SORGHUM TANNINS: INTERACTION WITH STARCH AND ITS EFFECTS ON IN VITRO STARCH DIGESTIBILITY

A Dissertation

by

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ABSTRACT

Most of the calories in cereal foods come from starch. Decreasing starch digestibility is fundamental to prevent obesity and diabetes. This study investigated interactions of condensed tannins (proanthocyanidins-PA) and other sorghum phenolic compounds with starch molecules and their effect on *in vitro* starch digestibility. High tannin (predominant in large molecular weight PA, 80%), black (monomeric polyphenols) and white (low in polyphenols) sorghum phenolic extracts were cooked with starches varying in amylose content. Starch pasting properties, polyphenol profile and *in vitro* starch digestibility were evaluated.

Unlike other treatments, samples with tannin phenolic extracts had significantly ($P \leq 0.05$) lower setback in the test using a Rapid Visco Analyser (RVA) compared to control. The same treatments had the least extractable phenol and PA contents after cooking with all starches. These evidences suggest interactions between starch molecules and PA. Furthermore, after mixing tannin phenolic extracts with pure amylose/amylopectin, extractable polymeric PA was in much lower concentration (62% less) in presence of amylose compared to amylopectin. This drop in concentration increased to 85% when purified tannin extract (90% polymeric PA) was used. This indicates a stronger interaction between amylose and large molecular weight PA.

When high amylose starch was used in an autoclave cooking/cooling technique, the RS content of control (26.4%) was similar ($P > 0.05$) to samples with black phenolic extracts (27%); samples with tannin phenolic extracts increased RS to about 40%. The

RS increased to 46% when purified tannin extract was used. All these evidences suggest that sorghum condensed tannins, specifically the polymeric PA, directly interacted with amylose, increasing RS content, whereas the monomeric polyphenols did not.

This study opens opportunities to use tannin sorghum to develop products for diabetics and weight control, high in dietary fiber and natural dark color.

In the other part of this project, polyphenols from black and tannin sorghum bran were extracted using an Accelerated Solvent Extractor (ASE) and eco-friendly solvents such as water, and mixtures ethanol/water. ASE at 120 and 150^{o}C using 50 and 70% ethanol/water was efficient in extracting as much phenols (45 mg GAE/g) and 12% more antioxidants (628 μmol TE/g) from black sorghum compared to conventional methods using aqueous acetone and acidified methanol. Therefore, ASE extracts from black sorghum could be used in beverages and in colorants containing high antioxidant content.

DEDICATION

To my parents Geraldo and Maria de Fatima, to my siblings Fabricio and Fabiana

and

To my wife, Lilian.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Overweight and obesity are the fifth leading risk factor for global mortality (WHO 2011) and are associated with increase incidence of diabetes, heart diseases, hypertension and some cancers (Bray 2004). In the United States, obesity rates more than doubled in the past decades and recent data showed that 35.7% of the adult population is obese (CDC 2010). Medical costs associated with obesity in the USA were estimated at $147 billion in 2008 (Finkelstein and Strombotne 2010). Thus, inexpensive alternatives to prevent obesity such as healthy diet and regular physical activities should be considered.

Diets that are low in fat and sugars, and rich in fruits, vegetables and whole grains help to prevent obesity (Kumar and Singh 2009). Regular consumption of whole grains is linked with lower risks of many chronic diseases such as obesity and type 2 diabetes. Complex carbohydrates, such as resistant starch, inulin, hemicellulose, and phenolic compounds are present in whole grains and are the main responsible in the prevention of such chronic diseases (Liu 2007).

Sorghum is ranked as the fifth leading crop in the world (FAO 2009). Sorghum is an important food crop in semi-arid parts of Africa and Asia and it is also finding increased use as an “ancient grain” and gluten free food ingredient in the USA (Asif et al 2010). This growth in popularity is mainly due to agronomic advantages such as high drought tolerance, high yields, low cost, and potential health benefits including

slow starch digestibility, cardiovascular disease reduction, antioxidant activity, anti-inflammatory and anti-carcinogenic properties (Awika and Rooney 2004; Awika et al 2009; Burdette et al 2010).

Special sorghum varieties are good sources of phenolic compounds such as condensed tannins (proanthocyanidins), 3-deoxyanthocyanins and other flavonoids concentrated in the sorghum bran (Awika et al 2005). Condensed tannins, specifically the high molecular weight ones, have more powerful antioxidant activity *in- vitro* and *in-vivo* than simple phenols and other natural antioxidants (Hagerman et al 1998; Tian et al 2012). Besides their high antioxidant activity, tannins reduce nutrient digestibility by interacting with proteins (Hagerman et al 1992) and digestive enzymes (Hargrove et al 2011; Davis and Hoseney 1979).

Starch is the major component of cereals and main source of calories in cereal products. Decreasing starch digestibility is important because it would help lower caloric intake, providing benefits against obesity and type 2 diabetes. Sorghum has the lowest raw starch digestibility among cereals due to strong association between starch granules and endosperm proteins (kafirins) which restrict accessibility to starch by α-amylase (Rooney and Pflugfelder 1986). Even after cooking, sorghum flour has lower starch digestibility compared to corn due to interaction between starch and cross-linked kafirins (Zhang and Hamaker 1998).

Other components such as polyphenols may decrease *in vitro* starch digestibility by inhibiting digestive enzymes (Hargrove et al 2011) and interacting with starch. There are limited studies showing interactions between starch and phenolic compounds.

Condensed tannins were demonstrated to be adsorbed by raw starch (Davis and Hoseney 1979; Bourvellec et al 2005). Small phenolic compounds including gallic acid, ferulic acid and catechins were reported to change functional properties of starch (Wu et al 2009; Zhu et al 2008; Beta and Corke 2004) by interacting with starch molecules.

Poor nutrient digestibility of sorghum has been seen as a negative aspect for animal feeding (Serna-Saldivar and Rooney 1995). However, it is advantageous for human health. Sorghum polyphenols, especially high molecular weight condensed tannins are known to bind with proteins, severely limiting their digestibility, however, interactions with starch have not been demonstrated.

The overall objective of this study is to investigate interactions of condensed tannins and other sorghum polyphenols with starch and their effects on *in vitro* starch digestibility. The specific objectives were:

1. To investigate interactions between sorghum polyphenols and starch and effect of such interactions on *in vitro* starch digestibility.
2. To evaluate the extent of interactions of purified condensed tannins from sorghum with starch molecules and their effects on resistant starch formation.
3. To determine the effect of subcritical water on extractability of sorghum polyphenols.

CHAPTER II

LITERATURE REVIEW

PREVALENCE OF OBESITY AND DIABETES

Obesity is a major health, social and economic problem (Sampsel and May 2007). Defined as an excess of body fat, obesity is a chronic disease that is strongly associated with other serious diseases such as diabetes, heart diseases and some cancers such as pancreas, colon, prostate and breast cancer (Bray 2004; Ryan 2009). Overweight and obesity are currently the fifth leading risk factor for global mortality, with 2.8 million deaths each year. Around 1.4 billion adults worldwide were overweight and 500 million were obese in 2008 and it is estimated that obesity rates will keep increasing (WHO 2011).

In the United States, obesity occurrence among adults has significantly increased during the past two decades. Approximately 35% of the adult population is obese and as this rate increased over time, health care costs also increased. The medical costs associated with obesity in the USA were estimated at $147 billion in 2008 (CDC 2010). Another major issue is that obesity rate is not increasing only among adults. The number of obese children and adolescents reached about 17% in 2009, which was three times higher since 1980 (CDC 2009). It is predicted that US life expectancy will decrease as a result of increasing childhood obesity (Olshansky et al 2005).

Obesity is a common condition in every continent. Increase prevalence of obesity is not confirmed to only United States, Latin America and Europe. A pronounced

increase in the prevalence of overweight and obesity has been observed in Japan, China, India and in the Middle East during the last two decades (Kopelman 2000; Kumar and Singh 2009).

Genetic factors contribute to obesity, however, according to Kopelman (2000), changes in life style such as physical inactivity, and diet rich in high calorie foods, which are cheaper and more accessible than healthy foods (Finkelstein and Strombotne 2010), are the most important contributors to the climbing in obesity rates. Poor diet and physical inactivity were ascribed as the root cause of 16.6% of US deaths, exceeded only by tobacco use, which was responsible for 18.1% of deaths in 2000 (Mokdad et al 2004).

As consequence of increased in obesity over the last 30 years, type 2 diabetes has significantly increased. The global numbers of people with diabetes rose from 151 million in 2000 to 221 million in 2010 (Zimmet et al 2001). In the United States, 25.8 million people were diagnosed with diabetes in 2010. This was a significant increase compared to 2002 (12.1 million) and 2007 (17.5 million). The total cost of diabetes in 2007 was around $174 billion of which $116 billion was spent as medical costs and $58 billion in loss of productivity, unemployment and mortality. Diabetes is a major cause of heart disease, blindness and kidney failure (CDC 2011).

Prevention and treatment of obesity could reduce chronic disease incidence for millions and save billions of dollars in health care. Treatments involve educational, behavioral, pharmacological and surgical support (Stern et al 2005). A large amount of money is spent on surgical procedures and drugs. Weight-loss drugs that promise easy, quick results are widely used but most of them have questionable safety, have

undesirable side effects and are not based on credible scientific evidence and may not help people achieve long-term weight management (Bachman 2007; Robinson and Niswender 2009). A nutritional based intervention is an inexpensive alternative to the aid weight loss and weight management (Swinburn et al 2004). Thus, lifestyle changes are the best way to prevent, treat obesity and related diseases. Regular physical activities and diets with less fat, sugar and more fiber, fruits, vegetables and whole grains are the key to success in fighting obesity.

WHOLE GRAINS CONSUMPTION AND HEALTH

Consumption of whole grains and whole grain products is associated with reduced risks of various types of chronic diseases such as obesity, type 2 diabetes, cardiovascular diseases (Lutsey et al 2007) and some types of cancer (Slavin 2000). The main effect is attributed to the fiber (i.e. resistant starch, inulin, hemicellulose and β-glucan) and micronutrients such as polyphenols, carotenoids, phytates, folates, in the outer layer of the grain and in the germ fraction (Slavin et al 1999).

The effect of fibers depends on their solubility. Soluble fiber reduces blood cholesterol (Braaten et al 1994) and post-prandial glycemic response (Casiraghi et al 2006). Insoluble fibers increase stool volume and decrease its transit time through the intestinal tract, thus decreasing contact between carcinogenic compounds and colon cells. Their fermentation may also produce significant amount of butyrate which is beneficial for colon health (Olmo et al 2007). The micronutrients found in whole grain specially polyphenols protect against chronic diseases due to their antioxidant activity (Slavin et al 1999).

The antioxidant capacity (dry basis) of many fruits and vegetables is generally higher than that of cereal products (Saura-Calixto and Goni 2006). However, when compared as daily antioxidant consumption, the differences between cereals, fruits and vegetables become less marked (Miller et al 2000). One explanation for this fact is that most polyphenols (major contributor of antioxidant activity) in fruits and vegetable are in free forms, easily extracted by solvents whereas the ones from cereals are mostly bound to the cell wall components such as cellulose and lignin. Thus, most polyphenols from cereals are not quantified in antioxidants/polyphenols in vitro assays. This shows that there is an underestimation of their amount in foods (Perez-Jimenez and Saura-Calixto 2005). Food processing, such as thermal processing, pasteurization and fermentation contribute to the release of these bound phenolic compounds (Dewanto et al 2002).

Because polyphenols in fruits and vegetables are mainly in free forms, they can be readily available in the upper gastrointestinal tract (Chu et al 2002; Sun et al 2002) and help prevent diseases in that region, whereas in cereals, polyphenols are mostly bound to the cell wall. Thus, they will be less hydrolyzed during gastrointestinal digestion and will reach the colon intact, where they will provide an antioxidant environment and may prevent colon cancer (Adom and Liu 2002; Perez-Jimenez and Saura-Calixto 2005).

Despite many polyphenols present high antioxidant activity *in vitro*, they may not present the same benefits *in vivo*. Biological properties of polyphenols depend on their bioavailability. Polyphenols in general have low bioavailability. Polyphenols such

as quercetin and rutin have lower bioavailability (0.3-1.4%) compared to catechins, phenolic acids, isoflavones and anthocyanidins (3-26%) (Scalbert and Williamson 2000). Deprez et al (2001) reported that procyanidins larger than trimers could not be absorbed by the intestinal cell monolayer. Compounds that are not absorbed in the stomach or small intestine are carried to the colon and the fermented products (smaller molecules) may be absorbed. For example, procyanidin polymer is degraded in the colon into low molecular weight phenolic acids that may be absorbed *in vivo* through the colon (Deprez et al 2000).

Type II diabetes can be prevented by consuming whole grains because of their ability to decrease insulin resistance and improve insulin sensitivity (Pereira et al 2002). There are evidences that whole grains also help to prevent many types of cancer such as colorectal, gastric and other digestive tract cancers (Jacobs et al 1998). Fermentation of dietary fiber produces short-chain fatty acids such as butyrate that has shown to be antineoplastic and cause aberrant cells to apoptose (Kim and Milner 2007). Short chain fatty acids also decrease the colonic pH, which is associated with lower carcinogenic potential (Alberts et al 2003).

Thus, intake of whole grain foods is highly recommended to prevent many types of chronic diseases and have a healthy life. Currently available whole-grain foods are not frequently consumed, and few children achieve the whole-grain intake recommendation. It is very important to focus on research to develop whole grain products for kids. Instead of eating products with refined flour, they could start eating foods with 100% whole grains or partially substituted. Whole grain pancakes had the same acceptance as

those using refined flour in school (elementary, middle, high). White whole wheat had more acceptance than the red one due to light color and mild flavor (Chu et al 2011). Thus, finding a product as vehicle of whole grains such as pancakes, bread and slowly substituting the amount of whole grains is a way to gain acceptance among kids.

SORGHUM

Sorghum is one of the most important cereal crops in the world (FAO 2009). Sorghum constitutes a major source of calories and protein for many people in Africa and Asia. Many sorghum products such as bread, porridges, tortillas and beers are consumed in these countries. Sorghum, especially the white types, flour and products such as expanded snacks and cookies are becoming popular in Japan (United States Grains Council 2011). In the United States, sorghum is mostly used for animal feeding and alcohol production. Use of sorghum for human consumption is still low in the US, but it is increasing (Asif et al 2010). This rise in the interest of sorghum is mainly due to its agronomic and potential health benefits, as well as the growing demand for gluten free products (Brannan et al 2001).

There has been a high interest for polyphenols due to their potential health benefits. Polyphenols protect plants against pests and diseases, and have antioxidant activity. Sorghum has many polyphenols including phenolic acids and flavonoids such as anthocyanins and tannins. Many health benefits of consuming sorghum polyphenols such as slow digestibility, reduction in cardiovascular disease, antioxidant activity, anti-inflammatory and anti-carcinogenic properties have been reported (Awika and Rooney 2004; Awika et al 2009; Burdette et al 2010).

All sorghums contain phenolic compounds. The amount present is affected by genetic variability and environment. Sorghum color and overall appearance is affected by pericarp color and thickness, presence of pigmented testa and endosperm color (Dykes et al 2005). Sorghum will have tannins if there is presence of a pigmented testa. Sorghums have been classified as type I, (not significant levels of tannins when extracted with 1% acidified methanol), type II (tannins that are extractable in 1% acidified methanol but are not extracted in methanol) and type III (tannins that are extractable in acidified methanol and methanol) (Hahn and Rooney 1986; Price et al 1978).

Black and brown sorghum grains and brans have a greater amount of phenols (5.5-63.8 mg/g GAE) than other cereals (0.2-3.3 mg/g). They also have higher antioxidant capacity (52-768 μmol TE/g product, ABTS assay) compared to other cereals (<0.1-34 μmol TE/g product, ABTS assay) (Awika et al 2004; Awika 2003). Furthermore, the antioxidant activity values of black and brown sorghum brans were higher than in fruits and vegetables (Awika and Rooney 2004). These high levels of antioxidants in sorghum bran demonstrate its potential as source of natural antioxidants. Sorghum bran is also rich in dietary fiber. Thus, the use of sorghum bran has potential health benefits and may be used as food ingredient in products such as bread, cookies and extrudates (Awika et al 2005).

Sorghum phenolic acids

Phenolic acids of sorghum exist as benzoic or cinnamic acid derivatives (Fig. 1). They are mostly concentrated in the bran. Most of them are in the bound form (esterified

to cell wall polymers), with ferulic acid being the most abundant bound phenolic acid in sorghum (Hahn et al 1983) and other cereals (Adom and Liu 2002).

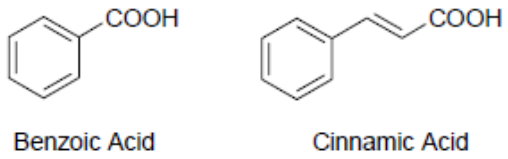


Fig. 1. Basic structure of phenolic acids

Other phenolic acids have been identified in sorghum including caffeic, syringic, protocatechuic, p-coumaric and sinapic as the more abundant (Hahn 1984). They all show good antioxidant activity *in vitro* and thus may contribute to the health benefits associated with whole grain consumption.

Phenolic acids are more absorbed than other phenols from food due to their small molecular sizes (Scalbert et al 2002; Lafay and Gil-Izquierdo 2008). In sorghum and other cereals, most phenolic acids are esterified to cell wall components which make them unavailable for absorption. Human colonic esterases are capable of releasing esterified phenolic acids from cereal brans increasing their bioavailability (Kroon et al 1997; Andreasen et al 2001).

Sorghum anthocyanins and other flavonoids

Anthocyanins have antioxidant properties and potential as natural food colorant. The anthocyanin in sorghum is the unique 3-deoxyanthocyanidins (Fig. 2) (Awika et al 2004). The common sorghum 3-deoxyanthocyanidins are apigeninidin and luteolinidin.

They are more stable in acidic solutions relative to the anthocyanidins found in fruits, vegetables and other cereals. Black sorghum bran has the highest level of anthocyanins (4.0-9.8 mg/g) among sorghum varieties, and fruits/vegetables (Awika et al 2004).

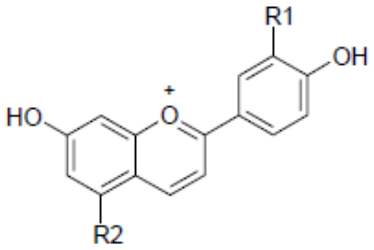


Fig. 2. Chemical structure of 3-deoxyanthocyanidins

Anthocyanins have low bioavailability (1.7-3.3%) (Bornsek et al 2012), however, comparable with some phenolic acids. Moreover, they may help preventing obesity. McDougall and Stewart (2005) reported that anthocyanins from berries inhibited alpha glucosidase activity and might reduce blood glucose levels.

Other flavonoids isolated and identified in sorghum grains include the flavones apigenin and luteolin; the flavanones eriodictyol, eriodictyol 5-glucoside and naringenin; the flavonol kaempferol 3-rutinoside-7-glucuronide; and the proanthocyanidins monomers (catechins), oligomers and polymers (large molecular weigh tannins) (Dykes and Rooney 2006).

Sorghum tannins

Tannins are high molecular weight polyphenols that have the ability to complex with carbohydrates and proteins. They are classified as hydrolysable or condensed tannins. Hydrolyzable tannins are gallic acid esters of glucose. Condensed tannins

(Fig. 3), which are also called proanthocyanidins, consist of polymers of flavan-3-ol. Molecular weights from 800-6000 were reported for condensed tannins isolated from many plant tissues (Hagerman and Butler 1981). Sources of tannins are berries, cocoa, wine, tea, nuts, sorghum, barley, finger millet (Serrano et al 2009).

There have been no tannic acid or hydrolysable tannins detected in sorghum (Awika and Rooney 2004). Catechin is the most commonly reported monomer present in sorghum. High molecular weight tannins are the most abundant forms of the tannins in sorghum (Hagerman and Butler 1980; Dykes et al 2005). Among cereals, sorghum has the highest levels of tannins (Serna-Saldivar and Rooney 1995). Tannin sorghum is widely consumed in some African countries because of prolonged satiety and unique color of the products (Awika and Rooney 2004).

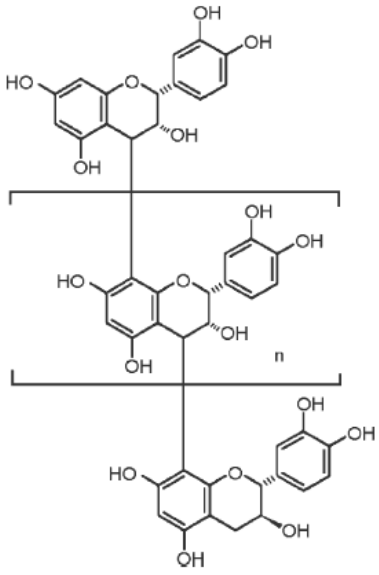


Fig. 3. Chemical structure of condensed tannins (proanthocyanidins) found in sorghum

Tannins are present in sorghum with a pigmented testa (classified as type II and III sorghums). These sorghums have B1_B2_ genes. For a pigmented testa, both genes must be dominant. When the S gene (spreader gene) is dominant along with the dominant B1 and B2 genes, pericarp color becomes brown and they contain tannins that are more easily extractable (Type III sorghum-extracted in methanol and 1% HCL methanol) than the ones with the recessive s gene (Type II sorghum- only extracted in 1% HCL methanol) (Hahn and Rooney 1986).

Tannins from sorghum have more powerful antioxidant activity *in vitro* than simple phenols. High molecular weight tannins have the greatest antioxidant activity in vitro among natural antioxidants (Hagerman et al 1998). Tannins retain at least 50% of their antioxidant activity even when complexed with proteins (Riedl and Hagerman 2001). In regards to bioavailability, Deprez et al (2001) reported that procyanidins up to trimers could be absorbed by the intestinal cell monolayer.

High molecular weight condensed tannins from persimmon were more effective antioxidants than grape seed proanthocyanidin (mostly oligomers) *in vitro*, *ex vivo* and *in vivo* (Gu et al 2008; Tian et al 2012). Large molecular weight tannins are not absorbed and then can bind compounds and act as antioxidant in the gastrointestinal tract, reducing oxidative stress, or go to the colon and be fermented, releasing compounds that can be absorbed and have potential health benefits (Saura-Calixto et al 2010; Tian et al 2012).

