase HPLC due to the possibility of fragmentation of major peaks into basic compound units by mass spectrometry leading to more comprehensive structure analysis. However, identification of compounds specific to sorghum and maize was hampered by the absence of standards and absence of compound information in the polyphenol database Phenol Explorer (INRA, 2013). More specific LC-MS phenolic compound identification in sorghum and maize is required in future research.

For the purpose of this study the effect of bran phenolic extracts on α-amylase was evaluated. The Megazyme Ceralpha method representing a simplified in vitro simulation of one digestion phase (small intestine digestion phase) was used. The assay principle involves the use of the oligosaccharide, non-reducing end-blocked *p*-nitrophenyl maltoheptaoside (BPNPG7) as substrate. After hydrolysis of the BPNPG7 by the endo-acting α-amylase into a blocked maltosaccharide and *p*-nitrophenyl maltosaccharide, further hydrolysis of *p*-nitrophenyl maltosaccharide to glucose and free *p*-nitrophenol takes place by excess quantities of α-glucosidase. The amount of released *p*-nitrophenol is directly related to the activity of the α-amylase and is determined spectrophotometrically (Megazyme, 2012). The method has a standard error of less than 5%. McCleary and Sheehan (1987) found that the method correlated well with established procedures for α-amylase assay and permits rapid and specific quantification of α-amylase activity. Therefore, for the purpose of this study, the method was adequate.

However, a possible limitation is that this is an *in vitro* assay and it may not accurately predict the effect that the flavonoids would have on the activity of pancreatic α-amylase in the human digestive tract if consumed as a nutraceutical or a food product. The digestion of food (specifically food

carbohydrates) involves a complex process which includes several phases (Woolnough, Monro, Brennan & Bird, 2008) and gastro-intestinal handling and utilization of carbohydrates are further influenced by the botanical origin of the food, food composition, food matrix and degree of processing (Englyst & Englyst, 2005). Furthermore, Tadera *et al.* (2006) found differences in the amount of inhibition flavonoids could have on α-amylase versus α-glucosidase activity, indicating that not all starch hydrolysing enzymes in the digestive tract will be inhibited equally. Brayer, Luo & Withers (1995) also found that four regions of polypeptide chain conformations of the porcine pancreatic α-amylase differ significantly from that of human pancreatic α-amylase which may lead to differences in substrate differentiation and cleavage patterns between porcine and human pancreatic α-amylase. However, in spite of these limitations, it is possible to draw some reasonable conclusions based on the *in vitro* study about the possible effect of flavonoids on α-amylase activity in the human digestive tract The effectiveness of the possible anti-diabetic characteristics of these flavonoids after consumption needs further research in which more comprehensive *in vitro* and *in vivo* digestion methodology needs to be applied.

When the enzyme inhibition assay was initially carried out using extracts acidified with HCl, very low inhibition (high enzyme activity) with very little variance between the different bran extracts were observed. It was suspected that the presence of Cl^- ions in the HCl may have affected the α-amylase activity, because the α-amylase activity is Cl-dependant. It has been hypothesized that the Cl^-ion is important for activation of the binding site of the enzyme (D'Amico, Gerday & Feller, 2000; Qian, Ajandouz, Payan & Nahoum, 2005). Qian *et al.* (2005) stated however, that the activity level of the enzyme in their study could also have been affected by other basal activity not dependant on chloride. Although it was not exactly clear what the reason was for the inhibition pattern of the enzyme when extracts acidified with HCl were used, it was decided to rather use acetic acid (weaker organic acid) acidified organic solvents for the extracts used in the enzyme inhibition assay only and HCl-acidified organic extracts for Folin Ciocalteu, HPLC and LC-MS analysis. It may be hypothesized that that the phenolic compounds identified in the HCL-acidified organic extracts could in all likelihood also be present in the acetic acid-acidified extracts as both acids essentially play the role of reducing the pH of the extraction solvent.

5.2 Discussion of main trends and mechanisms

The unextracted bran samples showed the highest levels of inhibitory activity against α-amylase. This might be due to the high concentration of phenolic compounds in the bran or the presence of phytates and fibre as mentioned in Chapter 4. Importantly however, this is an indication that the bran components that inhibit the α-amylase enzyme do not require extraction from the bran matrix to exert inhibitory activity. In other words, they can exert their inhibitory activity in situ and clearly demonstrates the anti-diabetic properties of the bran. These findings suggest that the incorporation of bran into the diet could be a potential strategy that can be used to assist in the control of blood glucose levels of people suffering from diabetes.

The results of this study indicated that there were positive correlations between the total phenolic content and the inhibitory activity of the bran and bran extracts on porcine pancreatic α-amylase as indicated by the correlation coefficients shown in Table 5.2.