There are many studies reporting that tannins have anti-carcinogenic and anti-inflammatory effects (Huang et al 2010). Awika et al (2009) demonstrated that phenolic

extracts from tannin sorghum were more potent inhibitors of esophageal and colon cancer cell proliferation compared to other phenolic extracts from different sorghum varieties. Burdette et al (2010) reported that tannin sorghum phenolic extracts presented significant anti-inflammatory activity. Hargrove et al (2011) showed that sorghum condensed tannins inhibited α-amylase and aromatase (target in breast cancer chemotherapy) more efficiently than polyphenols in black sorghum. These positive effects of condensed tannins are important because of the potential to decrease starch digestibility, and to reduce incidence of cancer.

The vanillin-HCL assay is widely used to quantify tannins in sorghum (Price et al 1978). However, HPLC is a better tool for more accurate and reliable measurements of tannins. Normal phase HPLC with fluorescence detection separates and quantifies condensed tannins according to the degree of polymerization. Gu et al (2002) have developed a widely used method. They effectively separated monomers to decamers and the polymers (degree of polymerization, DP, above 10) were resolved as a distinct peak. However, this method uses dichloromethane (chlorinated solvent), which raises concern with respect to laboratory exposure and environmental protection (Kelm et al 2006). Recently, Langer et al (2011) slightly modified the HPLC method developed by Kelm et al (2006) to separate and quantify dark chocolate tannins. They used non-toxic, environmentally favorable solvents and obtained an excellent separation of tannins (up to decamers) and a peak that represents the polymers (DP > 10).

Tannins in sorghum are mostly polymeric (DP ≥ 11). The level of polymeric proanthocyanidins in high-tannin sorghum (over 80%) is higher than in many foods such

as cocoa and blueberry (Awika et al 2003a; Prior and Gu 2005). Processing of tannin sorghum decreases the amount of tannins and their relative ratios of the different molecular weights (degree of polymerization). Awika et al (2003a) used tannin sorghum bran to make cookies and bread and they found a reduction in procyanidin levels. This effect was more pronounced in the case of higher molecular weight tannins. Extrusion of tannin sorghum grain increased procyanidin oligomers (DP ≤ 4) and decreased polymers, possibly due to break down of the high molecular weight polymers into lower weight compounds.

Lower starch and protein digestibility in sorghum

Sorghum has the lowest starch digestibility among cereals (Rooney and Pflugfelder 1986). Even after cooking, Zhang and Hamaker (1998) demonstrated that sorghum flour had lower starch digestibility compared to corn. In addition, sorghum has unique low protein digestibility, especially upon cooking (Duodu et al 2003). Cooked sorghum flour had lower protein digestibility compared to uncooked while there was no difference in cooked and uncooked maize (Hamaker et al 1986).

Low raw starch digestibility in sorghum is because of the interaction between starch and protein. Starch granules are embedded in a protein matrix which forms a physical barrier and prevents access of amylases to the starch (Rooney and Pflugfelder 1986). Upon cooking, the solubility of kafirin (sorghum protein) decreases due to disulphide crosslinking which causes the low sorghum protein digestibility by proteolytic enzymes (Duodu et al 2003). Moreover, sorghum protein plays a large role in starch digestibility upon cooking. Endosperm proteins restrict starch granule from fully

gelatinizing and there may be a protein-gelatinized starch interaction. These interactions reduce susceptibility of starch to enzyme hydrolysis (Zhang and Hamaker 1998; Rooney and Pflugfelder 1986).

Besides starch-protein interactions, other food components such as dietary fiber and polyphenols reduce sorghum protein and starch digestibility. High content of dietary fiber in sorghum flour was responsible for a lower starch digestibility (Bach Knudsen et al 1988). Phytic acid and tannins present in sorghum inhibit digestive enzymes and form complexes with proteins, carbohydrates and minerals. Thus, when present in sorghum, they lower nutrient digestion (Schons et al 2011; Duodu et al 2003).

Sorghum tannin is capable of binding and precipitating protein (Butler et al 1984). The interaction between them is governed by hydrogen bonding and hydrophobic interactions. Proteins that bind strongly to tannins are large, have loose, open structure and are rich in proline. Hagerman and Butler (1981) showed that proteins with molecular weight less than 20,000 have low affinities for tannins with exception of proline-rich protein. Furthermore, Hagerman et al (1992) reported that hydrolysable tannins do not precipitate proteins because they are easily degraded in the gut to small phenolics, thus, condensed tannin, for being more resistant to degradation, are the main responsible for interacting with proteins. It has been reported that the large the molecular weight of condensed tannin, the stronger the interaction with protein (Huang et al 2011).

Poor animal performance due to interactions between tannins with proteins and carbohydrates (such as fiber) has been widely reported (Serna-Saldivar and Rooney 1995; Rooney and Pflugfelder 1986). However, a recent study by Waghorn (2008)

showed that there may be an improvement in animal performance by consuming condensed tannins depending on the concentration of protein and fiber in the diet. When diet contains excess of proteins, condensed tannins may reduce protein degradation and then, performance is improved, however, when protein concentrations are low and fiber content is high, condensed tannins are always detrimental to animal performance.

Until recently, the poor nutrient digestibility of sorghum was seen as a negative aspect for animal feeding (Serna-Saldivar and Rooney 1995). Because tannins reduced feed value, breeding efforts eliminated tannins from sorghum. Processing technologies such as dry cooking, extrusion, malting and flaking increase nutrient digestibility (Duodu et al 2003). Another way to improve sorghum nutritional value is to use enzyme treatment. Schons et al (2011) showed that tannase reduced amount of tannins in sorghum in an in-vivo study. In regards to human health, the lower carbohydrate and protein availability of sorghum could be advantageous (Dicko et al 2006).

STARCH

Starch is the major dietary source of carbohydrates in the human diet and is the most abundant storage polysaccharide in plants. It occurs in plant tissues as granules. Chemically, starches are composed of amylose and amylopectin. The first one is essentially a linear polymer of glucose molecules linked with α-D-(1-4) linkages and the second is a highly branched molecule with α-D-(1-4) and α-D-(1-6) linkages (Parker and Ring 2001).

Starch properties

During cooking and presence of water, starch granules swell and lose their birefringence and crystallinity (gelatinization). Besides the loss of order, starch becomes more digestible and soluble after gelatinization. The viscosity of the starch solution keeps increasing as temperature increases (starch keeps swelling) up to a point where the granules disrupt and then viscosity decreases. Amylose and amylopectin are leached out into solution. Upon cooling, amylose molecules in solution will re-associate (retrogradation) and form a gel with consequent increase in viscosity.

Rapid Visco-Analyzer (RVA) is a cooking, stirring viscometer with controlled temperature and shear used for testing the viscous properties of starch. RVA is a useful research tool for studying starch cooking properties and also has potential as a product development aid (Almeida-Dominguez et al 1997; Ragaee and Abdel-Aal 2006).

RVA monitors viscosity change during cooking/cooling of starch. The major parameters measured by RVA are peak time (time at which peak viscosity occurs), peak viscosity (the maximum hot paste viscosity), holding strength (the trough at the minimum hot paste viscosity), final viscosity (viscosity at the end of the test after cooling), breakdown (difference between peak viscosity and holding strength) and setback (difference between final viscosity and holding strength) (Ragaee and Abdel-Aal 2006).

Peak time and peak viscosity are associated with degree of swelling during heating. Breakdown is associated to the ability of starches to withstand heating at high temperature and shear stress. Setback is related to retrogradation and reordering of starch

molecules. The low setback values indicate low rate of starch retrogradation and syneresis (Ragaee and Abdel-Aal 2006).

Starch molecular weight distribution

Size-exclusion chromatography (SEC) separates the polymeric molecules of different sizes. SEC columns have a porous structure of different sizes. The largest molecules in a mixture exit the column first and the smallest exit the column last. Amylopectin and amylose can be separated and characterized using SEC. Once separated, solubility and molecular weight distribution can be evaluated (Hoang et al 2008). This information can be used to learn more about the functionality of starch in food products. Moreover, it is possible to determine if there are interactions (cross-linking) between starch and other compounds based on an increase in amylose and amylopectin molecular weight.

Many studies on structure changes of starch have been done using SEC. Han et al (2003) studied thermal degradation of starch and showed that amylose had a higher degradation than amylopectin. Witt et al (2010) used SEC to monitor changes in starch molecular structure as it is subjected to *in vitro* digestibility. They measured the time evolution of the distributions of molecular weight and branch length of starch. Kaufman et al (2009) demonstrated that sorghum tannins can be efficiently analyzed by SEC. According to them, SEC allowed for an easy and rapid measurement of the tannin molecular weight distribution with results comparable to normal-phase HPLC.

Interactions of starch with other components

Tea catechins interact with starch, retarding retrogradation in rice starch (Wu et al 2009). Zhu et al (2008) showed that phenolic acids (gallic acid, ferulic acid and caffeic acid), flavonoids (quercetin, naringin and catechin) and tannins (proanthocyanidins from grape seed) changed functional properties of wheat starch. They suggested that functional groups could interact with amylose and amylopectin through hydrogen bonding. In another study, Wu et al (2011) reported an interaction between tea polyphenols and rice starch during gelatinization using RVA.

In regards to condensed tannins, Davis and Hoseney (1979) showed that sorghum condensed tannins are adsorbed and retained by uncooked starch and the fraction that is not retained acts as α-amylase inhibitor. In another study, Bourvellec et al (2005) showed that there is interaction of condensed tannins from fruits with uncooked starch and this affinity increased with the molecular weight of the tannins. They suggested that starch would be able to encapsulate procyanidins and to form inclusion complexes. Beta and Corke (2004) demonstrated that ferulic acid and catechin decreased starch final viscosity and setback viscosity suggesting that phenolic compounds interact with starch molecules during cooking.

Amylose presents a helical conformation. Hydroxyl groups of glucose residues are present on the outer surface of the helix while the internal cavity is a hydrophobic tube. It forms inclusion complexes with small hydrophobic molecules. Complexes with iodine, lipids and alcohol have been reported (Gelders et al 2005). There are many studies showing interactions between lipids and amylose can change properties and

functionality of starch and that there is an optimum concentration of fatty acids to form complexes with amylose (Tang and Copeland 2007). Furthermore, complexes between fatty acids and amylose can form rapidly under physiological conditions and contribute to the formation of RS (Crowe et al 2000). Liu et al (2011) demonstrated that catechins interacted with starch, specifically with amylose, lowering postprandial glycemic response. However, there is no study demonstrating interactions between amylose and polyphenols of different molecular weights and their effects on *in vitro* starch digestibility.

***In vitro* starch digestibility**

Starch can be classified according to its digestibility. It is classified as rapidly digestible starch (RDS) (glucose released after 20 min), slowly digestible starch (SDS) (glucose released between 20 and 120 min) and a portion that is resistant to digestion, resistant starch (RS) (Englyst et al 1992). RDS has a characteristic to provide rapid release of glucose into the blood. This glucose fluctuation leads to oxidative stress which may cause many chronic diseases (Monnier et al 2006). SDS provides slow and prolonged release of glucose which brings positive health effects in control of diabetes and cardiovascular diseases (Zhang and Hamaker 2009).

Resistant starch has been used in food products to deliver all health benefits of dietary fiber, mainly to prevent obesity and diabetes (Morrell et al 2004). Resistant starch functions as a prebiotic. It can be fermented (colonic bacteria) in the large intestine to short-chain fatty acids such as acetate, butyrate and propionate (Fassler et al

2007; Topping et al 2008). Butyrate plays an important role in suppressing tumor cells and decreasing the proliferation of colonic mucosal cells (Champ et al 2003).

There are 4 types of RS: RS1 (physically inaccessible trapped starch found in seeds, legumes and unprocessed cereals grains), RS2 (natural raw and ungelatinized starch granules resistant to digestion), RS3 (retrograded starch formed when starch present in food is heated and cooled), and RS4 (chemically modified starches) (Englyst et al 1992). Chemically modified starches are commonly used in food applications as they offer improved functional properties than native starches and exhibit greater resistance towards alpha amylase digestion (Han and BeMiller 2007). However, chemical modification is not desirable to many consumers. Thus, starch modification by sorghum phenolic extracts would be a natural and healthy way to increase resistant starch and antioxidant levels of foods.

ACCELERATED SOLVENT EXTRACTION (ASE): AN EFFICIENT AND ENVIRONMENTAL FRIENDLY EXTRACTION METHOD

Conventional methods for extraction of many compounds are usually based on organic solvents such as methanol, acetone and chloroform. In general, conventional extraction methods are performed at ambient temperature or at the boiling point of the solvent and are time consuming. Recently, alternative methods have gained increasing interest to allow for more environmentally sustainable and faster extraction (Sun et al 2012).

Accelerated Solvent Extraction (ASE) has become a popular green extraction technology and frequently used in extraction of antimicrobials, pesticides and bioactive/

nutritional compounds (Sun et al 2012). This type of extraction utilizes higher temperatures and pressures during the extraction process. Elevated pressures (>1000 psi) allow for solvents to be heated at temperatures higher than their normal boiling point, resulting in fast, efficient extractions (Richter et al 1996).

The main parameters affecting ASE efficiency are temperature and pressure. The use of high temperatures during the extraction process affects solvent properties such as dielectric constant and viscosity. It increases diffusion rates and the capacity to solubilize analytes. Moreover, it can disrupt the strong solute-matrix interactions and decrease liquid solvent viscosity allowing better penetration of matrix particles and enhanced extraction. Use of high pressure is mainly to keep solvents in a liquid state when temperatures are above the boiling point. It also improves the extraction efficiency by forcing the solvent into areas that would not normally be contacted using normal atmospheric conditions (Richter et al 1996).

Water is capable of extracting different classes of compounds depending on temperature using ASE (Soto- Ayala and Luque de Castro 2001). When water is in liquid state under pressure above 100^{o}C, but below its critical temperature of 374^{o}C it is referred to as superheated or subcritical water. The use of ethanol and water enable environmentally sustainable extraction process which agrees with the Twelve Principles of Green Chemistry (Co et al 2012). High levels of antioxidants from Spruce Bark were obtained when water and ethanol were used in an extraction system under high temperature and pressure (Co et al 2012). They concluded that the method was efficient and environmentally sustainable.

Other bioactive compounds such as anthocyanins and tannins have been extracted from fruits using ASE. Monrad et al (2010a) extracted anthocyanins from dried red grape pomace using ASE at different temperatures. The solvents used were mixtures of ethanol and water. They concluded that ethanol/water (50:50) and ethanol/water (70:30) extracted similar amounts of anthocyanins as the conventional method using methanol at room temperature and atmospheric pressure.

Monrad et al (2010b) extracted tannins from red grape pomace using ASE. Ethanol/water solvents (0, 10, 30, 50, 70 and 90%) were compared to conventional method of extraction (acetone/water/acetic acid 70:29.5:0.5). Overall, 50% ethanol/water extracted more total procyanidins than other ethanol/water compositions and more than the conventional method. Furthermore, it was more effective in extracting procyanidin monomers and oligomers than conventional method, but less effective to extract the high molecular weight ones.

This indicates that it is possible to rapidly extract anthocyanins, tannins and other phenolics using ASE, thus avoiding the need for expensive and toxic organic solvents. Moreover, these extracts can be used to modify starch, increasing resistant starch content and antioxidant potential. This would promote the expansion of starches with natural dark color, high in RS and antioxidants in the market.

CHAPTER III

EFFECT OF TANNINS AND OTHER SORGHUM POLYPHENOLS ON IN VITRO STARCH DIGESTIBILITY

MATERIALS AND METHODS

Sorghum samples

Three sorghum varieties grown in College Station, TX were chosen based on their different polyphenol concentration and profiles. High tannin sorghum (high in polymeric proanthocyanidins), and two other varieties without tannins: a white food-type sorghum (low in polyphenols) and black sorghum (TX430 black- high in monomeric polyphenols) were used. Sorghum brans were obtained by decorticating 1 kg batches in a PRL mini-dehuller (Nutama Machine Co., Saskatoon, Canada) and were separated with a KICE grain cleaner (Model 6DT4-1, KICE Industries Inc., Wichita, KS). The brans (approximately 10% of original grain weight) were milled to pass through a 0.5 mm screen using a UDY cyclone mill (Model 3010-030, UDY Corporation, Fort Collins, CO). They were kept at –20 ^{o}C until used.

Reagents

Gallic acid, catechin hydrate, Folin-Ciocalteu reagent, ethanolamine, amylose from potato and amylopectin from corn were obtained from Sigma (St. Louis, MO). All solvents were HPLC or analytical grade. Normal maize (amylose content = 23.9% ± 1.3), waxy (amylose content = 0.36% ± 0.04) and high amylose (amylose content=

66.5% ± 2.5) starches were obtained from National Starch Food Innovation (Bridgewater, NJ). Isoamylase (Cat. No. E-ISAMY, 1,000 units), total starch assay kit (Cat. No. K-TSTA) and resistant starch assay kit (Cat. No. K-RSTAR) were purchased from Megazyme International Ireland Ltd. (Bray Business Park, Bray, Co. Wicklow, Ireland).

Preparation of phenolic extracts from sorghum brans

Phenolic extracts from white, black and high tannin sorghums were obtained by extraction of the ground bran (15 g) with 70% (v/v) aqueous acetone (900 mL) with stirring for 2 hours. Extracts were then centrifuged (3100 *g*) for 10 min, and the acetone was immediately removed from the supernatant under vacuum at 40 ^{o}C; the aqueous extracts were freeze-dried and stored at 4 °C until used.

Material characterization

Total phenol content was measured using the modified Folin-Ciocalteu method of Kaluza et al (1980). Total starch was determined using the total starch kit (AACC method 76-13) and amylose content was determined using the amylose/amylopectin ratio kit, both from Megazyme. Crude protein percentage (% nitrogen multiplied by 6.25) was determined base on a combustion method (Sweeney 1989). SDS-PAGE (Laemmli 1970) was used to identify different molecular weight proteins in the phenolic extracts.

An Agilent 1100/1200 HPLC system with diode array and fluorescence detectors (Agilent Technologies, Santa Clara, CA) was used to profile polyphenols. The method described by Awika et al (2009) was used with modifications to profile phenolic acids

and anthocyanins in the samples. A reversed phase 150 x 2.00 mm, 5 μm, C-18 column (Phenomenex, Torrance, CA) was used. HPLC conditions were; injection volume, 10.0 μL; flow rate, 1.0 mL/min. The mobile phase consisted of (A) 2% formic acid in water and (B) 2% formic acid in acetonitrile. The 43 min elution gradient for B was as follows: 0-3 min, 10% isocratic; 3-4 min, 10-12%; 4-5 min, 12% isocratic; 5-8 min, 12-18%; 8-10 min, 18% isocratic; 10-12 min, 18-19%; 12-14 min, 19% isocratic; 14-18 min, 19-21%; 18-22 min, 21-26%; 22-28 min, 26-28%; 28-32 min, 28-40%; 32-34 min, 40-60%; 34-36 min, 60% isocratic; 36-38 min, 60-10%; 38-43 min, 10% isocratic.

A normal phase HPLC method described by Langer et al (2011) was used to separate proanthocyanidins based on degree of polymerization (DP) in the tannin sorghum phenolic extract. The column was a Develosil Diol (250 mm x 4.6 mm, 5 μm particle size; Phenomenex, U.K.). The mobile phase was a binary gradient with a flow rate of 0.6 mL/min and consisted of (A) acidic acetonitrile (Acetonitrile/acetic acid, 98:2; v/v) and (B) acidic aqueous methanol (Methanol/water/acetic acid, 95:3:2; v/v/v). The 83 min elution gradient for B was as follows: 0-3 min, 7% isocratic; 3-57 min, 7-37.6%; 57-60 min, 37.6-100%; 60-67 min, 100% isocratic; 67-73 min, 100-7%; 73-83 min, 7% isocratic.

Starch pasting properties

A Rapid Visco Analyzer (RVA) was used in order to investigate effects of sorghum phenolic extracts on starch pasting properties. Distilled water was added to normal corn starch (3.0 g, dry basis) and freeze-dried phenolic extracts at 4 levels (0%, 5%, 10% and 20% starch basis) in the RVA canister to obtain a total sample weight of

28 g. The slurry was manually homogenized to prevent lump formation and the pH was recorded with a portable pH meter model Russel RL060P (Thermo Scientific, Beverly, MA) before the RVA run. Pasting properties of corn starch and mixtures with freeze-dried sorghum phenolic extracts were determined using a Rapid Visco-Analyzer (RVA Model 4, Newport Scientific PTY Ltd, Warriewood, Australia).

The temperature profile used was the RVA Standard 2 provided by the instrument manufacturer. There was a sample equilibration at 50^{o}C for 1 min followed by a linear temperature increase from 50 – 95^{o}C in 7.5 min, then a holding step at 95^{o}C for 5 min, cooling to 50^{o}C within 7.5 min and another holding step at 50^{o}C for 2 min giving a total of 23 min. The viscosities were reported in Rapid Visco Units (RVU). Peak time (min), peak viscosity (RVU), final viscosity (RVU), breakdown (RVU) and setback (RVU) were determined using the Thermocline software version 2.2 (Newport Scientific PTY Ltd, Warriewood, Australia). Pastes obtained from the RVA were immediately frozen in liquid nitrogen, kept at -50^{o}C and then freeze-dried. The freeze-dried material was stored at 4^{o}C. Control (freeze-dried sorghum phenolic extracts in water without starch) was included to determine heat sensitivity of the sorghum phenolic extracts to RVA cooking.

Interactions of sorghum polyphenols with amylose/amylopectin

In order to demonstrate interactions between starch molecules and sorghum polyphenols, changes in phenol content and concentration of different molecular weight proanthocyanidins before/after cooking were evaluated. Pure amylose, pure amylopectin, waxy, normal and high amylose starches (10% w/v in distilled water) were mixed with

freeze-dried sorghum phenolic extracts (10% starch basis) in a shaker for 1 h. Samples were frozen in liquid nitrogen and freeze-dried. The freeze-dried material was analyzed for total phenol content (phenol concentration in the supernatant before cooking). In addition, the freeze-dried material containing tannin phenolic extracts (0.2 g) was mixed with methanol (10 mL) and the supernatant was filtered through a 0.45 μm membrane and injected (20 μL) in the HPLC to determine concentration of proanthocyanidins and their molecular weight profile (DP).

Total phenols and proanthocyanidins concentrations in the supernatant after cooking were also determined. Waxy and normal starches (10% w/v in distilled water) were mixed with freeze-dried sorghum phenolic extracts (10% starch basis) in a shaker for 1 h. Then, samples were cooked at 95^{o}C/20min to gelatinize starches, frozen and freeze dried. Same procedure was followed using high amylose starch (10% w/v in distilled water) mixed with sorghum phenolic extracts (10% starch basis). In order to gelatinize high amylose starch, mixture was cooked in an autoclave (121^{o}C/30min). Control (freeze-dried sorghum phenolic extracts in water, 3 mg/mL, without starch) was included for both cooking treatments. Total phenols assay and HPLC analysis for tannins were done on the freeze-dried material as described above (before cooking). For control, 5μL was injected in the HPLC.

In vitro starch digestibility

Rapidly digestible starch (RDS) and slowly digestible starch (SDS) of freeze-dried samples from RVA was measured based on the Englyst method (Englyst et al 1992) with modifications. Specifically, 300 U/mL α-amylase and 95 U/mL

amyloglucosidase were dissolved into 10 mL of sodium acetate (NaOAC) buffer (0.1 M, 4mM $CaCl_2$, pH 5.2, made with benzoic acid saturated distilled water) to hydrolyze 200 mg of sample in a shaking water bath at 37^{o}C with a shaking speed of 170 rpm. Samples (0.5 mL) were taken at different time intervals (20 min and 120 min), and the reaction was stopped with 5 mL of absolute ethanol. After centrifugation (8 000 rpm, 5 min), an aliquot of supernatant was mixed with GOPOD (glucose oxidase and peroxidase) and glucose concentration was determined by measuring absorbance of the mixture in a spectrophotometer at 510 nm. Percentage of hydrolyzed starch was calculated by multiplying a factor of 0.9 (162/180- factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch) to the glucose content. Rapidly digestible (20 min) and slowly digestible starches (between 20-120 min) were measured.

Resistant starch (RS) content of freeze-dried samples from RVA was directly measured using the resistant starch assay kit from Megazyme (AACC method 32-40). Briefly, samples (100 mg) were incubated in a shaking water bath with 4 mL mixture of pancreatic alpha amylase (10 mg/mL) and amyloglucosidase (3.3 U/mL) for 16 h at 37^{o}C to hydrolyze the non-RS fraction to glucose. The reaction was terminated with 4mL of absolute ethanol and undigested starch was recovered by centrifugation (9000 rpm, 10 min). The residue was washed with 50% ethanol twice and then dissolved with 2 mL of KOH by stirring in an ice water bath. The solution was neutralized with acetate buffer (pH 3.8) and starch was hydrolyzed to glucose with amyloglucosidase. Glucose released (measure of RS content) was determined spectrophotometrically at 510 nm with glucose oxidase-peroxidase reagent (GOPOD).

In order to better understand the effects of the polyphenols-starch molecules interactions on *in vitro* starch digestibility, normal, waxy and high-amylose starches (25% w/v in distilled water) were cooked with sorghum phenolic extracts (10% starch basis) in an autoclave at 121 ^{o}C for 30 min, cooled at room temperature and then stored at 4 ^{o}C overnight. This was repeated 2 more times (3 heating/cooling cycles) and the samples were freeze-dried and RS content determined. Compared to RVA, this method produces more RS because of the drastic heating conditions (more amylose and amylopectin in solution) and cooling at 4^{o}C, optimum temperature for starch (mainly amylose), retrogradation.

Furthermore, normal starch was pre-treated with isoamylase and the hydrolyzed material was subjected to 3 heating/cooling cycles as described above. Debranching of amylopectin by the action of isoamylase will produce more linear molecules and will help to understand possible interactions between linear molecules and sorghum tannins and their effects on RS formation. The pH of a slurry of normal starch at the concentration of 5% (w/v) in distilled water was adjusted to 4.5, instantly heated to 70 ^{o}C for gelatinization, and quickly reduced to 45 ^{o}C within 1 min. Isoamylase at 1% (based on starch weight) was added and hydrolysis took place for 24h. Then, the enzyme was deactivated (boiling temperature) and sorghum phenolic extracts added (10 % starch basis). Samples were subjected to 3 heating/cooling cycles, as described above, in an autoclave (121^{o}C/30 min) and then freeze-dried. RS content was determined in the freeze-dried material.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) to determine significant differences. Fisher's least significant difference (LSD) ($P \leq 0.05$) was used to compare multiple means. The software used was SPSS v 16.0 for windows (SPSS Inc., Chicago, IL). All tests were done in three replications.

RESULTS AND DISCUSSION

Properties of sorghum phenolic extracts

White, black and high-tannin freeze dried phenolic extracts had yields of 4, 12 and 11%, respectively, based on bran weight. Phenol content (mg GAE/g) of the sorghum phenolic extracts were 438 ± 25.4 (tannin), 366 ± 16.1 (black) and 48.1 ± 3.5 (white).

Starch was not detected in the sorghum freeze-dried phenolic extracts. About 50 mg/g crude protein was detected in the freeze-dried phenolic extracts. Since acetone was used in the phenolic extractions, these proteins were not expected to interact with tannins. Acetone inhibits formation of tannin-protein complexes (Hagerman and Robbins 1987) by precipitating high molecular weight proteins which are the ones that have high affinities for tannins (Hagerman and Butler 1981). Proteins with molecular weight less than 20,000 have low affinities for tannins (Hagerman and Butler 1981). SDS-PAGE showed that there were only small molecular weight (below 10,000) proteins present in the phenolic extracts (data not shown).

Phenolic acids such as caffeic and ferulic acid were previously identified in sorghum (Hahn et al 1983). The major 3-deoxyanthocyanins (Luteolinidin,

Apigeninidin, 5-Methoxyluteolinidin and 7-Methoxyapigeninidin) were not detected in white and tannin sorghum phenolic extracts, but were the major polyphenols in black phenolic extracts (Appendix, Fig. A1), which agrees with previous findings (Dykes et al 2009; Awika et al 2009). The tannin phenolic extract contained mostly proanthocyanidins (129 mg/g), with a high ratio (77%) of polymeric (DP >10) proanthocyanidins (Table I) as previously reported (Awika et al 2003a).

Effect of sorghum phenolic extracts on starch pasting properties

Black and tannin sorghum phenolic extracts significantly ($P \leq 0.05$) affected normal starch pasting properties and the effect was dependent on phenolic extract concentrations (Table II). Peak viscosity was higher ($P \leq 0.05$) for black and tannin treatments (376.3 - 393.4 RVU) at all levels compared to control (353.5 RVU) (Table II). Viscosity values increased as phenolic extract concentration increased. The same trend was observed for peak time which ranged from 8.3-8.8 min compared to 8.1 min for the control. Above 10%, black phenolic extract had slightly higher peak time than other treatments. There were no significant ($P > 0.05$) differences in pH among treatments (Table II).

As the concentration of phenolic extracts increased, the starch-phenolic extract mixture had higher solids content which may have affected the RVA parameters mentioned before. However, white sorghum phenolic extracts mixed at all levels with starch did not differ ($P > 0.05$) from control in peak viscosity and peak time (Table II) implying that solid content was not the major contributor.

Table I. Proanthocyanidin content[a] of tannin sorghum phenolic extract (TSPE): before and after cooking at 95°C/20 min and at 121°C/30 min [b]

DP[c]	TSPE	TSPE after cooking (95°C/20 min)	TSPE after cooking (121°C/30 min)
1	0.25 ± 0.01	0.78 ± 0.06	3.37 ± 0.18
2	0.58 ± 0.03	1.74 ± 0.09	3.43 ± 0.29
3	1.43 ± 0.10	1.88 ± 0.11	nd
4	2.21 ± 0.09	3.67 ± 0.26	nd
5	2.64 ± 0.11	3.96 ± 0.11	nd
6	4.67 ± 0.08	5.57 ± 0.47	nd
7	4.74 ± 0.35	5.16 ± 0.33	nd
8	5.13 ± 0.24	4.73 ± 0.37	nd
9	4.62 ± 0.32	4.18 ± 0.39	nd
10	3.96 ± 0.18	nd[e]	nd
P[d]	98.90 ± 4.30	91.10 ± 6.80	35.62 ± 1.60
Total	129.10 ± 5.81	122.80 ± 9.0	42.40 ± 2.07

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10; [e] Not detected.

Table II. Effect of sorghum phenolic extracts on normal starch pasting properties [w]

			Pasting properties				
Treatments	Phenol content [y]	pH	Peak viscosity (RVU)	Peak time (min)	Final Viscosity (RVU)	Breakdown (RVU)	Setback (RVU)
Control (corn starch)	-	5.6 [a]	353.5 [ab]	8.1 [ab]	347.5 [ab]	168.0 [a]	162.0 [bc]
White (5%)	0.87 [a]	5.7 [a]	338.4 [a]	8.1 [ab]	334.0 [a]	160.2 [a]	155.8 [b]
White (10%)	2.5 [bc]	5.5 [a]	337.2 [a]	8.0 [a]	348.0 [ab]	168.4 [a]	179.2 [d]
White (20%)	4.55 [d]	5.6 [a]	346.9 [ab]	8.2 [ab]	429.0 [d]	157.3 [a]	239.4 [e]
Black (5%)	2.61 [c]	5.6 [a]	376.7 [bc]	8.5 [c]	368.2 [b]	175.3 [a]	166.9 [b]
Black (10%)	5.82 [e]	5.5 [a]	378.5 [c]	8.7 [d]	382.4 [c]	169.7 [a]	173.6 [cd]
Black (20%)	14.2 [g]	5.5 [a]	392.3 [d]	8.8 [d]	424.8 [d]	158.2 [a]	190.6 [d]
High tannin (5%)	2.1 [b]	5.5 [a]	376.3 [bc]	8.3 [bc]	347.7 [ab]	180.2 [a]	151.6 [ab]
High tannin (10%)	4.7 [d]	5.6 [a]	379.4 [c]	8.3 [bc]	345.4 [ab]	178.3 [a]	144.2 [ab]
High tannin (20%)	8.9 [f]	5.5 [a]	393.4 [d]	8.6 [c]	369.0 [b]	160.8 [a]	136.4 [a]

[w] Means followed by the same letter in a column are not significantly different ($P \leq 0.05$)

[y] Measured after cooking (mg GAE/g)

RVU- Rapid Visco Units

As previously mentioned, black and tannin phenolic extracts had the highest concentration of phenols (about 10 times more than white phenolic extract). Thus, changes observed in starch pasting properties when black and tannin phenolic extracts were mixed with starch and cooked could be due to the presence of more phenols in solution which compete for water with starch for hydration (Zhu et al 2008) or possible interactions of black and tannin sorghum polyphenols with starch.

During cooling, final viscosity increased as black and white sorghum phenolic extract concentrations increased (Table II). The same trend was not observed for tannin phenolic extracts which had similar final viscosity to control. Setback increased as white and black phenolic extract concentrations increased; however, it tended to decrease as concentration of tannin phenolic extract increased (Table II). This suggests some interaction of tannins with leached amylose, which may help retard starch retrogradation. The evidence suggests that low molecular weight polyphenols (in white and black sorghum) and the proanthocyanidins (in tannin sorghum) interact with starch via different mechanisms.

There are a few reports on the effect of polyphenols on starch properties. Tea catechins were shown to interact with rice starch and retard its retrogradation (Wu et al 2009). Zhu et al (2008) demonstrated that a diverse set of phenolic compounds changed wheat starch functional properties; they suggested that the changes were due to possible alteration of solution pH by the polyphenols as well as hydrogen bonding. In this study, the sorghum phenolic extracts did not affect solution pH, thus the observed differences are mostly attributed to their phenolic composition.

Interactions between sorghum polyphenols and amylose/amylopectin

Changes in phenol content of starch-phenolic extract mixtures

Phenolic content of freeze-dried extracts before RVA cooking, as previously mentioned, was higher for tannin phenolic extracts (438 mg GAE/g) than black (366 mg GAE/g) and white (48.1 mg GAE/g). However, after RVA cooking, treatments with black phenolic extract had significantly ($P \leq 0.05$) higher extractable phenol content at all levels (Table II). The evidence indicates that sorghum proanthocyanidins may be interacting with starch molecules (forming insoluble complexes) to a greater extent than the simple phenolics in the black sorghum phenolic extracts. This may partly explain the observed differences in RVA pasting properties of the tannin sorghum phenolic extract compared to the black sorghum phenolic extract. There were no significant ($P > 0.05$) differences in the phenol content of the control (freeze-dried phenolic extracts cooked without starch) before and after RVA cooking (data not shown).

In order to completely gelatinize starch and investigate specific interactions of amylose and amylopectin with sorghum polyphenols, mixtures of waxy and normal starch with phenolic extracts were cooked at 95^{o}C/20 min, and the mixture with high amylose starch was cooked at 121^{o}C/30 min. Before cooking, adsorption of sorghum polyphenols to raw starch (the difference between added and extractable polyphenols) was significantly higher for the tannin sorghum phenolic extract (20.5 – 36.4%) than the black sorghum phenolic extract (10.8 – 16.2%) or the white sorghum phenolic extract (4.1 – 10.4%) (Table III).

Table III. Total phenol (extractable) content (mg GAE/g) before and after cooking of sorghum phenolic extracts (10% starch basis) mixed with waxy, normal and high amylose starches

	Phenol content (mg GAE/g) of sorghum phenolic extracts					
	White		Black		High tannin	
	Before cooking	After cooking	Before cooking	After cooking	Before cooking	After cooking
Controls						
Control 1 (95°C/20 min)	48.0 [a]	48.1 [a]	369.0 [b]	364.9 [b]	442.3 [c]	423.1 [c]
Control 2 (121°C/30 min)	48.0 [a]	47.4 [a]	369.0 [c]	351.8 [b]	442.3 [e]	381.9 [d]
Cooking 1 (95°C/20 min)						
Waxy starch	4.6 [b]	3.4 [a]	32.8 [d]	8.9 [c]	27.7 [d]	4.1 [ab]
Normal starch	4.3 [b]	2.9 [a]	30.7 [d]	9.4 [c]	35.0 [d]	4.2 [b]
Cooking 2 (121°C/30 min)						
High amylose starch	4.6 [b]	3.1 [a]	31.4 [d]	8.7 [c]	33.9 [d]	4.5 [b]

Means followed by the same letter in a row are not significantly different ($P \leq 0.05$).

Maize starch (and other cereal starches) contains large surface pores (up to 1 μm diameter) (Fannon et al 1992) which are likely sites for poyphenol adsorption into the intact granule. The larger tannin molecules are more likely to be physically trapped within the pores and thus become 'unextractable' compared to the smaller polyphenols. Additionally, hydrogen bonding is likely to increase the stability of the polyphenols within the starch granule.

Previous research has demonstrated that 40-60% condensed tannins are adsorbed on raw starches and this adsorption was dependent on the starch surface area with higher surface area having the highest condensed tannins adsorbed (Davis and Hoseney 1979). Bourvellec at al (2005) suggested that due to presence of pores containing amylose chains on raw starch granules, condensed tannins would not only be adsorbed on the starch surface but could interact with amylose forming inclusion complexes.

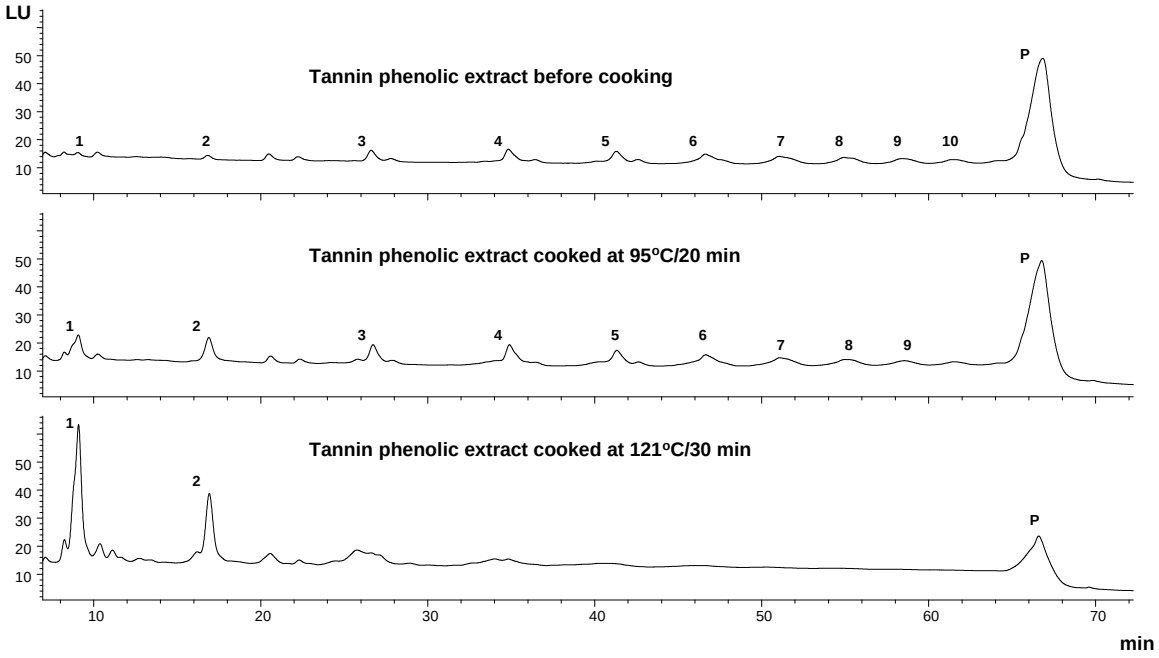
There was a large decrease in extractable phenols after cooking for all treatments (Table III). This difference was highest for tannin sorghum treatments which had a further average decrease in extractable phenols of 87% after cooking. The drop in extractable phenol content was around 70% for black sorghum treatments and 30% for white sorghum treatments (Table III). The result agrees with the RVA data, where lower extractable phenols were present in the tannin phenolic extract compared to black phenolic extract treatments after cooking. There was no significant ($P > 0.05$) difference in the phenol content of the control (freeze-dried phenolic extracts cooked without starch) before and after cooking at 95^{o}C/20 min (Table III). However, there was a slight

but significant ($P \leq 0.05$) decrease in the phenol content of control after cooking at 121°C/30 min (Table III).

The large changes in extractable phenols after cooking indicates that condensed tannins and the simple phenolic compounds in sorghum chemically interact with gelatinized amylose and amylopectin molecules. The increased swelling and opening of amylose and amylopectin chains likely enabled the polyphenols to bind to specific sites on the molecules via hydrogen bonds and, likely, hydrophobic interactions. The hydrophobic interactions are likely for 3-deoxyanthocyanins and sorghum proanthocyanidins which tend to be less polar than their analogs from fruits and vegetables. The chemical interactions were apparently strongest for the proanthocyanidin-containing phenolic extracts. The larger molecular weight proanthocyanidins provide more hydroxyl groups for hydrogen bonding, and also contain more hydrophobic domains that would promote stronger interactions with gelatinized starch.

Changes in molecular weight profile of proanthocyanidins cooked with starch

To better understand possible interactions of condensed tannins with starch molecules, the treatments with tannin sorghum phenolic extracts were profiled using normal phase HPLC. The controls (tannin phenolic extracts without starch) had significant ($P \leq 0.05$) increase in catechins (monomers) and dimers after cooking at 95°C/20 min, and even more so at 121°C/30 min (Table I; Fig. 4). The concentration of monomers and dimers after cooking at 95°C/20 min increased from 0.25 to 0.78 mg/g and from 0.58 to 1.74 mg CE/g respectively (Table I).



(control). Numbers on peaks denote degree of polymerization (DP) – polymers with DP >10.

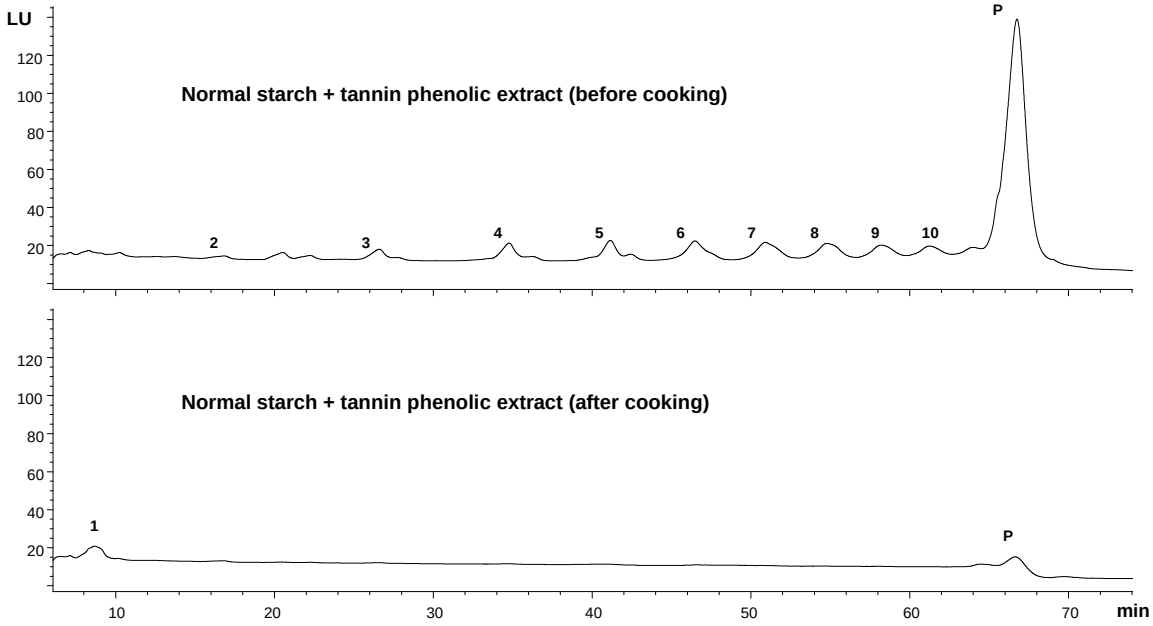
The concentration of monomers increased more than 10 times from 0.25 to 3.4 mg/g and dimers increased from 0.58 to 3.4 mg CE/g after cooking at 121^{o}C/30 min (Table I). In addition, the oligomeric proanthocyanidins up to DP 6 increased in the phenolic extract cooked at 95^{o}C/20 min. This indicates that even relatively mild heat treatment induces significant depolymerization of condensed tannins. The concentration of polymeric tannins decreased upon cooking at 95^{o}C/20 min (from 98.9 to 91.1 mg CE/g) and after autoclave cooking (from 98.9 to 35.6 mg CE/g) due to depolymerization, and thermal degradation (Table I; Fig. 4). Thermal-induced depolymerization of sorghum tannins after severe heat treatment was previously demonstrated (Awika et al 2003a). The heat induced depolymerization of tannins into monomers and dimers observed in this study may increase bioavailability of sorghum tannins (Deprez et al 2001).