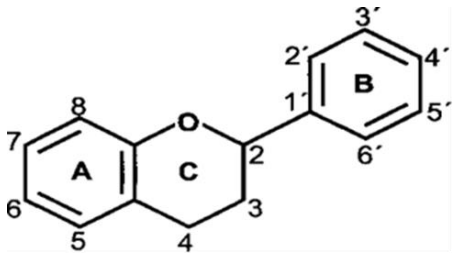
Table 5.2 Correlation coefficients (R-values) between total phenolic content and inhibition of porcine pancreatic α-amylase by bran extracts of white maize and white and red non-tannin sorghum

Grain Type	Correlation coefficient (r-Value)
White maize	0.798*
White non-tannin sorghum	0.457***
Red non-tannin sorghum	0.679**

*Significant at p<0.01; **Significant at p<0.05; *** Significant at p<0.5

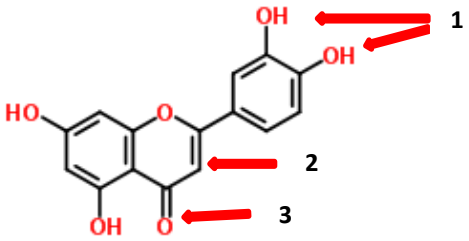
The unexpected high r-value of the white maize extracts can be ascribed to fewer values used to determine this r-value. Although a significant amount of total phenolics were detected in the water extracts from white maize, these did not show any inhibition against the enzyme activity while the acidified methanol and acetone extracts did. The observation of a significant amount of total phenolics in the maize bran water extracts may be due to an over-estimation of phenolic compounds in these crude extracts as a result of non-phenolic interfering substances and could have led to a false correlation between phenolic content and % inhibition of the water extracts. The observed positive correlation between total phenolic content and enzyme inhibition suggests that the ability of the extracts to inhibit the enzyme can be related to the presence of phenolic compounds such as flavonoids. The acidified methanol extract from red non-tannin sorghum bran which had the highest concentration of flavonoids with flavones (apigenin and luteolin) (Figure 5.1) and flavanones (eriodictyol and naringenin) (Figure 5.1) detected as the two main groups of flavonoids, also had the highest α-amylase inhibitory activity. Furthermore, the water extract of the red non-tannin sorghum (Mr BUSTER) which was the only water extract that contained flavanones like eriodictyol, was also the only water extract

that showed inhibition against α-amylase. These observations indicated that the flavone and flavanone compounds identified in the extracts are important for inhibition of the α-amylase enzyme.



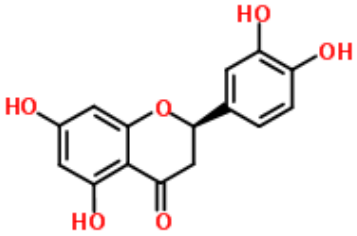
Basic flavonoid structure

FLAVONES

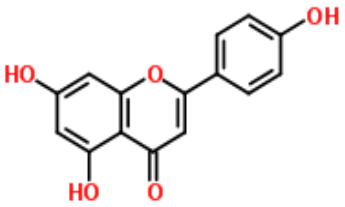


Luteolin

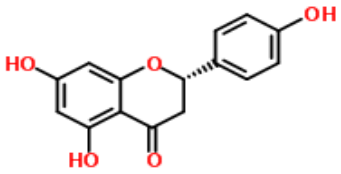
FLAVANONES



Eriodictyol



Apigenin



Naringenin

Structural feature	Interaction with amino-acids in enzyme binding site
OH-groups attached to B-ring (1)	• Hydrogen-bonding: with COOH-groups of amino acid residues (Lo Piparo *et al.*, 2008) • Ionic bonds: With NH-groups of amino acid residues (Papadopoulou *et al.*, 2004)
2,3-Double bond in the C-ring (2) 4-oxo-Group attached to C-ring (3)	• Hydrophobic interactions: between polyphenol aromatic ring and hydrophobic amino acid residues (Papadopoulou *et al.*, 2004) • Conjugation of C2-C3 double bond to 4-oxo-group enables conjugated π-system with amino acid residues which stabilizes the flavonoid-enzyme interaction in the enzyme active site (Lo Piparo *et al.*, 2008)

Figure 5.1 Basic flavonoid structure indicating the flavonoid numbering system (Reviewed by Bravo, 1998) and chemical structures of flavones and flavanones detected in extracts of red non-tannin sorghum showing the main structural features responsible for inhibition of porcine pancreatic α-amylase.

Interactions of polyphenols like flavonoids and proteins (enzymes) have been described by various literature sources (Reviewed by Bennick, 2002; Haslam, 1974; Jöbstl, O'Connell, Fairclough & Williamson, 2004; Reviewed by Le Bourvellec & Renard, 2011). These interactions may be affected by several factors including the concentrations of the polyphenols and enzyme, solvent composition, ionic strength, temperature and pH (Reviewed by Le Bourvellec & Renard, 2011). Chethan *et al.* (2008) reported that inhibition of millet malt amylases by phenolic compounds in crude extracts might be due to non-competitive inhibition where the phenolic compounds bind to the enzyme or to the enzyme-substrate complex at sites other than the binding site. Shobana *et al.* (2009) also reported a non-competitive type of inhibition by finger millet seed coat extracts on pancreatic α-amylase. As crude extracts were used in this study, enzyme kinetics analysis would have been required to establish the exact mode of inhibition, but a non-competitive mode of inhibition could be expected.

The inhibitory potential of flavonoids on the activity of α-amylase is based upon three structural requirements (Figure 5.1):

1) The presence of OH-groups attached to the flavonoid structure. This could be responsible for the formation of hydrogen bonds between the hydroxyl groups of the flavonoid and the COOH-groups of the amino acid residues in the binding site of the enzyme (Lo Piparo *et al.*, 2008) as well as the formation of ionic bonds between the NH-groups of amino acid residues and the flavonoid (Papadopoulou *et al.*, 2004);

2) The 2,3-double bond in the C-ring of the flavonoid providing delocalised electrons which can form covalent bonds with delocalised electrons in ring structures of some amino acid residues like tryptophan in the binding site of the enzyme (Lo Piparo *et al.*, 2008);

3) The 4-oxo/keto-group attached to the C-ring of the flavonoid which also provide delocalise electrons. Furthermore, due to the conjugation of the C2-C3 double bond to the 4-keto group, this feature enables the flavonoid to form a highly conjugated π-system with certain amino acids in the enzyme binding site which ensures the stabilization of the interaction of the flavonoid with the enzyme binding site (Lo Piparo *et al.*, 2008).

Besides the 2,3-double bond in the C-ring and hydroxylation of the B-ring, the presence of a 5-OH-group attached to the A-ring enhances the inhibitory activity of the flavonoid (Tadera *et al.*, 2006).

The flavonoids detected in the extracts had some or all of the above-mentioned structural features (Figure 5.1) important for enzyme inhibition. Luteolin and apigenin detected in the red non-tannin sorghum extracts contained the 2,3-double bond in the C-ring. All the flavonoids detected in the non-tannin sorghum extracts had OH-groups attached to their structures as well as the 4-oxo-group (keto-

group) attached to the C-ring. The flavonoids detected in the extracts of the red non-tannin sorghum had a 5-OH-group attached to the A-ring which could further increase their inhibitory activity. Figure 5.2 illustrates possible modes of interaction between flavonoids and proteins or enzymes, specifically hydrogen bonding, ionic bonding and hydrophobic interactions which may lead to inhibition of enzyme activity (Reviewed by Le Bourvellec & Renard, 2011).

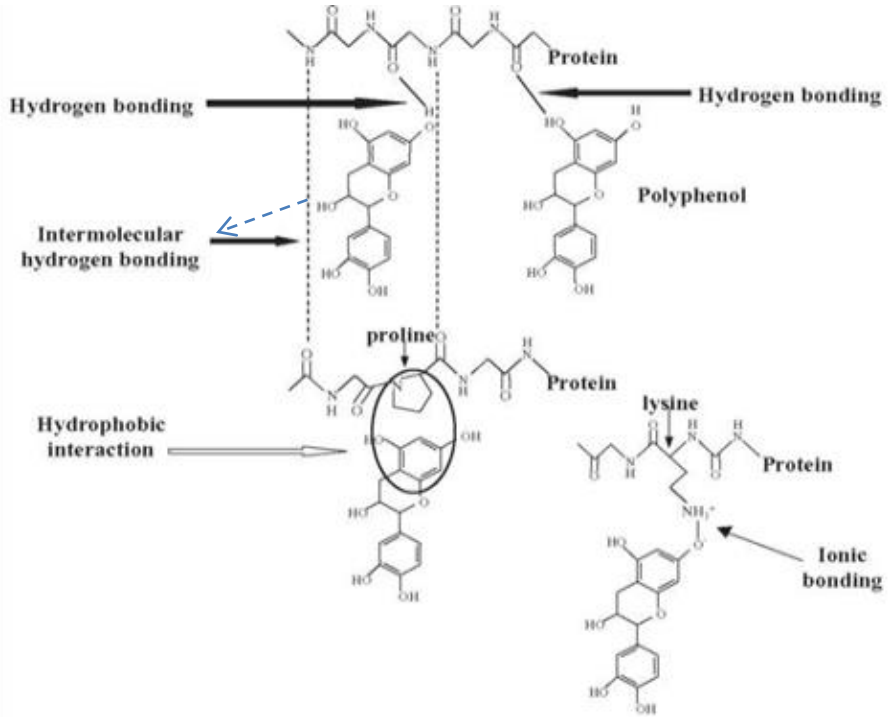


Figure 5.2 Illustration of modes of interactions between polyphenols and proteins (Reviewed by Le Bourvellec & Renard, 2011)

CHAPTER 6

6 Conclusions and Recommendations

Bran and bran extracts of red non-tannin sorghum contain higher levels of total phenolics and total flavonoids (flavones and flavanones) than white maize and white non-tannin sorghum. This correlates well with the inhibitory activity of the bran and bran extracts against porcine pancreatic α-amylase. Red non-tannin sorghum bran and bran extracts have higher inhibitory activity against porcine pancreatic α-amylase than bran and bran extracts from white maize and white non-tannin sorghum. The observed inhibitory activity of the unextracted bran samples indicates that the components that inhibit the enzyme do not need to be extracted and can exert inhibitory effects in situ. These findings suggest that the bran samples, especially from red non-tannin sorghum have the potential to be used for control of postprandial glucose levels in people suffering from diabetes due to their significant inhibitory activity against the starch-hydrolysing enzyme, porcine pancreatic α-amylase.