After cooking (95^{o}C/20 min) normal starch with tannin phenolic extracts, there was a decrease in the oligomeric and polymeric tannins to mostly undetectable levels (Table IV; Fig. 5) which indicated that almost all of the sorghum condensed tannins (oligomers and polymers) interacted with amylose/amylopectin in solution. Moreover, the appearance of monomeric (catechin) peak was observed (Table IV; Fig. 5). The same trend was observed for treatment with waxy starch (cooked at 95^{o}C/20 min) and high amylose starch (cooked at 121^{o}C/30 min). However, autoclaved treatment produced much higher levels of monomeric catechin (200 µg/g compared to cooking at 95^{o}C/20 min, 50 µg/g). Thus it is likely that during thermal treatment, depolymerization of 'free' proanthocyanidins proceeds simultaneously with their chemical interactions with gelatinized starch to form insoluble complexes.

Table IV. Proanthocyanidin content[a] of tannin sorghum phenolic extract (TSPE) mixed with normal starch (NS) before and after cooking (95°C/20min); and after mixing with pure amylose and amylopectin [b]

DP[c]	TSPE + NS before cooking	TSPE + NS after cooking	TSPE + amylopectin	TSPE + amylose
1	nd[e]	0.05 ± 0.0	nd	nd
2	0.031 ± 0.0	nd	nd	nd
3	0.12 ± 0.01	nd	0.07 ± 0.01	0.045 ± 0.0
4	0.17 ± 0.02	nd	0.094 ± 0.01	0.069 ± 0.0
5	0.24 ± 0.08	nd	0.12 ± 0.0	0.076 ± 0.0
6	0.50 ± 0.03	nd	0.20 ± 0.01	0.12 ± 0.01
7	0.54 ± 0.05	nd	0.22 ± 0.0	0.12 ± 0.01
8	0.53 ± 0.02	nd	0.27 ± 0.02	nd
9	0.46 ± 0.04	nd	0.20 ± 0.01	nd
10	0.38 ± 0.04	nd	0.19 ± 0.01	nd
P[d]	10.60 ± 0.94	0.52 ± 0.03	3.63 ± 0.23	1.32 ± 0.10
Total	13.60 ± 1.20	0.57 ± 0.03	5.0 ± 0.30	1.75 ± 0.13

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10. [e] Not detected.



(95°C/20 min) of tannin sorghum phenolic extracts mixed with normal starch. Numbers on peaks denote degree of polymerization. P = polymers with DP >10.

The fact that only monomeric forms of proanthocyanidins were detectable in the cooked mixtures indicates that oligomers and polymers are most strongly involved in tannin-starch interactions. Thus, like proteins, starch may be interacting with the proanthocyanidins through hydrogen bonding (Hagerman and Butler 1981; Butler et al 1984) as well as hydrophobic interactions as previously mentioned.

Reaction of pure amylose and amylopectin with sorghum phenolic extracts

To further investigate the relative interactions of sorghum polyphenols with amylose and amylopectin, pure amylose and pure amylopectin were mixed with sorghum phenolic extracts at room temperature. There was no significant ($P > 0.05$) difference between extractable phenol content of white or black phenolic extracts mixed with amylopectin or amylose (Fig. 6). However, phenol concentration of tannin phenolic extracts mixed with amylose was significantly ($P \leq 0.05$) lower (9.0 mg GAE/g) than when mixed with amylopectin (16.1 mg GAE/g) (Fig. 6). In addition, compared to starting material, the decrease in extractable phenol content was more dramatic for the tannin sorghum phenolic extract treatments than the black (or white) phenolic extract (Fig. 6). Furthermore, the concentration of different molecular weight proanthocyanidins (oligomers and polymers) were significantly ($P \leq 0.05$) lower in the presence of amylose compared to amylopectin (Table IV; Fig. 7).

The fact that only the sample with proanthocyanidins interacted more strongly with amylose compared to amylopectin suggests that the linear nature of amylose and the structure of sorghum proanthocyanidin polymers afford a more optimum configuration for stronger bond formation between starch and polyphenols in solution.

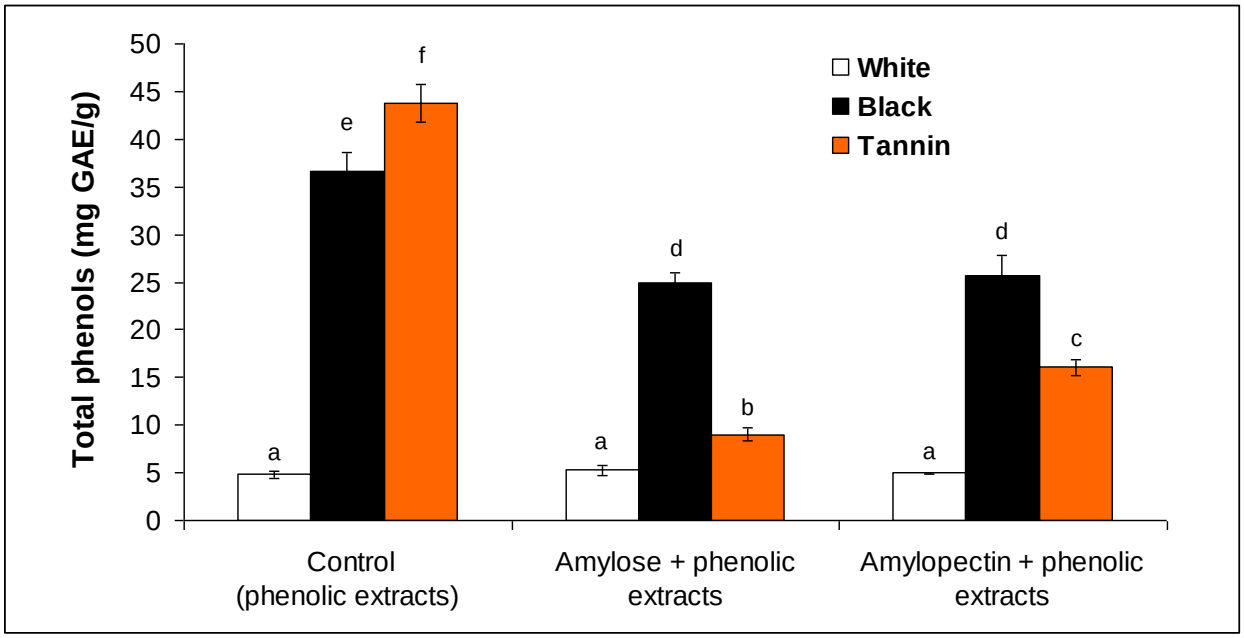
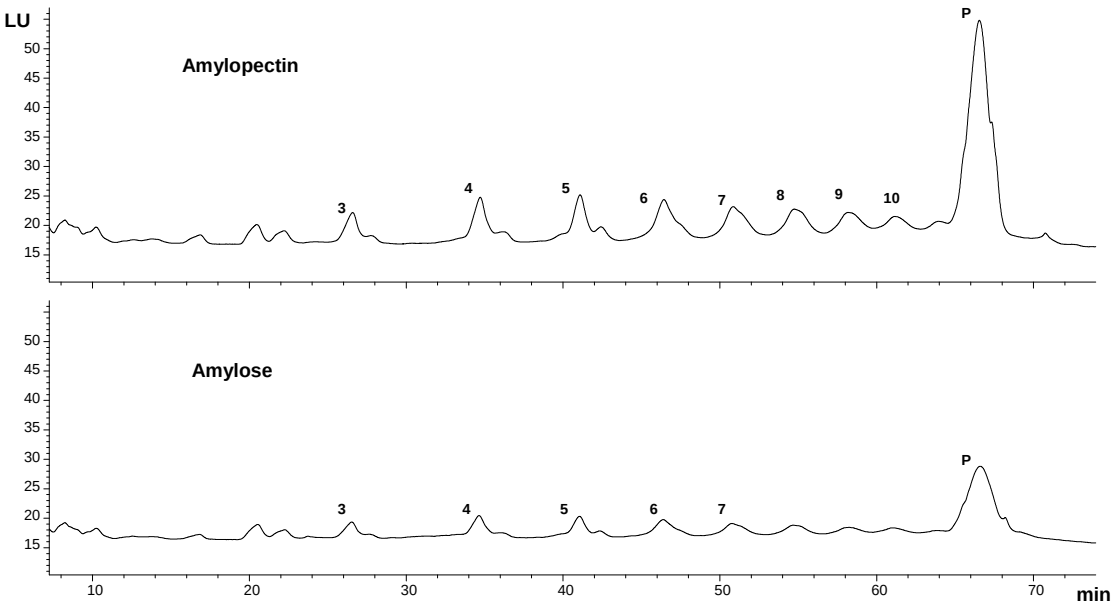


Fig. 6. Extractable phenol content (mg GAE/g) of freeze-dried sorghum phenolic extracts with and without the presence of amylose and amylopectin.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).



Numbers on peaks denote degree of polymerization. P = polymers with DP >10.

This suggests that hydrophobic interactions are a major contributor to the tannin-starch interactions, that has been demonstrated for proteins (Siebert et al 1996).

The physical conformation of the polymeric proanthocyanidins provides more hydrophobic sites than possible with the monomeric polyphenols, while the linear nature of amylose makes its hydrophobic core more accessible in solution compared to amylopectin. While amylopectin side chains not involved in double helix structure also provide limited hydrophobic sites, steric hindrance would likely interfere with its ability to efficiently interact with the polymeric tannins. Thus a portion of unextractable polymeric proanthocyanidins might be physically trapped within the bulky amylopectin matrix without necessarily chemically interacting with the starch. Obviously steric hindrance would be less of an issue for the monomeric polyphenols, which explains why black and white phenolic extracts polyphenols bound similarly to amylose and amylopectin. The hydrophobic interactions with amylose is likely to favor larger proanthocyanidin molecules; this was observed in this study (Fig. 7), which demonstrated that as proanthocyanidin DP increased, its apparent binding efficiency with amylose also increased (i.e., extractability decreased). Thus this work demonstrates for the first time, albeit indirectly, specific DP-dependent proanthocyanidin-starch interactions.

In vitro **starch digestibility**

Effect of RVA cooking on RDS, SDS and RS contents of starch-polyphenol mixtures

Starch mixed with tannin sorghum phenolic extract at 5% and 10% showed the lowest rapidly digestible starch (RDS) content (61.9% and 60.5% respectively) after 20

min digestion (Fig. 8). Compared to control (68.7%), this represents a 10% and 12% reduction in starch digestibility, respectively. In general, black and white sorghum phenolic extracts at similar levels (5 and 10%) did not present significant ($P > 0.05$) difference in RDS compared to control.

At 20% level, phenol concentration played a major role in starch digestibility. Tannin phenolic extracts at 20% had 56.0% RDS (19% reduction compared to control) whereas black phenolic extracts at 20% had 56.6 % RDS (Fig. 8). This is likely due to enzyme inhibition at such high levels of phenols.

Slowly digestible starch (SDS) content was about 3% for all samples and there were no significant differences among them. This was expected because the slow digestion property of starch is determined by the semi-crystalline structure of amylopectin (Zhang and Hamaker 2009) which is disrupted during cooking. Because samples in this work were frozen immediately after cooking, it should be assumed that no re-crystallization of starch/retrogradation took place.

Sorghum tannin phenolic extracts significantly increased resistant starch content of normal starch cooked in RVA (Fig. 9). There were no significant ($P > 0.05$) differences between control and white sorghum phenolic extract treatment. At 5% level, tannin phenolic extracts had almost double (5.5%) RS compared to control (2.9%) whereas no significant ($P > 0.05$) difference was observed for the black phenolic extracts (Fig. 9). At 10% level, tannin phenolic extracts still had the highest RS content (8.3%) and black phenolic extracts had 5.6%.

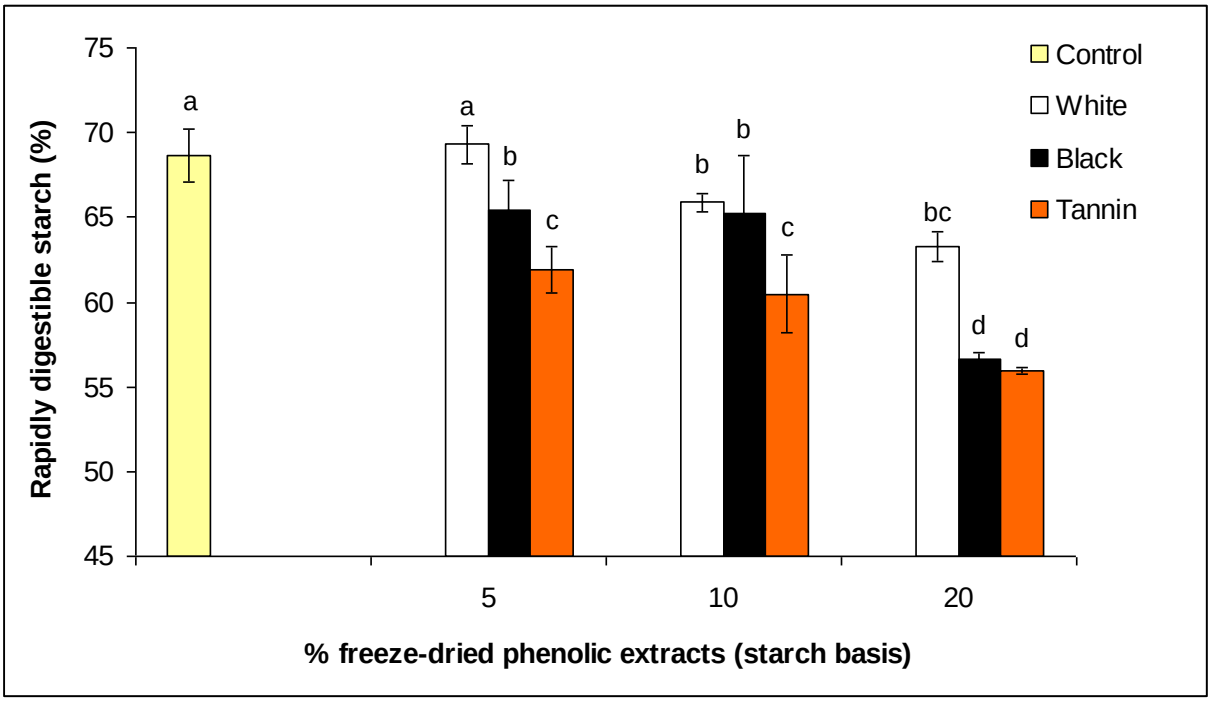


Fig. 8. Effect of sorghum phenolic extracts on rapidly digestible starch (RDS) content. Errors bars indicate standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

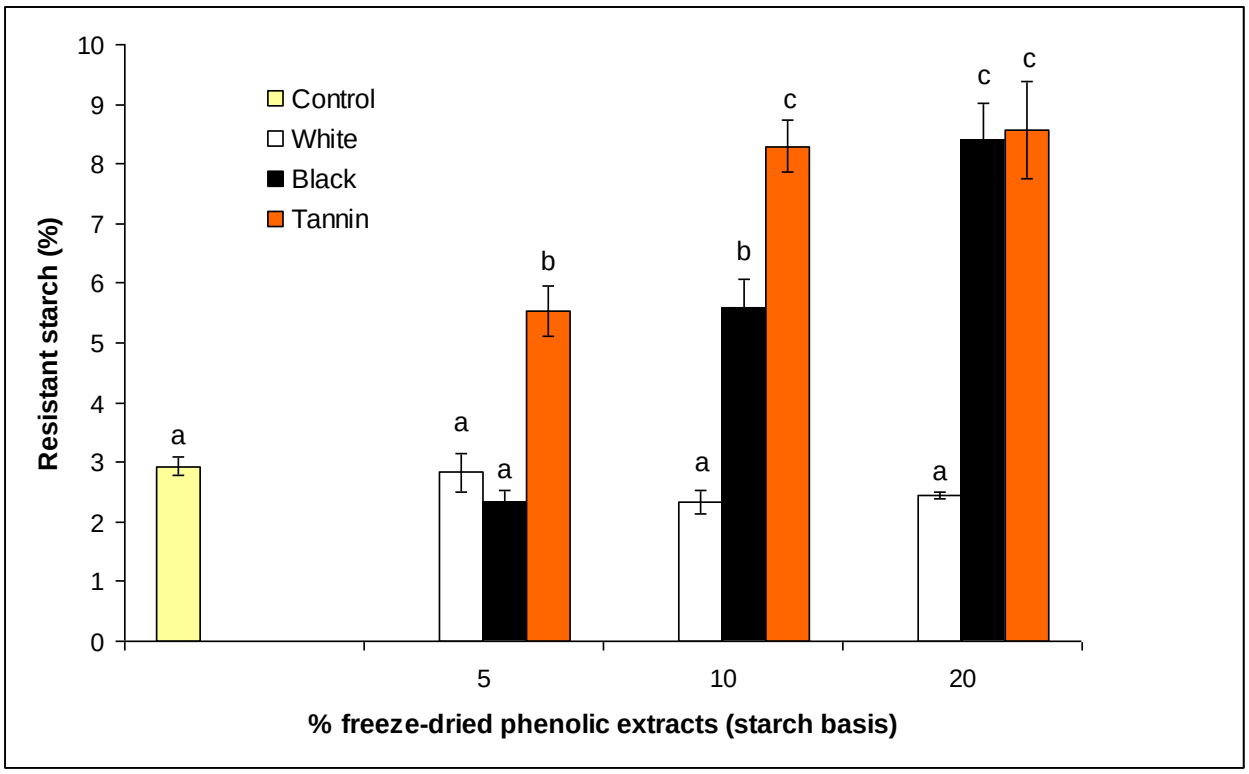


Fig. 9. Effect of sorghum phenolic extracts on resistant starch content of normal starch cooked in a RVA. Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

At 20% level, both tannin and black phenolic extracts had the same effect on resistant starch formation; the RS content was about 8.5% for both treatments (Fig. 9).

As previously mentioned in this study, black phenolic extracts cooked with starch in RVA had significantly smaller reduction in extractable phenols compared to the tannin phenolic extract treatments and still presented less RS content up to 10% level compared to tannin phenolic extract treatment. This shows that amount of extractable phenols in solution was not the most important cause of increase in RS and decrease in RDS.

Davis and Hoseney (1979) reported that condensed tannins can be adsorbed on raw starch and act as α-amylase inhibitor. Recently, Hargrove et al (2011) demonstrated that both tannin and black sorghum (without tannins) phenolic extracts inhibited α-amylase and this inhibition increased as concentration of phenolic extracts increased. Tannin phenolic extracts inhibited enzyme more strongly than black phenolic extracts; however, as concentration increased, the inhibition of both phenolic extracts became similar. This may explain the higher increase in RS and decrease in RDS when high concentration of black phenolic extract was used (20% level).

Thus, it was observed that condensed tannins played a greater role in the *in vitro* starch digestibility (formation of RS and decrease of RDS) compared to 3-deoxyanthocyanins and other simple phenols in sorghum. This is partly explained by the stronger interactions of polymeric proanthocyanidins with starch molecules observed in this study.

Effect of cooking-cooling cycles on resistant starch content of starch-phenol mixtures

Multiple heating/cooling treatments are known to increase RS content in foods (Yadav et al 2009). The goal was to use this technique to enhance retrogradation of amylose and optimize RS formation and to investigate how amylose content affects interaction with sorghum polyphenols and formation of RS. This helped to better understand the effect of interactions between tannin and amylose (linear molecule) on RS formation.

Sorghum tannin phenolic extract significantly increased resistant starch content of normal and high amylose starches (Fig. 10). Control and treatment containing white sorghum phenolic extract did not differ ($P > 0.05$) in RS content when normal starch was used (Fig. 10). Their RS content was around 6.7% which is more than twice higher than control cooked in the RVA (2.9%). Treatment containing black sorghum phenolic extracts had RS around 9% (Fig. 10), compared to 5.6% when it was cooked in the RVA. The highest value of RS (13.7%) was obtained with tannin phenolic extract treatment (Fig. 10); this value was around 8.4% when cooked in the RVA.

RS content reached over 40% when tannin phenolic extract was cooked with high amylose starch, whereas there were no significant ($P > 0.05$) differences among control and treatments containing white and black phenolic extracts (RS content around 26%) (Fig. 10). This further supports the theory that hydrophobic interactions are dominant in explaining polyphenol-starch interactions. Both amylose and polymeric proanthocyanidins from sorghum have relatively strong hydrophobic regions which are more readily exposed during heat treatment, allowing for more efficient interactions.