The inhibitory activity of the red non-tannin sorghum bran can be related to the concentration and specific types of flavonoids present in the bran which have the potential to inhibit porcine pancreatic α-amylase activity due to their structural features. The high inhibitory activity of extracts from red non-tannin sorghum bran appears to be related to the presence of flavones and flavanones which were the two main groups of flavonoids detected in the bran extracts of the non-tannin sorghum by LC-MS. The red non-tannin sorghum bran extracts yielded higher amounts of flavonoids than the other extracts in general. Flavanones and derivatives including eriodictyol and naringenin were present only in extracts from red non-tannin sorghum bran while the flavones and derivatives including apigenin and luteolin were present in extracts from red non-tannin sorghum and white non-tannin sorghum bran. In contrast, bran extracts from white maize with generally low inhibition or no inhibition at all contained no flavonoids. The results of this study provide a basis for the application of red non-tannin sorghum bran in nutraceutical type preparations or in functional foods in controlling blood glucose levels of people suffering from diabetes.

More comprehensive *in vitro* and *in vivo* digestion studies are needed to investigate the effectiveness of the flavonoids towards the activity of human pancreatic α-amylase as well as other starch-hydrolysing enzymes like α-glucosidase in the human digestive tract. In future, enzyme kinetic analysis could enhance understanding of the mode of inhibition by the flavonoids against amylase activity. With the aim of utilization as anti-diabetic nutraceuticals, encapsulation of the flavonoids into appropriate delivery systems to protect them during the digestive processes in the mouth and stomach could be investigated. The effect of increased sorghum bran intake on the prevalence of diabetes in developing regions of the world can be investigated through nutritional intervention studies. Future

research can follow with regard to development of products which could be incorporated in the diets of people in these developing regions, utilising non-tannin sorghum bran as a cost effective anti-diabetic components of functional foods with good sensory properties.

CHAPTER 7

7 References

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ABUBAKARI, A. R. & BHOPAL, R. S. 2008. Systematic review on the prevalence of diabetes, overweight/obesity and physical inactivity in Ghanaians and Nigerians. *Public Health,* 122**,** 173-182.

AHERNE, S. A. & O'BRIEN, N. M. 2002. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition,* 18**,** 75-81.

AOKI, H., KUZE, N., KATO, Y. & GEN, S. 2002. Anthocyanins isolated from purple corn (Zea mays L.). *Foods and Food Ingredients Journal of Japan***,** 41-45.

AWIKA, J. M. 2011. Sorghum flavonoids: Unusual compounds with promising implications for health. *In:* AWIKA, J. M., PIIRONEN, V. & SCOTT, B. (eds.) *Advances in Cereal Science: Implications to Food Processing and Health Promotion* 1st ed. USA: American Chemical Society, Pages 107-200.

AWIKA, J. M., MCDONOUGH, C. M. & ROONEY, L. W. 2005. Decorticating sorghum to concentrate healthy phytochemicals. *Journal of Agricultural and Food Chemistry,* 53**,** 6230-6234.

AWIKA, J. M. & ROONEY, L. W. 2004. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry,* 65**,** 1199-1221.

AWIKA, J. M., ROONEY, L. W. & WANISKA, R. D. 2004. Properties of 3-deoxyanthocyanins from sorghum. *Journal of Agricultural and Food Chemistry,* 52**,** 4388-4394.

BANNON, M. 2011. Challenges for diabetic care: rural Africa & type 1 brittle diabetes. *QJM,* 104**,** 559-560.

BELTON, P. S. & TAYLOR, J. R. N. 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology,* 15**,** 94-98.

BEMILLER, J. N. & HUBER, K. C. 2008. Carbohydrates. *In:* DAMODARAN, S., PARKIN, K. L. & FENNEMA, O. R. (eds.) *Fennema's Food Chemistry.* Fourth ed. USA: CRC Press, Pages 120-149.

BENNICK, A. 2002. Interaction of plant polyphenols with salivary proteins. *Critical Reviews in Oral Biology & Medicine,* 13**,** 184-196.

BOYER, C. D. & SHANNON, J. C. 2003. Carbohydrates of the kernel. *In:* WHITE, P. J. & JOHNSON, L. A. (eds.) *Corn Chemistry and Technology.* Minnesota, USA: American Association of Cereal Chemists Inc., Pages 289-354.

BRAVO, L. 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews,* 56**,** 317-333.

BRODY, T. 1999. *Nutritional Biochemistry.* 2nd Edition. Academic Press. London, Pages 103-180.

BUTLER, A. E., JANSON, J., BONNER-WEIR, S., RITZEL, R., RIZZA, R. A. & BUTLER, P. C. 2003. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes,* 52**,** 102-110.

BUTTERWORTH, P. J., WARREN, F. J. & ELLIS, P. R. 2011. Human α-amylase and starch digestion: An interesting marriage. *Starch - Stärke,* 63**,** 395-405.

CHETHAN, S., SREERAMA, Y. N. & MALLESHI, N. G. 2008. Mode of inhibition of finger millet malt amylases by the millet phenolics. *Food Chemistry,* 111**,** 187-191.

CHIREMBA, C., TAYLOR, J. R. N., ROONEY, L. W. & BETA, T. 2012. Phenolic acid content of sorghum and maize cultivars varying in hardness. *Food Chemistry,* 134**,** 81-88.

CHIRINOS, R., ROGEZ, H., CAMPOS, D., PEDRESCHI, R. & LARONDELLE, Y. 2007. Optimization of extraction conditions of antioxidant phenolic compounds from mashua (Tropaeolum tuberosum Ruíz & Pavón) tubers. *Separation and Purification Technology,* 55**,** 217-225.

COULTATE, T. 2009. *Food The Chemistry of its Compounds.* Fifth ed. Cambridge, UK: Royal Society of Chemistry, Pages 51-60.

CUYCKENS, F. & CLAEYS, M. 2004. Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry,* 39**,** 1-15.

D'AMICO, S., GERDAY, C. & FELLER, G. 2000. Structural similarities and evolutionary relationships in chloride-dependent α-amylases. *Gene,* 253**,** 95-105.

DA SILVA, L. S. & TAYLOR, J. R. N. 2004. Sorghum bran as a potential source of kafirin. *Cereal Chemistry* 81**,** 322-327.

DAI, J. & MUMPER, R. J. 2010. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules,* 15**,** 7313-7352.

DAVIES, G. & HENRISSAT, B. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure,* 3**,** 853-859.

DONATH, M. Y. & SHOELSON, S. E. 2011. Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology,* 11**,** 98-107.

DYKES, L. & ROONEY, L. W. 2006. Sorghum and millet phenols and antioxidants. *Journal of Cereal Science,* 44**,** 236-251.

DYKES, L. & ROONEY, L. W. 2007. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World,* 52**,** 105-111.

DYKES, L., ROONEY, L. W., WANISKA, R. D. & ROONEY, W. L. 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. *Journal of Agricultural and Food Chemistry,* 53**,** 6813-6818.

DYKES, L., SEITZ, L. M., ROONEY, W. L. & ROONEY, L. W. 2009. Flavonoid composition of red sorghum genotypes. *Food Chemistry,* 116**,** 313-317.

EARP, C. F., MCDONOUGH, C. M. & ROONEY, L. W. 2004. Microscopy of pericarp development in the caryopsis of Sorghum bicolor (L.) Moench. *Journal of Cereal Science,* 39**,** 21-27.

EVERETTE, J. D., BRYANT, Q. M., GREEN, A. M., ABBEY, Y. A., WANGILA, G. W. & WALKER, R. B. 2010. Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. *Journal of Agricultural and Food Chemistry,* 58**,** 8139-8144.

FAHRNHAM, D. E., BENSON, G. O. & PEARCE, R. B. 2003. Corn perspective and culture. *In:* WHITE, P. J. & JOHNSON, L. A. (eds.) *Corn Chemistry and Technology.* Minnesota, USA: American Association of Cereal Chemists, Inc., Pages 1 - 33.

FAOSTAT. 2012. Available: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567 [Accessed 17 April 2012].

GARCIA-SALAS, P., MORALES-SOTO, A., SEGURA-CARRETERO, A. & FERNÁNDEZ-GUTIÉRREZ, A. 2010. Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules,* 15**,** 8813-8826.

GONÇALVES, R., MATEUS, N. & DE FREITAS, V. 2011. Inhibition of α-amylase activity by condensed tannins. *Food Chemistry,* 125**,** 665-672.

GRIFFITHS, D. W. 1981. The polyphenolic content and enzyme inhibitory activity of testas from bean (Vicia faba) and pea (Pisum spp.) varieties. *Journal of the Science of Food and Agriculture,* 32**,** 797-804.

GRIFFITHS, D. W. 1986. ABSTRACT. The inhibition of digestive enzymes by polyphenolic compounds. *In:* FRIEDMAN, M. (ed.) *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods.* Springer US, Pages 509-516.

GUENZI, W. D. & MCCALLA, T. M. 1966. ABSTRACT. Phenolic acids in oats, wheat, sorghum, and corn residues and their phytotoxicity. *Agronomy Journal,* 58**,** 303-304.

GUJER, R., MAGNOLATO, D. & SELF, R. 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. *Phytochemistry,* 25**,** 1431-1436.

GUPTA, R. K. & HASLAM, E. 1978. Plant proanthocyanidins. Part 5. Sorghum polyphenols. *Journal of the Chemical Society, Perkin Transactions 1,* 8**,** 892-896.

HAHN, D. H. & ROONEY, L. W. 1985. Effect of genotype on tannins and phenols of sorghum. *Cereal Chemistry* 63**,** 4-8.

HANSEN, S. H., JENSEN, A. G., CORNETT, C., BJØRNSDOTTIR, I., TAYLOR, S., WRIGHT, B. & WILSON, I. D. 1999. High-performance liquid chromatography on-line coupled to high-field NMR and mass spectrometry for structure elucidation of constituents of hypericum perforatum L. *Analytical Chemistry,* 71**,** 5235-5241.

HARGROVE, J. L., GREENSPAN, P., HARTLE, D. K. & DOWD, C. 2011. Inhibition of aromatase and a-amylase by flavonoids and proanthocyanidins from Sorghum bicolor bran extracts. *Journal of Medicinal Food,* 14**,** 799-807.

HASLAM, E. 1974. Polyphenol–protein interactions. *Biochemical Journal,* 139**,** 285–288.

HENRISSAT, B. & DAVIES, G. 1997. Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology,* 7**,** 637-644.

IDF. 2011. *IDF diabetes atlas – Fifth Edition* [Online]. Available: http://www.idf.org/diabetesatlas/5e/the-global-burden [Accessed 15 February 2012].