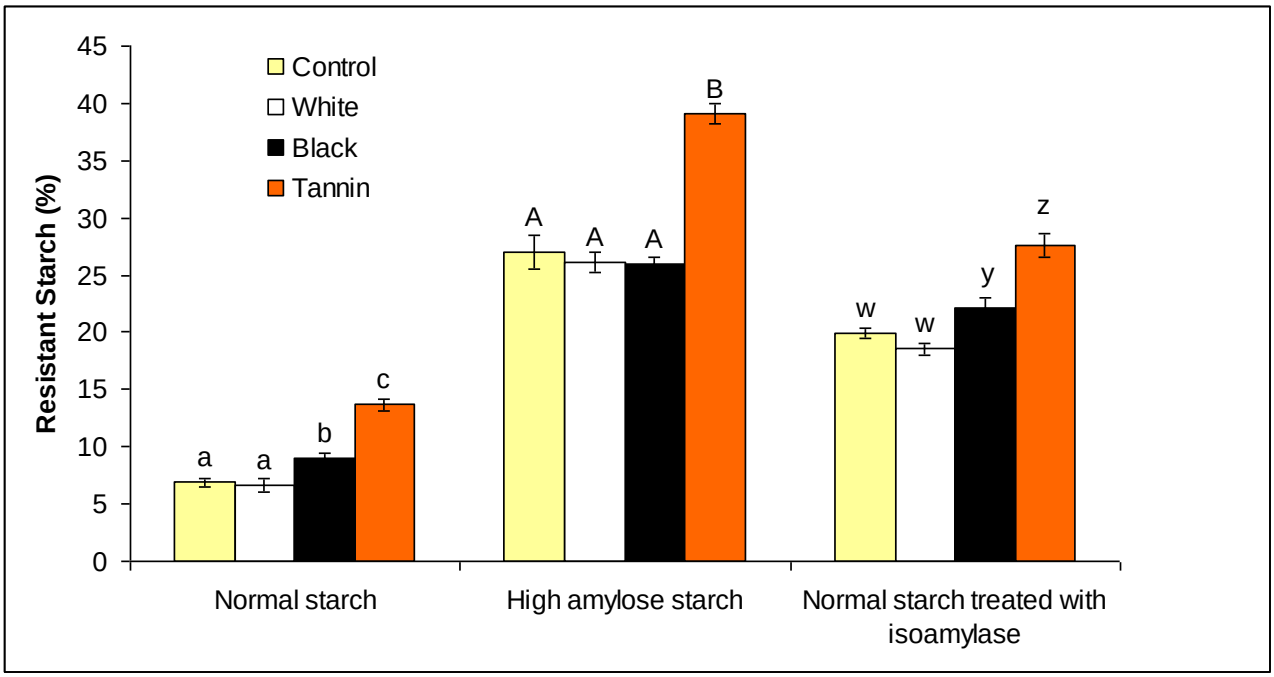


Fig. 10. Effect of sorghum phenolic extracts (10 % starch basis) on resistant starch content of normal starch (with and without treatment with isoamylase) and high amylose starch cooked in an autoclave (121°C/30 min) and cooled (4°C) overnight (3 heating/cooling cycles). Errors bars indicate ± standard deviation. Means followed by the same letter within treatment (each starch type) are not significantly different ($P \leq 0.05$).

Repeated heating-cooling cycles allows for further alignment of these regions, and thus increase in formation of RS over and beyond that observed from amylose-amylose interaction.

RS content below 1% was observed when waxy starch was cooked with all sorghum phenolic extracts. This suggests that enzyme inhibition does not play a role in the RS formation observed in this study. Interaction between sorghum tannins and amylose during cooking was the main reason for the observed increase in RS.

Effect of isoamylase pre-treatment of normal starch on resistant starch formation

In order to understand the effect of amylopectin debranching on polyphenol-starch interaction and RS formation, normal starch was treated with isoamylase and the autoclave heating-cooling cycle treatment as previously described. Isoamylase was to produce linear chains from amylopectin. The product of this debranching process is a starch solution with long (amylose) and short (from amylopectin branches) linear molecules. The highest RS content (28.6%) was obtained when tannin phenolic extract was cooked with the debranched starch (Fig. 10). There was no significant ($P > 0.05$) difference between control and treatment with white phenolic extract (RS around 20%) (Fig. 10). Treatment with black phenolic extract had RS content of 22% (Fig. 10).

To reconcile all the RS data, it is important to consider 'net RS' formation in presence of the sorghum phenolic constituents, i.e., RS formation beyond those observed for corresponding controls. Interestingly, the net formation of RS attributable to the monomeric polyphenols in black sorghum versus polymeric polyphenol-containing tannin sorghum followed different trends. In black sorghum treatments (10% starch

basis), net RS was 27 mg/g in RVA cooked normal starch versus 23 mg/g in heating-cooling cycle treated normal starch (Figs. 9 and 10). When isoamylase treatment was added to the heating-cooling cycle, the net RS formation declined modestly to 20 mg/g (Fig. 10). By contrast, tannin sorghum phenolic extract (10% starch basis) produced a net RS of 55 mg/g in RVA-cooked normal starch (Fig. 9). In heating-cooling cycle treated normal starch, net RS formation increased to 70 mg/g (Fig. 10); debranching treatment further increased the RS formation to 86 mg/g (Fig. 10).

As previously explained, repeated heating-cooling cycles of starch will favor increased RS formation attributed largely to increased amylose crystallinity due to of double helical crystallite formation (Zobel 1988). The polymeric condensed tannins may likely form complexes with the single helical amorphous regions of amylose, stabilized by hydrophobic and hydrogen bonding. Repeated heating-cooling would improve alignment of the starch-tannin hydrophobic regions and thus increase the formation of such complexes. The starch-tannin complexed regions would obviously be resistant to enzyme attack. The fact that net RS formation decreased for monomeric polyphenol starch in RVA treatment compared to autoclave cooking-cooling cycle treatment suggests the simple polyphenols probably complex with starch primarily via hydrogen bonds which can be disrupted by high heat treatment (Siebert et al 1996). Furthermore, the fact that debranching only increased net RS in the tannin sorghum treatments further demonstrates the involvement of linear starch molecules in starch-tannin interactions.

Another interesting observation which confirms the different specific interaction of the polymeric sorghum tannins (as opposed to monomeric ones) with amylose was a

large increase in net RS formation observed for high amylose starch treated with tannin sorghum phenolic extract (140 mg/g) (Fig. 10). This is in sharp contrast to no net RS formation in presence on monomeric polyphenol extracts (Fig. 10). Increasing amylose content increased the available amorphous hydrophobic domains to which the polymeric tannins could complex. Thus, it is apparent from the data that the polymeric sorghum tannins are more likely to increase RS content of starch than the monomeric polyphenols, probably due to the added advantage of strong hydrophobic interactions with starch not possible for the monomeric molecules.

This is the first study that demonstrates specific interactions between condensed tannins and starch molecules (amylose and amylopectin). Sorghum condensed tannins are more effective in interacting with amylose possibly through hydrophobic and hydrogen bonding, significantly increasing RS content of normal and high amylose starches compared to monomeric sorghum polyphenols such as phenolic acids and 3-deoxyanthocyanins. Thus high molecular weight polyphenols may provide new opportunities to produce functional food ingredients that reduce caloric density of starch-containing products while providing added health benefits.

CHAPTER IV

SORGHUM CONDENSED TANNINS: CHARACTERIZATION AND EFFECT ON RESISTANT STARCH FORMATION

MATERIALS AND METHODS

Sorghum samples

Two tannin sorghum varieties that differ in proanthocyanidin profiles, high-tannin and sumac, grown in College Station, TX were used. Freeze-dried crude extracts were prepared as described in Chapter III.

In addition, a sample of purified condensed tannins from high-tannin sorghum was used in order to obtain higher percentage of polymeric proanthocyanidins and eliminate effects of small phenolic compounds. The method to purify condensed tannins was used as described by Hagerman and Butler (1980) with modifications. Absolute ethanol (300 mL) containing 10 mM ascorbic acid was added into 30 g of high tannin sorghum bran and then stirred for 45 min and filtered. The residue was extracted four times with 200 mL methanol containing 10 mM ascorbic acid. Supernatant was saved after each extraction. An equal volume of 0.05 M acetate pH 4.0 was added to the extract and methanol removed by rotary evaporation at 35^{o}C. Then, the extract free of methanol was extracted (liquid-liquid extraction) three times with ethyl acetate. The sample was rotary evaporated at 35^{o}C to a volume about 50 mL and absolute ethanol was added to make the final sample solvent 80% ethanol.

The sample was applied to about four volumes of Sephadex LH20 column (300 mm height x 40 mm O.D). The column was equilibrated in 80% ethanol. Then, 95% ethanol was added until the eluate no longer absorbed light in the UV. Lastly, the column was washed with 50% aqueous acetone and the eluate (large molecular weigh tannins) was saved. The column was then re-equilibrated in 80% ethanol and the extract was rotary evaporated at 35^{o}C to remove acetone and then it was freeze-dried.

Reagents

Chemicals and reagents were the same as those used in Chapter III with addition of ethyl acetate that was purchased from Fisher (Fisher Scientific, Fair Lawn, NJ) and Sephadex LH20 from Sigma (St. Louis, MO). Starches and enzymes were the same as the ones from chapter III. A chromatography column was purchased from VWR (cat # 60001-986).

Material characterization

The normal phase HPLC method described by Langer et al (2011) was used in order to identify differences in proanthocyanidin concentration and molecular weight distribution among samples. Freeze dried extracts (crude phenolic extracts and purified tannin extract) were dissolved in methanol (5 mg/mL) and 15 µL was injected in the HPLC.

Interactions of sorghum condensed tannins with starch molecules

Changes in molecular weigh profile of proanthocyanidins in amylose/amylopectin-tannin extract mixtures

In order to understand interactions between amylose/amylopectin and polymeric proanthocyanidins, pure amylose and pure amylopectin (10% w/v in distilled water) were mixed with freeze-dried sorghum tannin extracts containing different concentrations of polymeric proanthocyanidins (crude extracts from high-tannin and sumac and purified extract from high-tannin) (10% starch basis) in a shaker for 1 h. Samples were frozen in liquid nitrogen and freeze-dried. The freeze-dried material (0.4 g) was mixed with methanol (5 mL) and the supernatant filtered through a 0.45 μm membrane and injected (20 μL) in the HPLC to determine concentration of proanthocyanidins and their molecular weight profile (DP).

Size exclusion chromatography

Freeze-dried tannin sorghum extracts (crude and purified tannins) were cooked with normal and high amylose starches at 121^{o}C/30 min in a 3 heating/cooling cycles and then freeze-dried and ground as described in Chapter III. These samples were analyzed in a SEC-MALS (Size exclusion chromatography with multi-angle light scattering) to determine changes in the radius of amylose molecules. It was demonstrated in Chapter III that stronger interactions happened between amylose and sorghum tannins and it was hypothesized that these interactions are stabilized by hydrogen and hydrophobic bonding. An increase in amylose radius would indicate a possible cross-linking (covalent interactions).

Samples (0.05 g), including controls (normal and high amylose starch without tannin extracts) were solubilized using 1 mL of DMSO (90%) under heat (100^{o}C) for 60 min with frequent vortexing. An aliquot (0.5 mL) was mixed with ethanol (1.5 mL) and then centrifuged (6750g/15 min). The residue was solubilized in KOH (1 mol/L) for 90 min at 100^{o}C. An aliquot of 1 mL was mixed with HCL (1 mL, 1 mol/L) to neutralize the solution. Then it was filtered with 1µm syringe filter and injected in the HPLC (Agilent 1200 HPLC system with Chemstation software).

Injection volume was 100 µL. Three columns were used in series (Shodex SB807, SB806, SB805). Mobile phase (0.1 M $NaNO_3$) flow rate was 0.50 mL/min. Absolute molecular weight and radius were obtained using a light scattering detector (Wyatt Heleos MALS detector with ASTRA software).

In vitro starch digestibility

RS content was measured in the cooked/cooled/freeze dried samples described above. Moreover, in the attempt to increase RS, samples were oven dried (105^{o}C/2h) instead of freeze-dried after autoclave cooking/cooling. Resistant starch (RS) was directly measured using the resistant starch assay kit from Megazyme (AACC method 32-40).

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) to determine significant differences. Fisher's least significant difference (LSD) ($P \leq 0.05$) was used to compare multiple means. The software used was SPSS v 16.0 for windows (SPSS Inc., Chicago, IL). All tests were done in three replications.

RESULTS AND DISCUSSION

Properties of sorghum purified tannin extract and crude phenolic extracts

The two tannin sorghum varieties studied had different concentrations and proanthocyanidin profiles. Sumac phenolic extracts presented higher concentration of total (228.1 mg/g) and lower of polymeric proanthocyanidins (degree of polymerization above 10) (72%) compared to high-tannin phenolic extracts (204.9 mg/g and 80% respectively) (Table V), which agrees with previous findings (Awika et al 2003a). Purified tannin extract had mostly polymeric proanthocyanidins (87%) (Table V). Furthermore, sumac phenolic extracts presented much higher concentration of catechins (2.2 mg/g), dimers (1.97 mg CE/g) and trimers (3.67 mg CE/g) compared to high-tannin sorghum (0.16, 0.42 and 1.22 mg CE/g respectively) (Table V). This is important because catechins and B-type proanthocyanidin dimers and trimers were reported to have high absorption *in vitro* and showed to be present in the blood of humans (Ou et al 2012; Deprez et al 2001). Tannin sorghum contains mostly B-type proanthocyanidins (Prior and Gu 2005; Krueger et al 2003). Thus, naturally, sumac sorghum has more bioavailable proanthocyanidins than high-tannin sorghum (Table V).

It was demonstrated in Chapter III that concentration of catechins and dimers in a phenolic extract from high-tannin sorghum increased upon heating. In this chapter, sumac phenolic extract (higher concentration of catechins, dimers and trimers), high-tannin phenolic extract and purified tannin extract were subjected to the same heat treatment (95^{o}C/20 min and 121^{o}C/30 min) and concentration of catechins and proanthocyanidin were determined. There was a significant increase in catechins and

dimers and decrease in polymers (DP > 10) after cooking (Tables V, VI, VII and VIII; Figs. 11,12 and13). Heating sumac phenolic extracts at 121^{o}C/30 min increased catechins from 2.2 mg/g to 6.91 mg/g. Concentration of dimers went from 1.97 mg CE/g to 7.46 mg CE/g (Tables V and VII; Fig. 12). Thus, heat treatment may increase bioavailability of proanthocyanidins in tannin sorghums, by depolymerizing high molecular weight polymers into the monomers and dimers.

Interactions between sorghum condensed tannins and starch

Reaction between sorghum condensed tannins and amylose/amylopectin

In order to investigate interactions of sorghum condensed tannins with amylose and amylopectin, pure amylose and pure amylopectin were mixed with sorghum phenolic extracts and purified tannin extract. The concentration of different molecular weight proanthocyanidins in the supernatant were significantly ($P \leq 0.05$) lower in the presence of amylose compared to amylopectin (Table IX; Appendix Figs. A2, A3 and A4). This difference was greater for the polymeric (DP >10) proanthocyanidins. Their concentration decreased 69% in the treatment containing high-tannin phenolic extract, 60% in the treatment containing sumac phenolic extract and 85% in the treatment containing purified tannin extract (Table IX).

Purified tannin extract had the highest level of polymeric proanthocyanidins (87%) followed by high-tannin phenolic extract (80%) and sumac phenolic extract (72%) (Table V).

Table V. Proanthocyanidin content[a] of crude tannin phenolic extracts of high tannin, sumac and purified tannin extract [b]

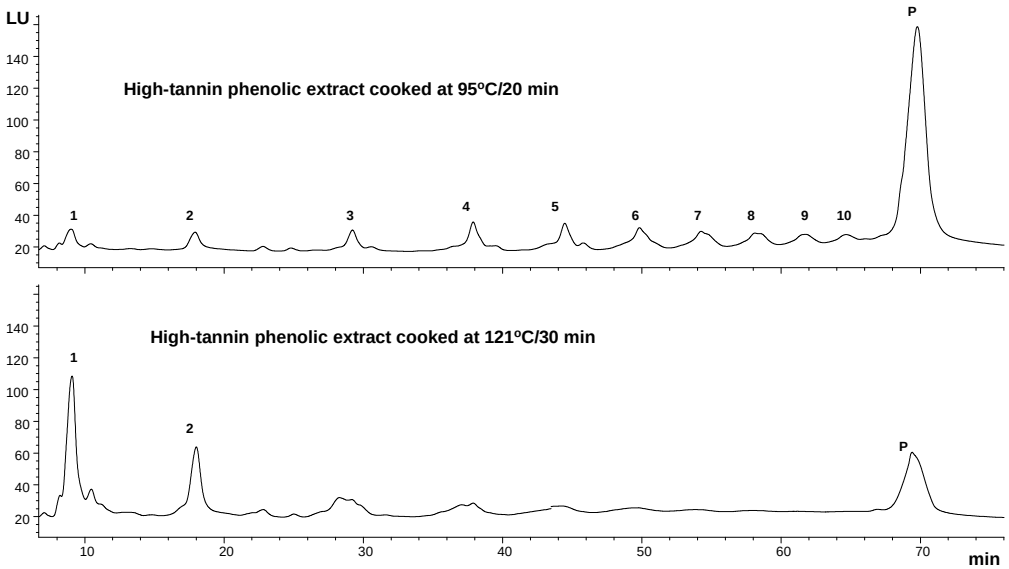
DP[c]	High-tannin	Sumac	Purified tannin
1	0.16 ± 0.01	2.2 ± 0.10	0.24 ± 0.02
2	0.42 ± 0.03	1.97 ± 0.13	0.35 ± 0.03
3	1.22 ± 0.09	3.67 ± 0.18	1.19 ± 0.08
4	2.39 ± 0.15	4.17 ± 0.25	1.87 ± 0.12
5	3.47 ± 0.21	4.93 ± 0.33	2.63 ± 0.21
6	6.93 ± 0.44	9.94 ± 0.49	4.56 ± 0.34
7	7.17 ± 0.49	9.94 ± 0.77	4.96 ± 0.15
8	7.23 ± 0.57	9.88 ± 0.55	5.2 ± 0.40
9	6.62 ± 0.61	9.0 ± 0.87	3.4 ± 0.23
10	5.62 ± 0.15	7.7 ± 0.41	4.01 ± 0.29
P[d]	163.7 ± 9.3	164.7 ± 6.9	176.7 ± 5.2
% polymers [e]	80	72	87
Total	204.9 ± 12.05	228.1 ± 10.98	205.07 ± 7.07

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10. [e] % polymers (DP > 10) as a percent of total.

Table VI. Proanthocyanidin content[a] of high-tannin phenolic extract (HTPE) after cooking at 95°C/20 min and at 121°C/30 min [b]

DP[c]	HTPE after cooking (95°C/20 min)	HTPE after cooking (121°C/30 min)
1	0.76 ± 0.05	3.37 ± 0.15
2	1.75 ± 0.06	3.84 ± 0.22
3	2.74 ± 0.10	nd[e]
4	4.79 ± 0.26	nd
5	5.95 ± 0.12	nd
6	8.37 ± 0.44	nd
7	7.89 ± 0.31	nd
8	7.42 ± 0.33	nd
9	6.30 ± 0.29	nd
10	5.75 ± 0.44	nd
P[d]	144.4 ± 7.4	47.5 ± 1.95
Total	196.1 ± 9.8	54.7 ± 2.32

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10.
[e] Not detected

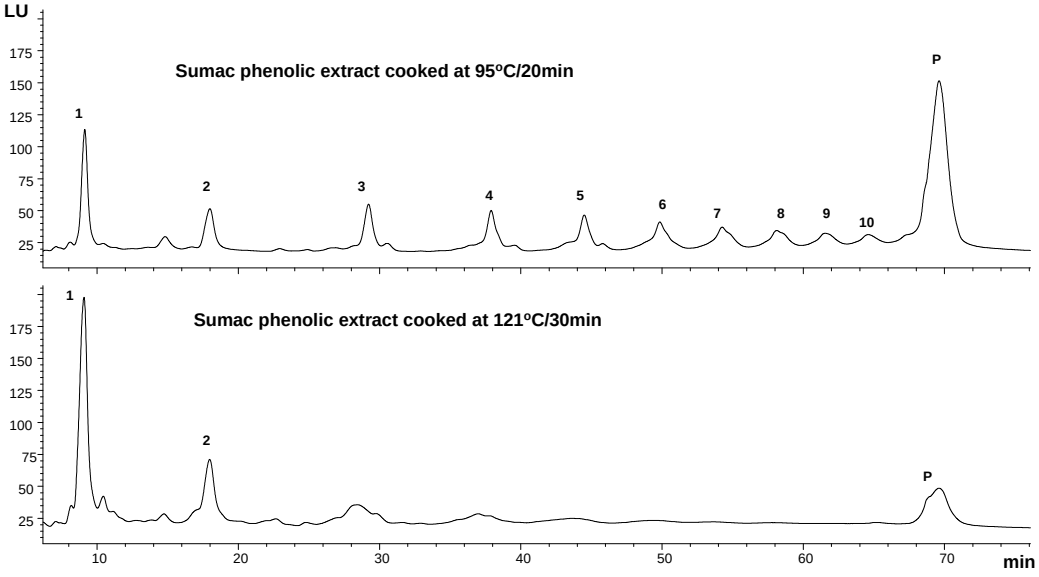


ract

Table VII. Proanthocyanidin content[a] of sumac phenolic extract (SPE) after cooking at 95°C/20 min and at 121°C/30 min [b]

DP[c]	SPE after cooking (95°C/20 min)	SPE after cooking (121°C/30 min)
1	3.26 ± 0.10	6.91 ± 0.33
2	3.60 ± 0.08	7.46 ± 0.43
3	5.41 ± 0.12	nd[e]
4	7.72 ± 0.29	nd
5	8.81 ± 0.19	nd
6	12.59 ± 0.55	nd
7	11.83± 0.54	nd
8	10.94 ± 0.44	nd
9	9.40 ± 0.55	nd
10	8.10 ± 0.51	nd
P[d]	138.9 ± 7.1	39.9 ± 1.78
Total	220.6 ± 10.5	54.3 ± 2.54

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10. [e] Not detected

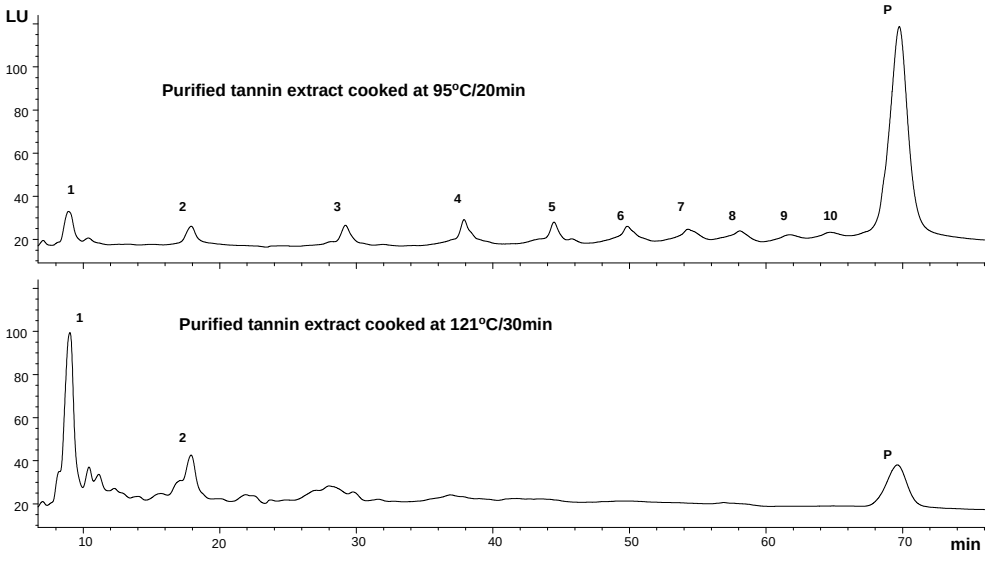


Fi
cc
pc

Table VIII. Proanthocyanidin content[a] of purified tannin extract (PTE) after cooking at 95°C/20 min and at 121°C/30 min [b]

DP[c]	PTE after cooking (95°C/20 min)	PTE after cooking (121°C/30 min)
1	1.04 ± 0.07	3.20 ± 0.20
2	1.52 ± 0.04	3.50 ± 0.21
3	2.67 ± 0.15	nd[e]
4	3.96 ± 0.31	nd
5	4.02 ± 0.22	nd
6	5.86 ± 0.34	nd
7	5.51 ± 0.35	nd
8	5.91 ± 0.26	nd
9	4.53 ± 0.23	nd
10	4.35 ± 0.29	nd
P[d]	116.6 ± 7.23	27.40 ± 1.99
Total	156.1 ± 9.49	34.1 ± 2.40

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10. [e] Not detected.