INRA. 2013. *Phenol-Explorer Version 3.0* [Online]. Available: www.phenol-explorer.eu/compounds [Accessed 17 July 2013].

JÖBSTL, E., O'CONNELL, J., FAIRCLOUGH, J. P. A. & WILLIAMSON, M. P. 2004. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules,* 5**,** 942-949.

KANDRA, L., GYÉMÁNT, G., ZAJÁCZ, Á. & BATTA, G. 2004. Inhibitory effects of tannin on human salivary α-amylase. *Biochemical and Biophysical Research Communications,* 319**,** 1265-1271.

KENT, N. L. & EVERS, A. D. 1994. *Technology of Cereals.* Fourth ed. UK: Elsevier Science Ltd, Pages 21-25.

KHODDAMI, A., WILKES, M. & ROBERTS, T. 2013. Techniques for analysis of plant phenolic compounds. *Molecules,* 18**,** 2328-2375.

KIM, J.-S., HYUN, T. K. & KIM, M.-J. 2011. The inhibitory effects of ethanol extracts from sorghum, foxtail millet and proso millet on α-glucosidase and α-amylase activities. *Food Chemistry,* 124**,** 1647-1651.

KIM, J.-S., KWON, C.-S. & SON, K. H. 2000. Inhibition of alpha-glucosidase and amylase by luteolin, a flavonoid. *Bioscience, Biotechnology, and Biochemistry,* 64**,** 2458-2461.

KIM, M. J., HYUN, J. N., KIM, J. A., PARK, J. C., KIM, M. Y., KIM, T. G., LEE, S. J., CHUN, S. C. & CHUNG, I. M. 2007. Relationship between phenolic compounds, anthocianins content antioxidant activity in colored barley germplasm. *Journal of Agricultural and Food Chemistry,* 55**,** 4802-4809.

KOTARSKI, S. F., WANISKA, R. D. & THURN, K. K. 1992. Starch hydrolysis by the ruminal microflora. *The Journal of nutrition,* 122**,** 178-190.

KRUEGER, C. G., VESTLING, M. M. & REED, J. D. 2002. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [Sorghum bicolor (L.) Moench]. *Journal of Agricultural and Food Chemistry,* 51**,** 538-543.

KUSANO, R., OGAWA, S., MATSUO, Y., TANAKA, T., YAZAKI, Y. & KOUNO, I. 2010. α-Amylase and lipase inhibitory activity and structural characterization of acacia bark proanthocyanidins. *Journal of Natural Products,* 74**,** 119-128.

KWON, Y.-I., APOSTOLIDIS, E. & SHETTY, K. 2008. Inhibitory potential of wine and tea against α-amylase and α-glucosidase for management of hyperglycemia linked to type 2 diabetes. *Journal of Food Biochemistry,* 32**,** 15-31.

LARSON, R. L. 1971. Glucosylation of quercetin by a maize pollen enzyme. *Phytochemistry,* 10**,** 3073-3076.

LE BOURVELLEC, C. & RENARD, C. M. G. C. 2011. Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Critical Reviews in Food Science and Nutrition,* 52**,** 213-248.

LEDER, I. 2004. *Sorghum and millets. Cultivated plants, primarily as food sources.* [Online]. Available: http://www.eolss.net/ebooks/Sample%20Chapters/C10/E5-02-01-04.pdf [Accessed 1 March 2012].

LEMLIOGLU-AUSTIN, D., TURNER, D. T., MCDONOUGH, C. M. & ROONEY, L. W. 2012. Effects of sorghum [Sorghum bicolor (L.) Moench] crude extracts on starch digestibility, estimated glycemic index (EGI), and resistant starch (RS) contents of porridges. *Molecules,* 17**,** 11124-11138.

LO PIPARO, E., SCHEIB, H., FREI, N., WILLIAMSON, G., GRIGOROV, M. & CHOU, C. J. 2008. Flavonoids for controlling starch digestion: Structural requirements for inhibiting human α-amylase. *Journal of Medicinal Chemistry,* 51**,** 3555-3561.

MATTILA, P., PIHLAVA, J.-M. & HELLSTROM, J. 2005. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry,* 53**,** 8290-8295.

MAURO, D. J., ABBAS, I. R. & ORTHOEFER, F. T. 2003. Corn starch modification and uses. *In:* WHITE, P. J. & JOHNSON, L. A. (eds.) *Corn Chemistry and Technology.* Second ed. Minnesota, USA: American Association of Cereal Chemists, Pages 605-634.

MCCLEARY, B. V. & SHEEHAN, H. 1987. Measurement of cereal α-amylase: A new assay procedure. *Journal of Cereal Science,* 6**,** 237-251.

MCCUE, P. & SHETTY, K. 2004. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro. *Asia Pacific Journal of Clinical Nutrition,* 13**,** 101-106.

MCCUE, P., VATTEM, D. & SHETTY, K. 2004. Inhibitory effect of clonal oregano extracts against porcine pancratic amylase *in vitro. Asia Pacific Journal Clinical Nutrition,* 13**,** 401-408.

MCDOUGALL, G. J., SHPIRO, F., DOBSON, P., SMITH, P., BLAKE, A. & STEWART, D. 2005. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *Journal of Agricultural and Food Chemistry,* 53**,** 2760-2766.

MEGAZYME. 2012. *Alpha-amylase assay procedure (Ceralpha Method K-CERA 01/12) for the measurement of plant and microbial alpha-amylases.* [Online]. Available: http://management.ebooks6.com/ALPHA-AMYLASE-ASSAY-PROCEDURE---(CERALPHA-METHOD)-pdf-e8287.html [Accessed 1 June 2012].

NACZK, M. & SHAHIDI, F. 2004. Extraction and analysis of phenolics in food. *Journal of Chromatography A,* 1054**,** 95-111.

NACZK, M. & SHAHIDI, F. 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis,* 41**,** 1523-1542.

NDERITU, A. M., DYKES, L., AWIKA, J. M., MINNAAR, A. & DUODU, K. G. 2013. Phenolic composition and inhibitory effect against oxidative DNA damage of cooked cowpeas as affected by simulated in vitro gastrointestinal digestion. *Food Chemistry,* 141**,** 1763-1771.

PAPADOPOULOU, A., GREEN, R. J. & FRAZIER, R. A. 2004. Interaction of Flavonoids with Bovine Serum Albumin: A Fluorescence Quenching Study. *Journal of Agricultural and Food Chemistry,* 53**,** 158-163.

PINNEY, S. E. & SIMMONS, R. A. 2010. Epigenetic mechanisms in the development of type 2 diabetes. *Trends in Endocrinology & Metabolism,* 21**,** 223-229.

QIAN, M., AJANDOUZ, E. H., PAYAN, F. & NAHOUM, V. 2005. Molecular basis of the efects of chloride ion on the acid−base catalyst in the mechanism of pancreatic α-amylase. *Biochemistry,* 44**,** 3194-3201.

ROBARDS, K. 2003. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *Journal of Chromatography A,* 1000**,** 657-691.

ROONEY, L. W. & PFLUGFELDER, R. L. 1986. Factors affecting starch digestibility with special emphasis on sorghum and corn. *Journal of Animal Science,* 63**,** 1607-1623.

SERNA-SALDIVAR, S. & ROONEY, L. W. 1995. Structure and Chemistry of Sorghum and Millets. *In:* DENDY, D. A. V. (ed.) *Sorghum and Millets: Chemistry and Technology.* Minesota, USA: American Association of Cereal Chemists, 69-168.

SHAW, J. E., SICREE, R. A. & ZIMMET, P. Z. 2010. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice,* 87**,** 4-14.

SHOBANA, S., SREERAMA, Y. N. & MALLESHI, N. G. 2009. Composition and enzyme inhibitory properties of finger millet (Eleusine coracana L.) seed coat phenolics: Mode of inhibition of α-glucosidase and pancreatic amylase. *Food Chemistry,* 115**,** 1268-1273.

ŠKERGET, M., KOTNIK, P., HADOLIN, M., HRAŠ, A. R., SIMONIČ, M. & KNEZ, Ž. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry,* 89**,** 191-198.

STALIKAS, C. D. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science,* 30**,** 3268-3295.

STYLES, E. D. & CESKA, O. 1977. ABSTRACT. The genetic control of flavonoid synthesis in maize. *Canadian Journal of Genetics and Cytology,* 19**,** 289-302.

TADERA, K., MINAMI, Y., TAKAMATSI, K. & MATSUOKA, T. 2006. Inhibition of alpha-glucosidase and alpha-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology,* 52**,** 149-153.

TAPAS, A. R., SAKARKAR, D. M. & KAKDE, R. B. 2008. Flavonoids as nutraceuticals: A review. *Tropical Journal of Pharmaceutical Research,* 7**,** 1089-1099.

TAYLOR, J. R. N. 2005. Non-starch polysaccharides, protein and starch: Form function and feed - Highlight on sorghum. *Australian Poultry Science Symposium,* 17**,** 9-16.

TAYLOR, J. R. N. & DEWAR, J. 2001. Developments in sorghum food technologies. *Advances in Food and Nutrition Research,* 43**,** 217-264.

TESTER, R., KARKALAS, J. & QI, X. 2004. Starch structure and digestibility enzyme-substrate relationship. *World's Poultry Science Journal,* 60**,** 186-195.

TSUDA, T., HORIO, F., UCHIDA, K., AOKI, H. & OSAWA, T. 2003. Dietary cyanidin 3-O-β-d-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *The Journal of Nutrition,* 133**,** 2125-2130.

UCHIDA, R., NASU, A., TOKUTAKE, S., KASAI, K., TOBE, K. & YAMAJI, N. 1999. Synthesis of new N-containing maltooligosaccharides, a-amylase inhibitors and their biological activities. *Chemical and Pharmaceutical Bullitin,* 47**,** 187-193.

WAGMAN, A. S. & NUSS, J. M. 2001. Current therapies and emerging targets for the treatment of diabetes. *Current Pharmaceutical Design,* 7**,** 417-450.

WATERMAN, P. G. & MOLE, S. 1994a. *Analysis of Phenolic Plant Metabolites.* First ed. London: Blackwell Scientific Publications, Pages 1-20.

WATERMAN, P. G. & MOLE, S. 1994b. *Analysis of Phenolic Plant Metabolites.* First ed. London: lackwell Scientific Publications, Pages 66-103.