Fi after
cc
polymerization. P – polymers with DP >10.

Table IX. Proanthocyanidin content[a] of crude tannin phenolic extracts and purified tannin extract mixed with pure amylose and amylopectin [b]

	High Tannin phenolic extract		Sumac phenolic extract		Purified tannin extract	
DP[c]	Amylose	Amylopectin	Amylose	Amylopectin	Amylose	Amylopectin
1	nd[e]	nd	0.11 ± 0.0	0.15 ± 0.01	nd	nd
2	0.02 ± 0.0	0.03 ± 0.0	0.10 ± 0.0	0.15 ± 0.0	nd	nd
3	0.05 ± 0.0	0.08 ± 0.0	0.16 ± 0.01	0.24 ± 0.02	0.08 ± 0.0	0.12 ± 0.01
4	0.10 ± 0.0	0.15 ± 0.01	0.19 ± 0.01	0.27 ± 0.02	0.12 ± 0.0	0.16 ± 0.01
5	0.12 ± 0.0	0.21 ± 0.02	0.17 ± 0.01	0.23 ± 0.01	0.13 ± 0.0	0.20 ± 0.0
6	0.21 ± 0.01	0.41 ± 0.02	0.29 ± 0.02	0.58 ± 0.04	0.21 ± 0.01	0.35 ± 0.02
7	0.19 ± 0.01	0.39 ± 0.03	0.25 ± 0.01	0.53 ± 0.05	0.23 ± 0.02	0.33 ± 0.02
8	0.17 ± 0.0	0.39 ± 0.01	0.22 ± 0.02	0.49 ± 0.03	0.13 ± 0.0	0.39 ± 0.03
9	0.14 ± 0.0	0.29 ± 0.01	0.18 ± 0.01	0.48 ± 0.03	0.15 ± 0.01	0.34 ± 0.01
10	0.12 ± 0.0	0.27 ± 0.02	0.16 ± 0.0	0.34 ± 0.01	0.14 ± 0.01	0.30 ± 0.02
P[d]	1.25 ± 0.08	4.1 ± 0.31	1.81 ± 0.09	4.53 ± 0.35	1.17 ± 0.10	7.98 ± 0.67
Total	2.37 ± 0.10	7.42 ± 0.43	3.34 ± 0.18	7.99 ± 0.57	2.36 ± 0.15	10.17 ± 0.79

[a] mg/g, expressed in catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; [c] Degree of polymerization; [d] Mixture of polymers with DP > 10.
[e] Not detected

Thus, it was demonstrated that extracts containing higher concentration of polymeric proanthocyanidins had stronger interaction with amylose, which was shown by the larger decrease in extractable polymeric proanthocyanidins in presence of amylose compared to amylopectin.

Size exclusion chromatography

There was no significant ($P > 0.05$) difference in amylose radius, which indicates the volume occupied by the molecule in solution and correlates with molecular weight, among treatments and control (Table X). The hypothesis was that tannin would interact with amylose not only through hydrogen and hydrophobic bonding, but also by possible cross-linking (covalent interactions).

Amylose radius did not change after mixing and cooking starch with sorghum tannin extracts (Table X) indicating that there was no covalent bonding involved. However, due to high variability presented by the data, no conclusion could be made. This high variability was probably because of the method used. It was necessary to dissolve freeze-dried starch and starch/tannins and then analyze them in the HPLC. Samples were subjected to treatments with DMSO and KOH under heat ($100^{o}C$) before injecting in the HPLC. Thus, due to the drastic conditions used to solubilize the material, high variability in the data was observed (Table X).

Further studies should focus on identifying mild methods to dissolve starch and starch/tannin. SEC/MALS is a good and widely used technique to measure molecular weight of starch and could help to answer the question whether or not there is a strong interaction (covalent) between starch and tannins.

Table X: Radius moments (nm) of amylose molecules with and without tannin extracts obtained from size exclusion chromatography with light scattering detection

	Radius (nm)
Test 1	
Normal starch (NS)	92.5 a
NS + high tannin phenolic extract	96.3 a
NS + sumac phenolic extract	109 a
NS + purified tannin extract	77.3 a
Test 2	
High amylose starch (HA)	69.0 A
HA + high tannin phenolic extract	79.2 A
HA + sumac phenolic extract	76.9 A
HA + purified tannin extract	90.8 A

For each test, means followed by the same letter are not significantly different ($P \leq 0.05$).

Effect of sorghum condensed tannins on resistant starch formation

Sorghum condensed tannins significantly increased resistant starch content of normal and high amylose starches (Fig. 14). When normal starch was cooked with tannin extracts, the highest value of RS (16.2%), among tannin extracts used, was obtained with purified tannin extract (Fig. 14). Cooked normal starch without tannin extract (control) had RS of 6.9% (Fig. 14).

RS content reached 46.5% when purified tannin extract was cooked with high amylose starch (Fig. 14). Control had RS of 25.7%. High-tannin and sumac phenolic extract cooked with high amylose starch had a RS content of 40.2% and 33.1% respectively. It was observed that the higher the percentage of polymeric (DP > 10) proanthocyanidins, the higher the RS content of the sample. Purified tannin extract had the highest concentration of high molecular weight proanthocyanidins and the highest RS content, especially when cooked with high amylose starch. This supports the theory that there is a strong interaction between large molecular weight proanthocyanidins and amylose, and it is stabilized by hydrophobic interactions and hydrogen bonding. Therefore, as explained in Chapter III, due to strong hydrophobic interactions, large molecular weight proanthocyanidins interact more with amylose than low molecular weight polyphenols, and such strong interaction favor RS formation.

In this study, samples were freeze-dried after heating/cooling cycles. However, for practical applications, oven drying the samples would be more suitable and produce more RS. It was reported that oven dried samples after heating/cooling cycles had significantly higher RS content compared to free-dried samples (Ozturk et al 2011).

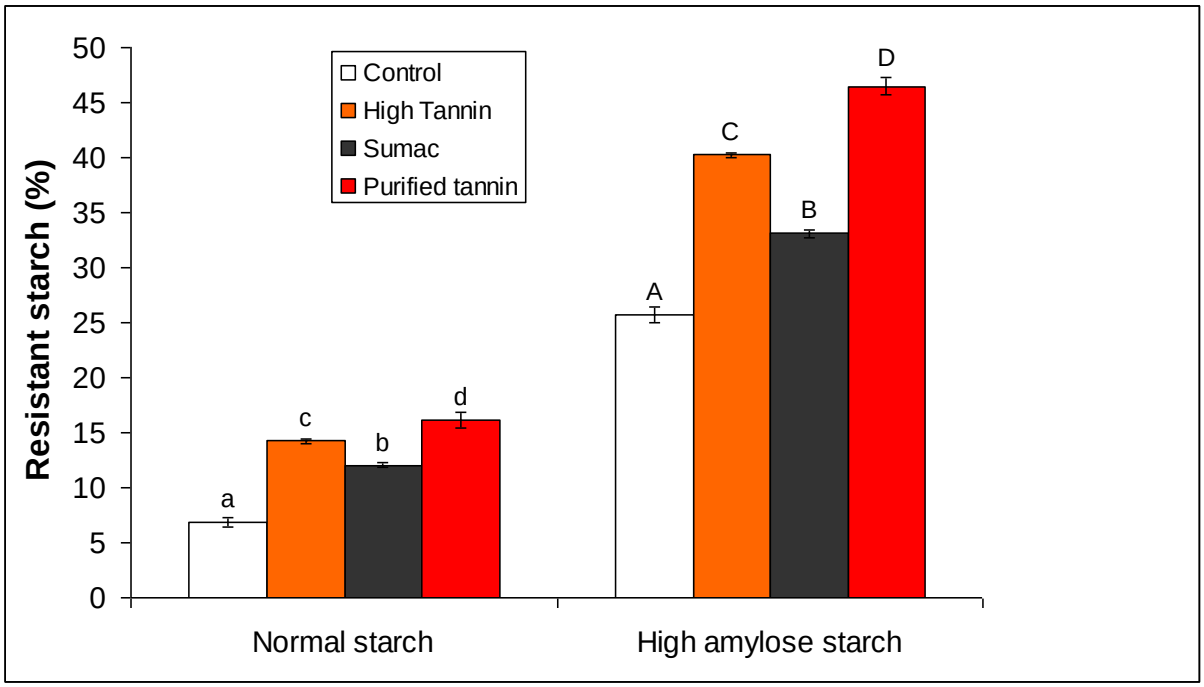


Fig. 14. Effect of tannin sorghum phenolic extracts (10 % starch basis) and purified tannin extract on resistant starch content of normal starch and high amylose starch cooked in an autoclave (121^{o}C/30 min), cooled (4^{o}C) over night (3 heating/cooling cycles) and freeze dried.

Errors bars indicate ± standard deviation. Means followed by the same letter within treatment are not significantly different ($P \leq 0.05$).

Thus, RS was determined in samples after heating/cooling cycles and oven dried (105^{o}C/2h) instead of freeze-dried in order to demonstrate effect of oven drying on RS content. A significant increase in RS content was observed (Fig. 15). RS increased from 6.8% (freeze-dried) to 7.5% (oven dried) of normal starch (control) (Figs. 14 and 15). When normal starch was cooked with high-tannin phenolic extract, RS content went from 14.5% (freeze-dried) to 16.2% (oven dried). Same trend was observed for treatments with sumac phenolic extracts (RS from 12.1% to 13.4%) and purified tannin extract (RS from 16.2% to 18.1%) (Figs. 14 and 15).

RS content went from 46.5% (freeze-dried) to 53.1% when purified tannin extract was cooked with high amylose starch and oven dried (Fig. 15). Treatments after oven drying containing high-tannin and sumac phenolic extracts had RS of 48.6% and 38.9% respectively (Fig. 15). Their RS content after freeze drying was 40.2% and 33.1% respectively (Fig. 14). RS in the control (high amylose without tannin extracts) increased from 25.7% (freeze-dried) to 28.1% (oven dried) (Figs. 14 and 15). This increase in RS due to oven drying (105^{o}C) was probably because of oxidation of compounds which produce new bonds not recognizable by digestive enzymes or maybe due to retrogradation of starch during drying (Ozturk et al 2011).

High-tannin sorghum has comparable concentration of polymeric proanthocyanidins (about 80%, table V) compared to fruits such as blueberries and black currant, and has higher concentrations compared to other fruits, legumes and nuts (Prior and Gu 2005).

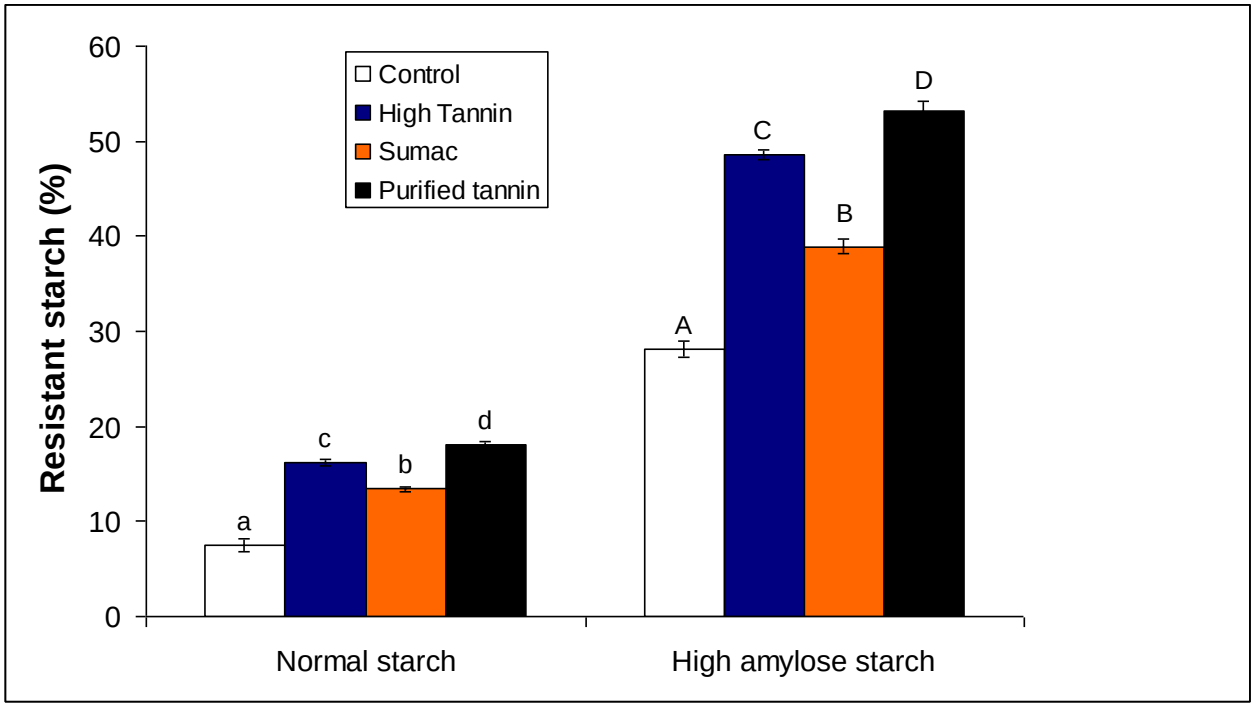


Fig. 15. Effect of tannin sorghum phenolic extracts (10 % starch basis) and purified tannin extract on resistant starch content of normal starch and high amylose starch cooked in an autoclave (121°C/30 min), cooled (4°C) overnight (3 heating/cooling cycles) and oven dried (105°C/2h).

Errors bars indicate ± standard deviation. Means followed by the same letter within treatment are not significantly different ($P \leq 0.05$).

High-tannin sorghum may be used to make breads, cookies, breakfast cereals and other dark colored products containing higher RS content and antioxidant activity.

RS is manufactured by a heating-cooling process and chemical modification (Mun and Shin 2006). However, chemical modification may have safety problems. Moreover, RS content may not be high enough just by heating-cooling process (retrogradation). It was demonstrated in this study (chapter III and IV) that sorghum condensed tannins, specifically the large molecular weight ones, interact with starch, especially with amylose, and increase RS content. More studies should be done on how baking/drying processes affect RS content of products containing tannin sorghum flour/bran and high amylose starch.

CHAPTER V

ACCELERATED SOLVENT EXTRACTION (ASE) OF POLYPHENOLS FROM SORGHUM BRAN

MATERIALS AND METHODS

Sorghum samples

High tannin sorghum and black sorghum (TX430 black) were used. Brans were obtained by decorticating 1 kg batches in a PRL mini-dehuller (Nutama Machine Co., Saskatoon, Canada) and separated with a KICE grain cleaner (Model 6DT4-1, KICE Industries Inc., Wichita, KS). The brans (approximately 10% of original grain weight) were milled to pass through a 1.0 mm screen using a UDY cyclone mill (Model 3010-030, UDY Corporation, Fort Collins, CO). They were kept at -20 ^{o}C until used.

Reagents

Chemicals and reagents were the same as the ones used in Chapter III with addition of ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium phosphate, sodium chloride and citric acid which were purchased from Sigma-Aldrich (St. Louis, MO). Luteolinidin chloride and apigeninidin chloride were obtained from ALSACHIM (Strasbourg, France) and 7-methoxyapigeninidin chloride was obtained from ChromaDex (Santa Ana, CA, USA). Sea send was purchased from EMD Millipore Chemicals (Billerica, MA). Enzymes for the *in vitro* starch digestibility were the same as those in chapter III.

Extraction method

Dionex model ASE 200 equipped with a solvent controller (Dionex Corp., Sunnyvale, CA) (Fig. 16) was used in this study.

A portion (0.5 g) of black and high tannin sorghum brans were mixed with 30 g of sand and then placed into 22 mL extraction cell containing cellulose paper filter at the bottom of each cell. The cell containing the sample was prefilled with extraction solvent, pressurized and then heated. ASE variables were pressure (1500 psi), one extraction cycle, flush volume (70%), nitrogen purge time (120 sec), static time (1 min), and preheat time (0 min). After extraction, the volume of each collection tube was adjusted to 35 mL with deionized water. Samples were immediately centrifuged for 8 min at 8,000 rpm. The supernatant was recovered and stored in plastic centrifuge tubes at - 20 ^{o}C.

Different temperatures (60, 120 and 150^{o}C) and solvents (0, 50 and 70% v/v ethanol/water and citric acid in water pH 2.5) were tested.

Conventional extractions using same solvents mentioned above with addition of acidified (1% HCL) methanol and 70% acetone/water were done to compare effectiveness of ASE extractions. Sorghum bran (0.5g) was mixed with solvents (35 mL) and mixture was shaken for 2 hours at 1 atm and room temperature. Extracts were centrifuged for 8 min at 8,000 rpm. The supernatant was recovered and stored in plastic centrifuges tubes at - 20 ^{o}C.

Fig. 16. Accelerated Solvent Extractor.

Extract characterization

Extracts from ASE and from conventional extraction methods were analyzed for total phenols and antioxidant capacity (ABTS). Moreover, quantification and identification of catechins, proanthocyanidins and 3-deoxyanthocyanins in the extracts were done by HPLC analyses.

Total phenols and antioxidant capacity

Total phenols assay, as described in Chapter III was performed. Antioxidant capacity of the extracts was measured *in vitro* by the ABTS assay as described by Awika et al (2003b). Briefly, The ABTS stock solution was prepared by reacting equal volume of 8 mM ABTS with 3 mM potassium persulfate in the dark for at least 12 h. The original absorbance of ABTS working solution at 734 nm should be around 1.5. Aqueous sample (0.1 mL) was added with 2.9 mL ABTS working solution then allowed to react in the dark for 30 min at room temperature. The absorbance was read at 734 nm. Antioxidant capacity was calculated and expressed as μmol Trolox equivalent /g sample.

HPLC analyses

The method by Awika et al. (2009) described in Chapter III was used to profile 3-deoxyanthocyanins in the black sorghum phenolic extracts. Extracts were filtered (0.45 μm) and 20.0 μL was injected onto a reversed phase 150 x 2.00 mm, 5 μm, C-18 column (Phenomenex, Torrance, CA). Quantification of 5-methoxyluteolinidin was determined using the calibration curve for luteolinidin along with the appropriate molecular weight correction factor (Dykes et al 2009).

A normal phase HPLC method by Langer et al. (2011) described in Chapter III was used to separate proanthocyanidins based on degree of polymerization (DP) in the tannin sorghum phenolic extracts. Extracts were filtered (0.45 μm) and 20.0 μL was injected onto a Develosil Diol (250 mm x 4.6 mm, 5 μm particle size; Phenomenex, U.K.).

In vitro starch digestibility

Freeze-dried tannin sorghum extracts (from ASE and conventional extractions) were cooked with high amylose starch at 121^{o}C/30 min in a 3 heating/cooling cycles and then freeze-dried and ground as described in chapter III. Resistant starch (RS) was measured using the resistant starch assay kit from Megazyme (AACC method 32-40).

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) to determine significant differences among them. Fisher's least significant difference (LSD) ($P \leq 0.05$) was used to compare multiple means. The software used was SPSS v 16.0 for windows (SPSS Inc., Chicago, IL). All tests were done in three replications.

RESULTS AND DISCUSSION

Total phenols and antioxidant activity

Black sorghum extracts

ASE and conventional extracts are shown in figs 17 and 18.

The effect of conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures on total phenol content of black sorghum bran is shown in Fig. 19.

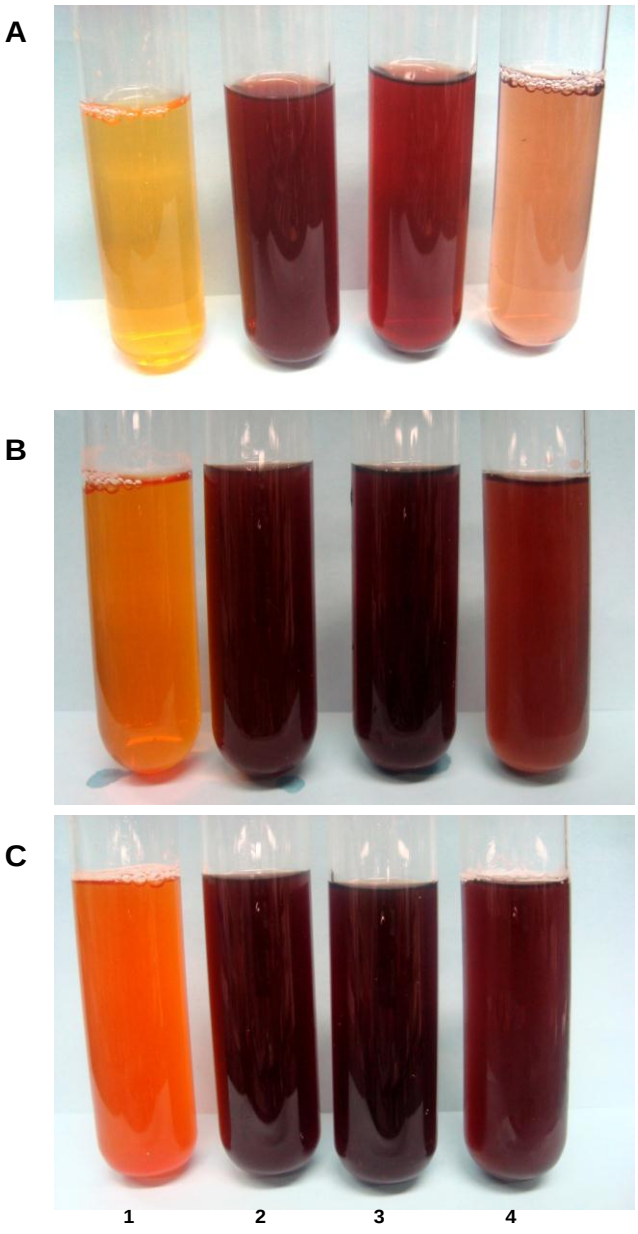


Fig. 17. Phenolic extracts from black sorghum bran extracted by ASE at: A (60°C), B (120°C), C (150°C).