WATSON, S. E. 2003. Description, development, structure and composition of the corn kernel. *In:* WHITE, P. J. & JOHNSON, L. A. (eds.) *Corn Chemistry and Technology.* Second edition ed. Minnesota, USA: American Association of Cereal Chemists, Pages 69 - 106.

WHITNEY, E. & ROLFES, S. R. 2011. *Understanding Nutrition* Twelfth ed. Wadsworth. USA: Wadsworth CENGAGE Learning, Pages 103-105.

WHO. s.a. *Chapter 1. Burden, mortality, morbidity and risk factors* [Online]. Available: http://www.who.int/nmh/publications/ncd_report_chapter1.pdf [Accessed 1 March 2012].

XU, B. J. & CHANG, S. K. C. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science*, 72**,** S159-S166.

YOON, J. H., THOMPSON, L. U. & JENKINS, D. J. 1983. The effect of phytic acid on in vitro rate of starch digestibility and blood glucose response. *The American Journal of Clinical Nutrition*, 38**,** 835-42.

ZHAO, B. & HALL, C. A. 2008. Composition and antioxidant activity of raisin extracts obtained from various solvents. *Food Chemistry*, 108**,** 511-518.

ŽILIĆ, S., SERPEN, A., AKıLLıOĞLU, G., GÖKMEN, V. & VANČETOVIĆ, J. 2012. Phenolic compounds, carotenoids, anthocyanins, and antioxidant capacity of colored maize (Zea mays L.) kernels. *Journal of Agricultural and Food Chemistry*, 60**,** 1224-1231.

8 APPENDICES

8.1 Appendix A

POTENTIAL OF SORGHUM AND OTHER CEREAL GRAIN PHENOLICS TO PREVENT AND ALLEVIATE METABOLIC SYNDROME AND TYPE 2 DIABETES

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There is substantial evidence that cereal grains rich in polyphenolic phytochemicals, such as most sorghum and millet types, can have anti-Metabolic Syndrome and antidiabetic actions (reviewed by Taylor *et al.,* 2013; reviewed by Taylor and Duodu 2014). Several activities have been indicated, including: inhibition of digestive amylase activity, modification of starch digestibility, reduction in starch availability, improvement in insulin sensitivity and prevention of protein glycation. Human intervention trials have assessed the glycaemic response of healthy and Type 2 diabetic subjects after consumption of foods from these grains, particularly finger millet (*Eleucine coracana)*. Unfortunately, the findings have been inconclusive, primarily due to weak and out-dated experimental methodology (reviewed by Shobana *et al.,* 2013).

The most convincing evidence of antimetabolic syndrome and antidiabetic effects involves inhibition of the key digestive amylases, α-amylase and α-glucosidase by polyphenolic-rich extracts from tannin sorghum (Kim *et al.,* 2011; Lemlioglu-Austin *et al.,* 2012). In recent research, we have shown that isolated sorghum tannins can inhibit α-glucosidase at a far lower Inhibitory Concentration (IC^{50}) than the antidiabetic drug amylase inhibitor, acarbose. Further, we have also shown that aqueous extracts from polyphenol-rich non-tannin sorghums are inhibitory against α-amylase.

Currently, it seems that the best way to utilise the potential anti-Metabolic Syndrome and antidiabetic activities of these cereal grains is through development of phytochemical-rich digestive amylase inhibitory nutraceuticals. In determining whether consumption of food and beverage products from these grains is actually protective against Metabolic Syndrome and Type 2 Diabetes, there is a chronic need for better designed studies. Products investigated need to be fully characterised in terms of their chemical and physical composition. In animal model experimentation, food and beverage products need to be used as they are typically consumed by people and not just in the form of flours or extracts. Most importantly, well-controlled human clinical studies and intervention trials are required.

Key words: antidiabetic, anti-Metabolic Syndrome, digestive amylase inhibition, millets, polyphenols, sorghum

References:

Kim J-S, Hyun TK, Kim MJ, The inhibitory effects of ethanol extracts from sorghum, foxtail millet and proso millet on α-glucosidase and α-amylase activities. *Food Chem* **124:** 1647-1651 (2011).

Lemlioglu-Austin D, Turner ND, McDonough CM and Rooney LW, Effects of sorghum [*Sorghum bicolor (L.) Moench*] crude extracts on starch digestibility, estimated glycemic index (EGI) and resistant starch (RS) contents of porridges. *Molecules* **17:** 11124-11138 (2012).

Shobana S, Krishnaswamy K, Sudha V, Malleshi NG, Anjana RM, Palaniappan L and Mohan V, Finger millet (*Ragi, Eleucine coracana* L.): A review of its nutritional properties, processing and plausible health benefits. *Adv Food Nutr Res* **69:** 1-39 (2013).

Taylor JRN and Duodu KG, Mini-review: Functional and health-promoting foods and beverages from sorghum and millets. *J Sci Food Agric* (invited review submitted) (2014).

Taylor JRN, Belton PS, Beta T and Duodu KG, Review: Increasing the utilisation of sorghum, millets and pseudocereals: Developments in the science of their phenolic phytochemicals, biofortification and protein functionality. *J Cereal Sci* http://dx.doi.org/10.1016/j.jcs.2013.10.009 (2013).9999999