1= Citric Acid (pH 2.5); 2= Water; 3= 50% ethanol in water; 4= 70% ethanol in water

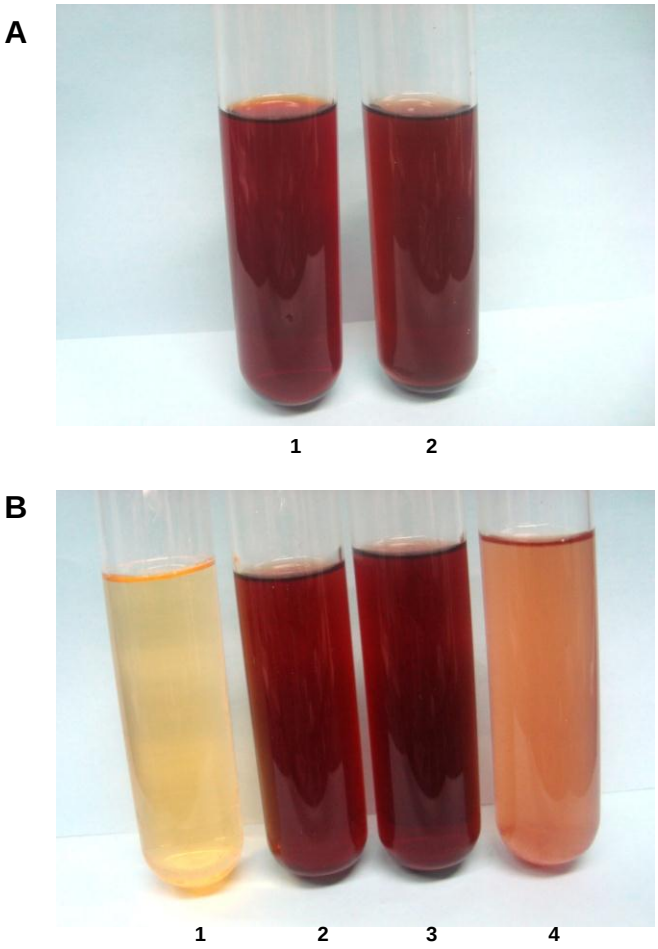


Fig. 18. Phenolic extracts from black sorghum bran extracted by conventional method (1 atm/25^{o}C).

A- 1 (1% HCL in methanol); 2 (70% acetone in water)

B- 1 (Citric acid pH 2.5); 2 (50% ethanol in water); 3 (70% ethanol in water); 4 (water)

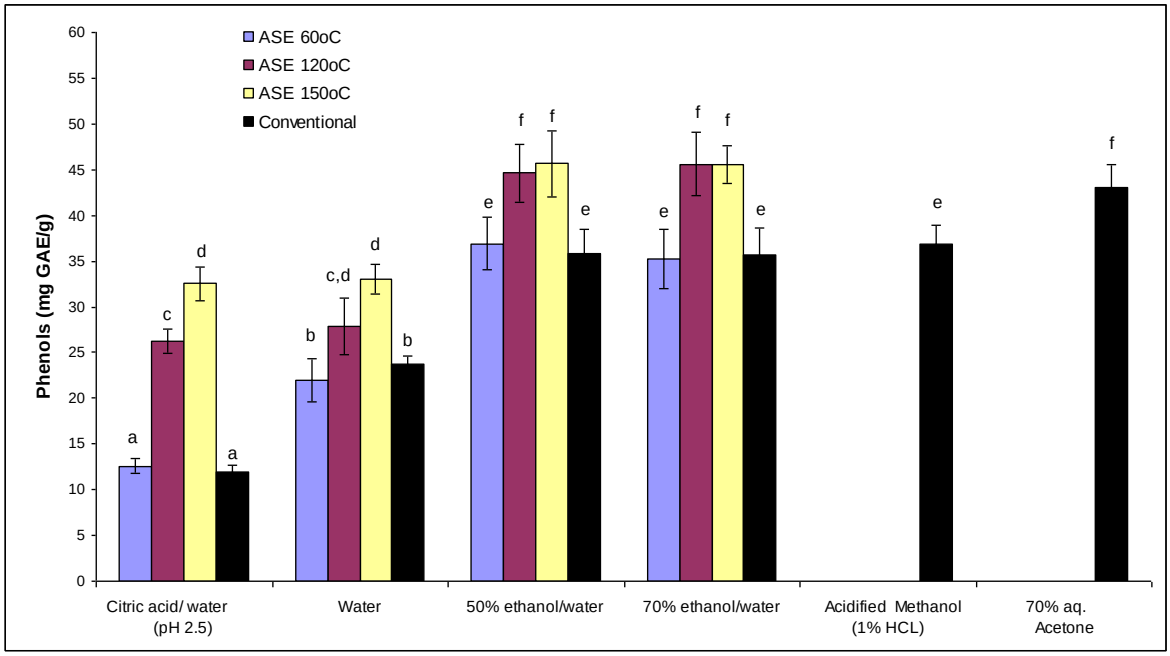


Fig. 19. Total phenol content (mg GAE/g) of black sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Total phenols (about 35 mg GAE/g) of ASE extractions at 60^{o}C using ethanol (50 and 70%) in water did not significantly differ ($P > 0.05$) from conventional extraction using the same ratios of ethanol/water and acidified methanol; however, conventional extraction with 70% acetone in water had highest phenol content (43 mg GAE/g) (Fig. 19). Total phenols of ASE extraction using water and citric acid/water (pH 2.5) were 22 and 12.5 mg GAE/g respectively which were statistically the same ($P > 0.05$) compared to the conventional extraction using either solvents (Fig. 19).

By increasing the temperature to 120^{o}C, total phenols of ASE extractions using ethanol (50 and 70%) in water increased to about 45 mg GAE/g (increase of 29%) which was significantly higher ($P \leq 0.05$) than conventional extraction using ethanol/water and acidified methanol (about 35 mg GAE/g), and it was statistically the same ($P > 0.05$) concentration as conventional extraction using 70% acetone/water (Fig. 19). Total phenols of ASE extractions using water and citric acid/water (pH 2.5) increased to about 26 mg GAE/g respectively, which was higher than extractions at 60^{o}C and than conventional extraction using water and citric acid/water (Fig. 19).

Total phenol concentration of ASE extractions at 150^{o}C were similar to 120^{o}C when ethanol (50 and 70%) in water were used (Fig. 19).

Phenol concentration of about 32 mg GAE/g was obtained when water and citric acid/water were used in the ASE extraction at 150^{o}C (Fig. 9). This concentration was slightly lower ($P \leq 0.05$) than conventional extraction using acidified methanol (36.8 mg GAE/g) (Fig. 9).

Thus, ASE extractions at 120°C and 150°C were effective in extracting more phenols from black sorghum extracts compared to conventional methods. ASE extracts using ethanol (50 and 70%) in water at 120°C and 150°C had as high phenol content as conventional extraction with 70% acetone/water (about 45 mg GAE/g). ASE extractions using water and citric acid/water (pH 2.5) at 150°C significantly increased phenol concentration (around 32 mg GAE/g).

The effect of conventional extraction (25°C/1 atm) and ASE extraction at different temperatures on antioxidant capacity of black sorghum bran is shown in Fig. 20. Antioxidant capacity of ASE extracts using water and citric acid/water at 60°C (361 and 257 μmol TE/g respectively) significantly ($P \leq 0.05$) increased compared to conventional extractions using same solvents (267 and 178 μmol TE/g respectively) (Fig. 20). ASE extracts of ethanol/water treatments at 60°C had statistically the same ($P > 0.05$) antioxidant capacity compared to conventional extractions using ethanol/water (about 500 μmol TE/g) (Fig. 20). Conventional extractions using acidified methanol and 70% acetone in water had higher ($P \leq 0.05$) antioxidant capacity (about 540 μmol TE/g) compared to other treatments at 60°C (Fig. 20).

Higher antioxidant capacity was obtained in the ASE extracts at 120°C and 150°C (Fig. 20). Water and citric acid extracts (from ASE) had similar antioxidant capacity (about 400 μmol TE/g) which was much higher than ASE extracts at 60°C and conventional extractions using these solvents (Fig. 20).

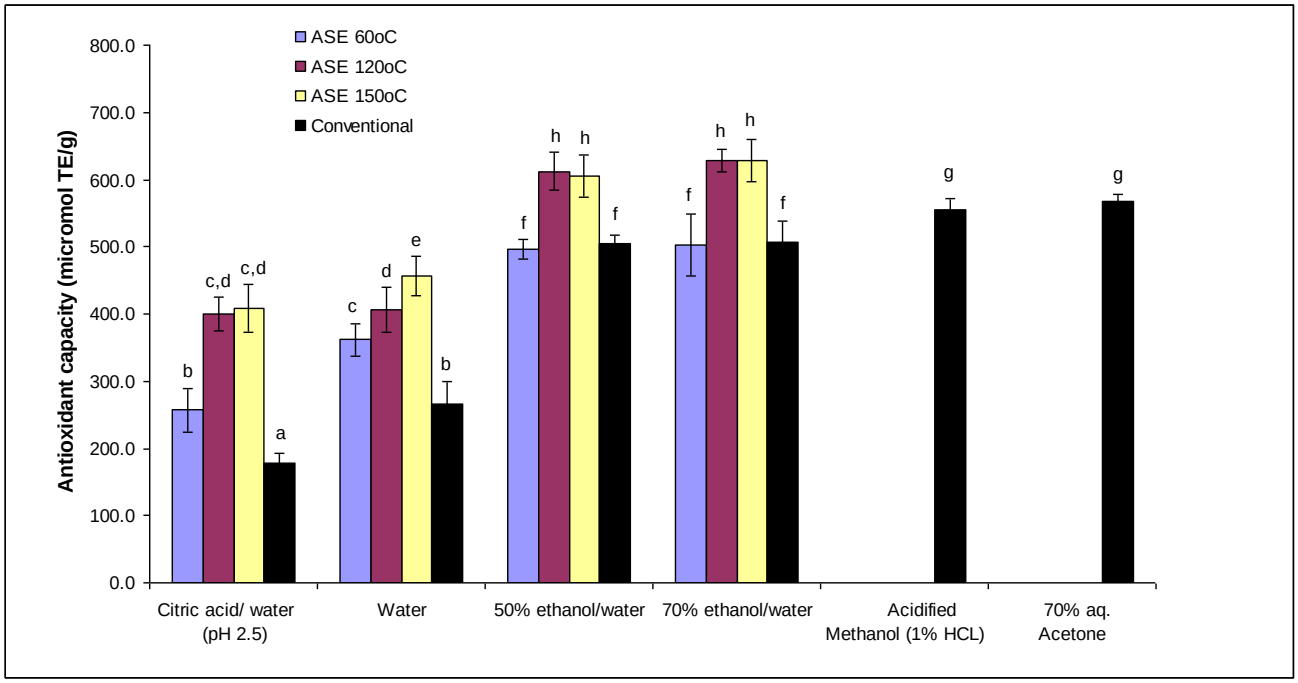


Fig. 20. Antioxidant capacity (μmol TE/g) of black sorghum bran extracted by conventional extraction (25°C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

ASE extracts of ethanol (50 and 70%) in water had antioxidant capacity of about 620 μmol TE/g which was higher than all treatments, including conventional extractions using acidified methanol and 70% acetone in water (Fig. 20). The higher antioxidant levels in the ASE treatments using ethanol/water at 120 and 150^{o}C may be due to the formation of thermal degradation compounds that have antioxidant capacity such as the ones formed in Maillard reaction (Eichner, 1981; Anese et al. 1999).

Thus, ASE extraction at temperatures above 100^{o}C (120^{o}C and 150^{o}C) was effective in increasing antioxidant activity of black sorghum extracts using "friendly" solvents. Despite the short time of extraction (10 min), high temperature and pressure enhanced extraction of phenols and antioxidants. High temperature and pressure increase diffusion rates and disrupt some of the solute-matrix interactions which help to increase extraction rate (Lou et al. 1997; Richter et al 1996).

High tannin sorghum extracts

ASE and conventional extracts are shown in Figs 21 and 22.

The effect of conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures on total phenol content of tannin sorghum bran is shown in Fig. 23. ASE extractions using ethanol/water was not effective in increasing total phenol content compared to conventional extractions using the same solvents. Similar or lower phenol content was observed in ASE extractions compared to conventional methods using ethanol/water (Fig. 23).

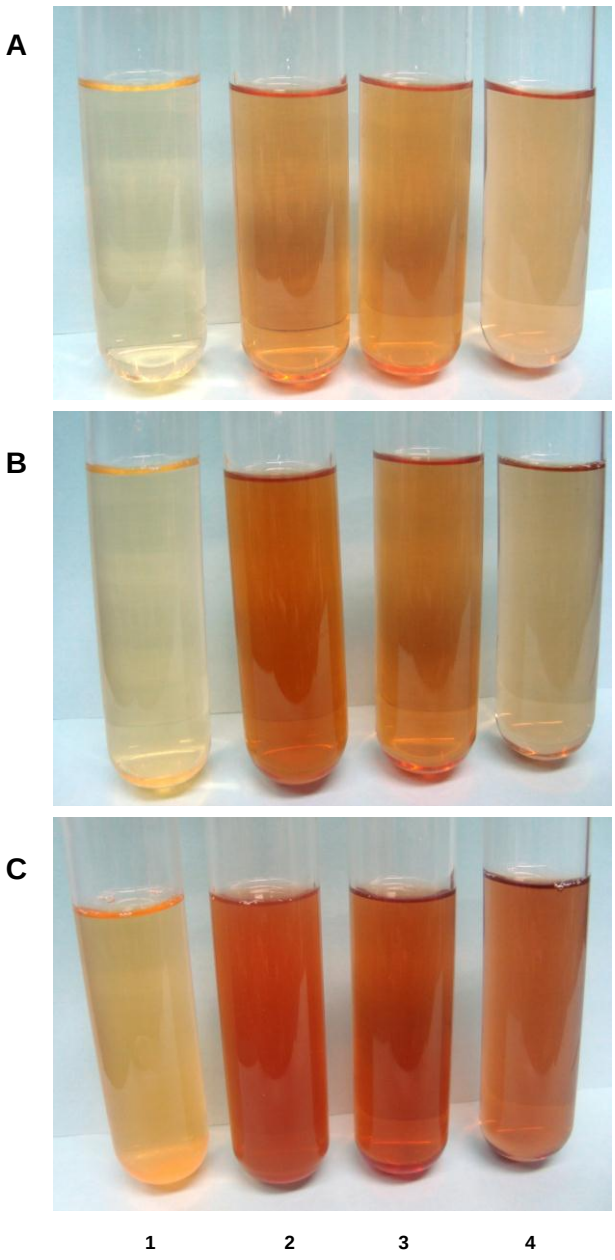


Fig. 21. Phenolic extracts from tannin sorghum bran extracted by ASE at: A (60°C), B (120°C), C (150°C).

1= Citric Acid (pH 2.5); 2= Water; 3= 50% ethanol in water; 4= 70% ethanol in water

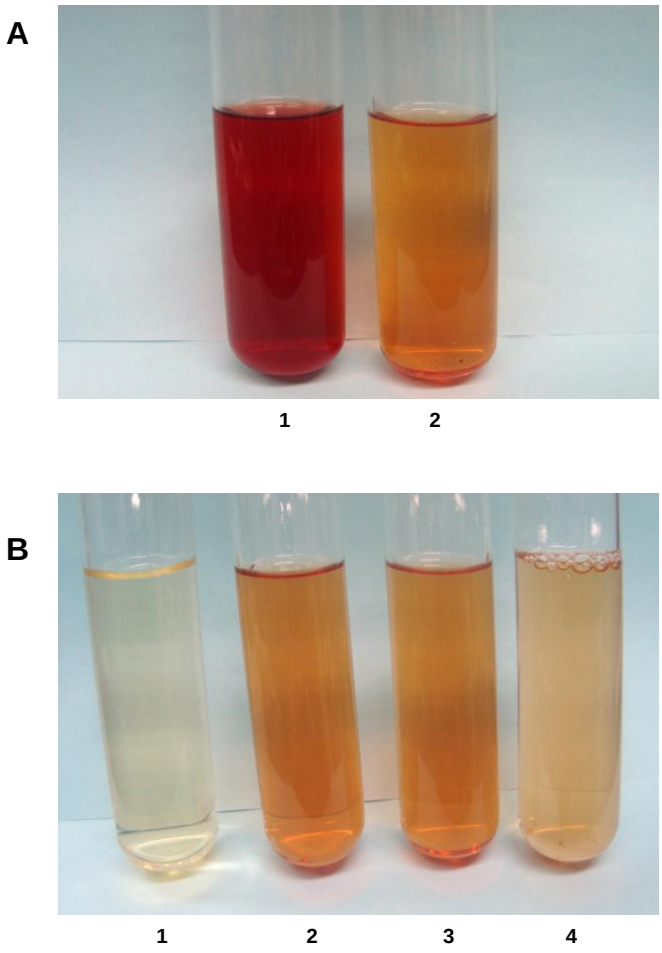


Fig. 22. Phenolic extracts from tannin sorghum bran extracted by conventional method (1 atm/25^{o}C).

A- 1 (1% HCL in methanol); 2 (70% acetone in water)

B- 1 (Citric acid pH 2.5); 2 (50% ethanol in water); 3 (70% ethanol in water); 4 (water)

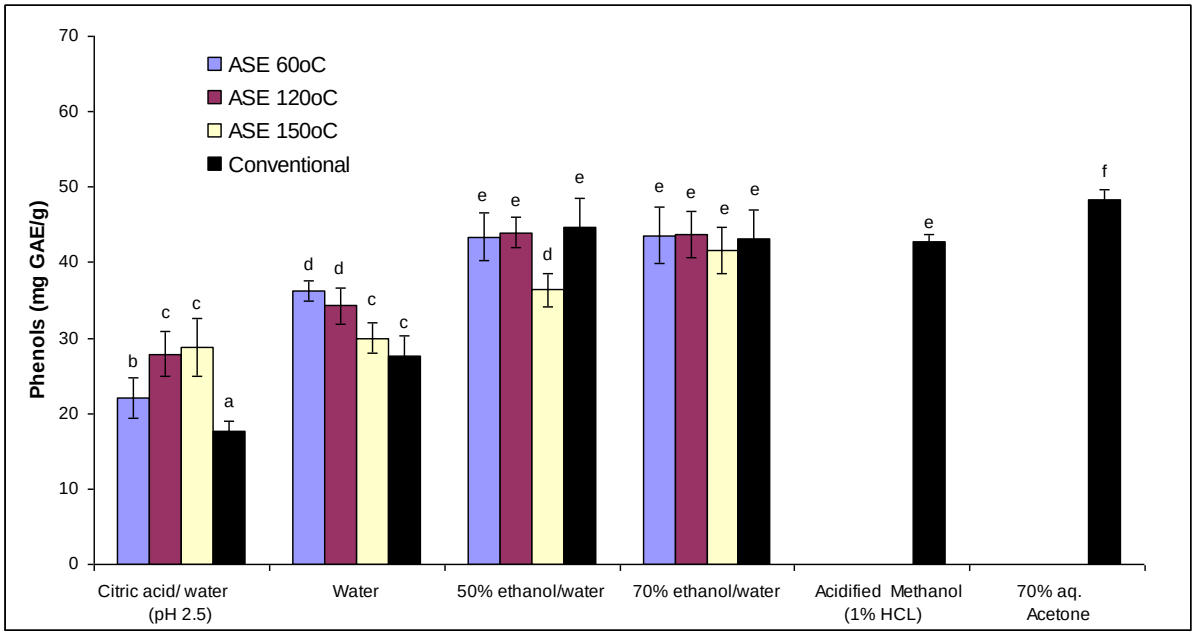


Fig. 23. Total phenol content (mg GAE/g) of tannin sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

However, total phenols of ASE extraction using water and citric acid were slightly higher compared to conventional extraction using these solvents (Fig. 23). Overall, a different trend was observed in the total phenol data of tannin sorghum bran compared to black sorghum bran. Tannin sorghum extract with high phenol content, comparable to acidified methanol and 70 % acetone in water, was obtained using ethanol (50 or 70%) in water at 1 atm/25^{o}C (conventional extraction) (Fig. 23).

Despite ASE extracts using ethanol/water had similar total phenols as conventional extracts, the extraction time in ASE is around 10 min, versus 2 h used for conventional extraction which may make ASE extraction preferred over the conventional.

The effect of conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures on antioxidant capacity of tannin sorghum bran is shown in Fig. 24. ASE extracts using 70% ethanol in water at 150^{o}C had statistically the same ($P > 0.05$) antioxidant capacity (about 625 μmol TE/g) than conventional method using 70% acetone in water (Fig. 24). Antioxidant capacity of ASE extracts with water and citric acid had maximum value at 120^{o}C (423 and 370.2 μmol TE/g, respectively) which was significantly higher ($P \leq 0.05$) than conventional extracts with water and citric acid (340 and 288 μmol TE/g, respectively) (Fig. 24). As previously explained in this study, this high antioxidant levels may be due to the formation of thermal degradation compounds having antioxidant capacity.

The increase in antioxidant capacity and decrease in phenols as ASE temperature increased has also been reported by Ju and Howard (2005).

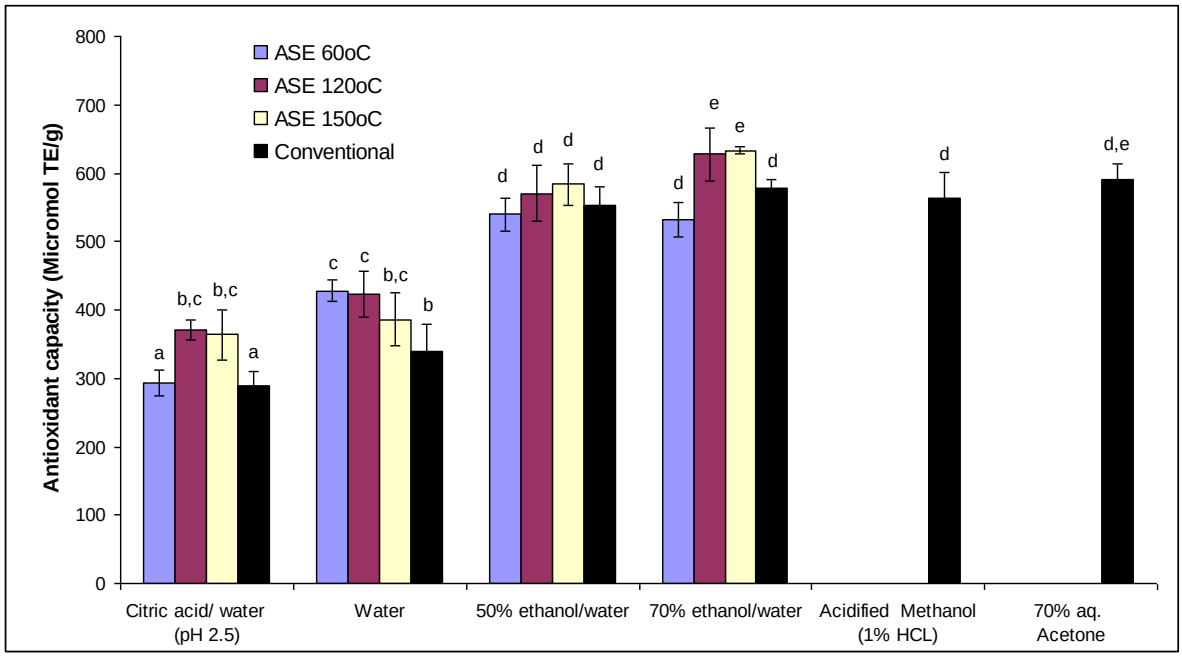


Fig. 24. Antioxidant capacity (µmol TE/g) of tannin sorghum bran extracted by conventional extraction (25°C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

Furthermore, they demonstrated that water alone was able to extract, using ASE at temperatures above 100^{o}C, same concentration of phenols and more antioxidants than conventional extraction using 60% methanol. In our study, ASE extractions with water increased phenol and antioxidant contents compared to conventional extraction using water, but they were always lower than conventional method using acetone in water and acidified methanol. This could be due to the fact that phenolic compounds in fuits/vegetables are in free forms, whereas the ones in cereals are mostly bound to the cell wall which make them more difficult to extract (Perez-Jimenez and Saura-Calixto, 2005).

Overall, ASE extractions were more effective for black sorghum than tannin sorghum bran. The percentage of increase in phenols and antioxidants using ASE at 120^{o}C and 150^{o}C compared to conventional extraction using same solvents was higher for black sorghum than tannin sorghum. Phenol content of ASE extracts of black sorghum bran using citric acid in water, water and ethanol/water at temperatures above 100^{o}C increased 169%, 35% and 29%, respectively, compared to conventional extraction using same solvents. Antioxidant capacity increased 124%, 69% and 24%, respectively. The highest phenol and antioxidant content obtained by ASE were 45.6 mg GAE/g and 628 μmol TE/g using ethanol/water as solvent. Much lower increase was observed for tannin sorghum bran extracts. Phenol content increased 59%, 23 and 0%, respectively, and antioxidant capacity increased 28%, 26% and 5%, respectively. The highest phenol and antioxidant content obtained by ASE were 44 mg GAE/g and 630 μmol TE/g using ethanol/water as solvent. These are promising data since it is possible to obtain

significant higher concentration of phenols and antioxidants by extracting sorghum bran, especially black sorghum, using water, acidified water and ethanol/water in ASE.

Tannin sorghum is known to have higher concentration of phenols and antioxidants than black sorghum. However, highest phenols obtained was by using either ASE or conventional method with 70% ethanol in water (48 mg GAE/g) and highest antioxidant activity was similar to black sorghum bran (about 630 μmol TE/g). ASE did not increase phenols and antioxidants in tannin sorghum as in black sorghum. This could be because of structure differences between the 2 samples, in other words, how strong the phenolic compounds are bound in the cell wall material in black compared to tannin sorghum bran. Based on this study, it seems like to be easier to extract phenolic acids and monomeric polyphenols such as 3-deoxyanthocyanins (major phenolic compounds present in black sorghum) than larger molecular weight polyphenols such as oligomeric and polymeric proanthocyanidins (major phenolic compounds present in tannin sorghum) which may be trapped in the cell wall more strongly and extraction becomes difficult even under high pressure and temperature. Another explanation is that maybe the tannins precipitate into insoluble complexes under heat, thus they could be extracted but not measured.

3-deoxyanthocyanins content in black sorghum

The effect of conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures on 3-deoxyanthocyanins concentration of black sorghum bran is shown in Figs. 25-28.

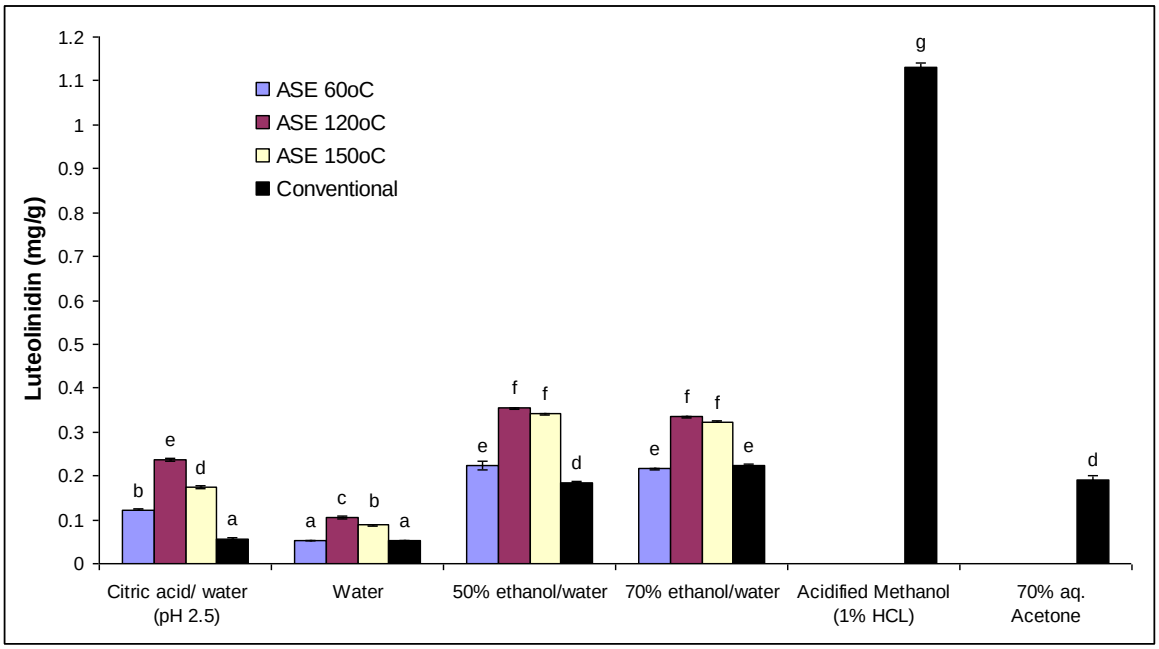


Fig. 25. Luteolinidin (mg/g) in black sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

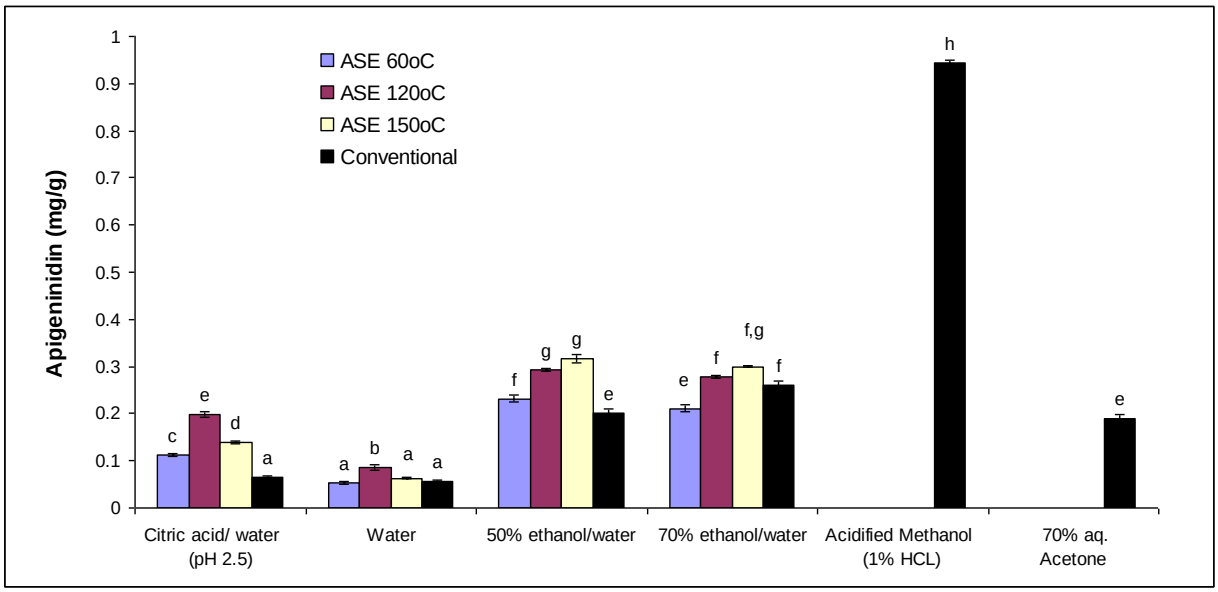


Fig. 26. Apigeninidin (mg/g) in black sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

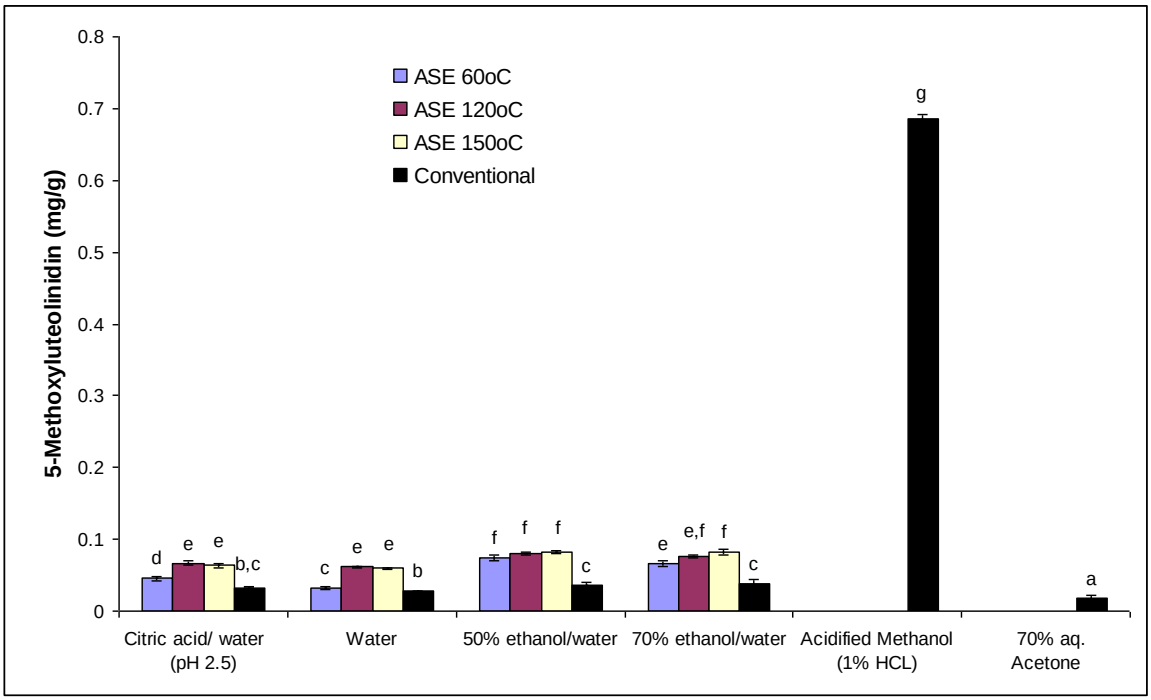


Fig. 27. 5-Methoxyluteolinidin (mg/g) in black sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

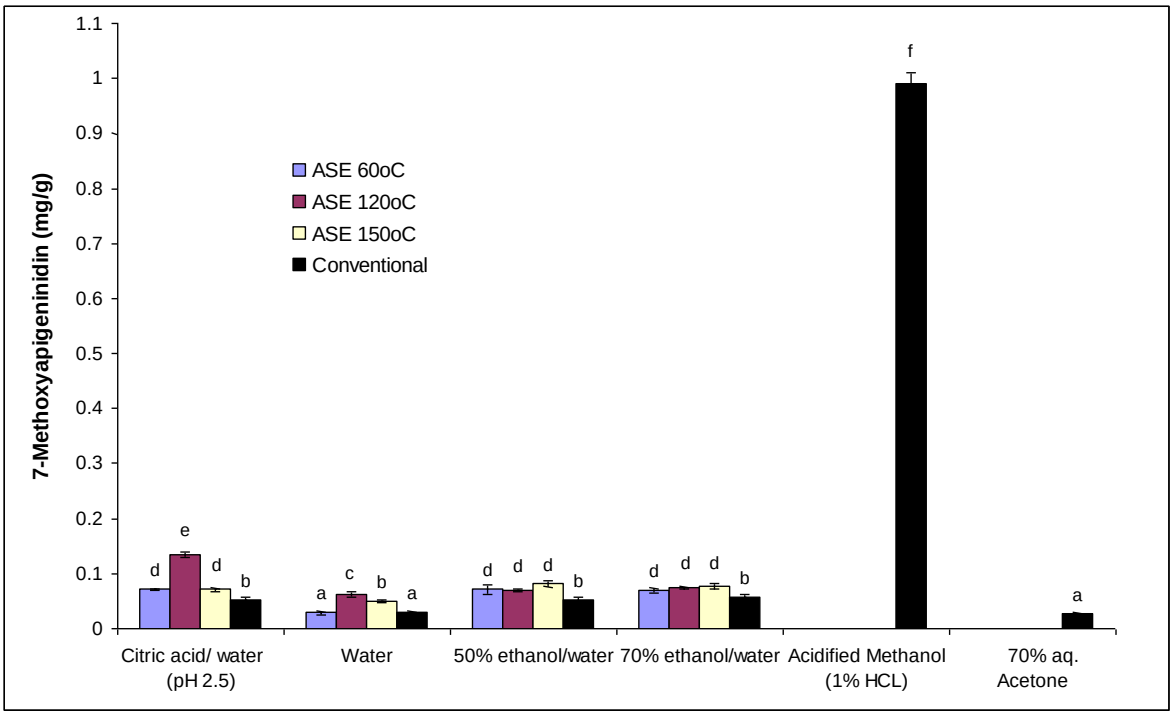


Fig. 28. 7-Methoxyapigeninidin (mg/g) in black sorghum bran extracted by conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures.

Errors bars indicate ± standard deviation. Means followed by the same letter are not significantly different ($P \leq 0.05$).

It is known that acidity is important for extraction of 3-deoxyanthocyanins in sorghum (Awika, 2003; Dykes et al. 2009). The most used solvent is the acidified (1% HCL) methanol. In this study, conventional extraction with acidified methanol had the highest values for all 3-deoxyanthocyanins (Figs. 25-28). It is also known that acetone is not a good solvent to extract anthocyanins because a reaction that take place between them decreasing anthocyanin concentration (Lu and Foo, 2001). Very low levels of all 3-deoxyanthocyanins were observed when 70% acetone in water was used (conventional extraction) (Fig. 25-28).

Overall, citric acid in water (pH 2.5) extracted more 3-deoxyanthocyanins than water (Fig. 25-28) due to lower pH. ASE extractions at all temperatures were effective to extract luteolinidin compared to conventional extractions (using same solvents) (Fig. 25). The highest concentration was obtained when 50% and 70% ethanol in water were used in the ASE extraction (Fig. 25). Same trend was observed in the extraction of apigeninidin (Fig. 26), 5-methoxyluteolinidin (Fig. 27) and 7-methoxyapigeninidin (Fig. 28).

Thus, ASE extraction with ethanol/water significantly increased concentration of 3-deoxyanthocyanins from black sorghum compared to conventional extractions using citric acid in water, water and (50, 70%) ethanol in water, but were lower than extractions with acidified methanol (conventional). Moreover, concentration of 3-deoxyanthocyanins tended to increase as temperature of extraction increased. The highest concentrations were obtained at 120^{o}C and 150^{o}C using 50 and 70% ethanol in

water. This shows that the 3-deoxyanthocyanins were thermo-stable under conditions used in the ASE.

Monrad et al. (2010a) reported that high levels of ethanol (50 to 70%) in water were necessary to optimize extraction of anthocyanins from red grape pomace using ASE and the optimum temperatures were above 100^{o}C. All extracts from ASE collected from 40 to 140^{o}C contained comparable amounts of anthocyanins as the conventional extraction (methanol/water/formic acid). From our study, in order to obtain more anthocyanins in solutions, acidified solvents should be used because of the bound characteristic of cereal polyphenols.

Catechin and proanthocyanidin content of tannin sorghum

The effect of conventional extraction (25^{o}C/1 atm) and ASE extraction at different temperatures on catechin (monomer) and proanthocyanidin (oligomer and polymer) concentrations of tannin sorghum bran is shown in Table XI.

It was demonstrated that as temperature increased in the ASE extractions, catechin concentration increased (Table XI). It was shown in Chapter III and IV that catechin concentration increased under heat treatment due to depolymerization of polymeric proanthocyanidins. Low pH is also known to break down polymeric and oligomeric proanthocyanidins producing catechin and dimers (Esatbeyoglu and Winterhalter, 2010). The conventional method using acidified methanol had higher concentration of catechins but very low polymeric proanthocyanidins (Table XI).

Table XI: Concentration (mg/g) [a] of proanthocyanidin monomer (catechin), oligomers and polymers in tannin sorghum bran extracts as affected by ASE at different temperatures compared to conventional extraction (25°C/ 1 atm) [b]

Solvent	Temperature (°C)	Catechin	Oligomers (2≤DP≤10)	Polymers (DP >10)
ASE				
Citric acid in water (pH 2.5)	60	0.04 ± 0.0	3.06 ± 0.11	7.16 ± 0.43
	120	0.12 ± 0.0	3.41 ± 0.15	5.06 ± 0.22
	150	0.49 ± 0.01	5.00 ± 0.21	4.24 ± 0.37
Water	60	0.05 ± 0.0	3.47 ± 0.10	8.00 ± 0.61
	120	0.10 ± 0.0	0.26 ± 0.01	2.89 ± 0.09
	150	0.38 ± 0.01	0.58 ± 0.03	3.43 ± 0.15
50% ethanol/water	60	-	5.65 ± 0.37	17.1 ± 0.55
	120	0.10 ± 0.0	5.88 ± 0.33	18.8 ± 0.95
	150	0.31 ± 0.02	5.21 ± 0.22	15.0 ± 0.66
70% ethanol/water	60	-	5.21 ± 0.43	15.92 ± 0.49
	120	0.06 ± 0.0	5.77 ± 0.33	17.83 ± 0.87
	150	0.50 ± 0.02	5.71 ± 0.18	14.98 ± 1.10
Conventional				
Citric acid in water (pH 2.5)	-	0.04 ± 0.0	3.16 ± 0.23	4.26 ± 0.21
Water	-	0.05 ± 0.0	0.28 ± 0.01	4.76 ± 0.19
50% ethanol/water	-	0.09 ± 0.0	5.87 ± 0.24	23.10 ± 0.11
70% ethanol/water	-	0.09 ± 0.0	5.73 ± 0.34	25.55 ± 0.42
Acidified methanol (1%HCL)	-	0.58 ± 0.03	2.45 ± 0.16	1.84 ± 0.06
70% aq. Acetone	-	0.07 ± 0.0	7.12 ± 0.52	26.40 ± 1.31

[a] Expressed as catechin equivalent (corrected by molecular weight); [b] Values are means ± standard deviation; DP= Degree of polymerization

From the ASE extractions, treatments containing citric acid in water (pH 2.5) and 70% ethanol in water at 150^{o}C had the highest level of catechins (0.5 mg/g) (Table XI). Water extraction by ASE at 150^{o}C produced 0.38 mg/g of catechin.

Conventional extraction of tannin sorghum bran using 70% acetone in water had the highest level of polymeric proanthocyanidin (26.4 mg CE/g) (Table XI). Aqueous acetone is known as the best solvent to extract high molecular weight proanthocyanidins (Prior and Gu, 2005). Conventional extractions containing ethanol/water had relative high levels of polymeric proanthocyanidin but lower than acetone extraction (Table XI). ASE ethanol/water extraction had less (15 to 18 mg CE/g) of the large molecular weight proanthocyanidin than conventional extraction (23 to 26 mg CE/g), maybe because of heat depolymerization, since conventional extraction was done at room temperature and ASE at high temperatures (60, 120 and 150^{o}C).

Monrad et al. (2010b) showed that optimal extraction of proanthocyanidins of red grape pomace using ASE was at temperatures above 80^{o}C using 50% ethanol in water. However, they also observed that aqueous acetone was more efficient to extract polymeric proanthocyanidins in a conventional extraction compared to treatments using ethanol/water in ASE.

Although ASE was less effective than conventional extraction using aqueous acetone in extracting high molecular weight proanthocyanidins, it extracted relatively high concentration of catechins, and low molecular weight proanthocyanidins.

***In vitro* starch digestibility**

The effect of tannin sorghum phenolic extracts (by ASE and conventional extraction) on resistant starch content of high amylose starch cooked (121^{o}C/30min)/cooled (4^{o}C) (3 heating/cooling cycles) is shown in Fig. 29.

It was observed that no treatment had such high RS by using other solvents in the conventional extractions and in ASE compared to aqueous acetone extraction (40% RS) (Fig. 29). Acetone extracts had more of the polymeric proanthocyanidins and thus presented higher RS content. Ethanol/water extracts when cooked with high amylose starch had about 33% RS, which was higher than control (25%, showed in Chapters III and IV) but lower than conventional extraction using aqueous acetone. Other treatments using citric acid in water and water cooked with high amylose starch had less than 30% RS (Fig. 29).

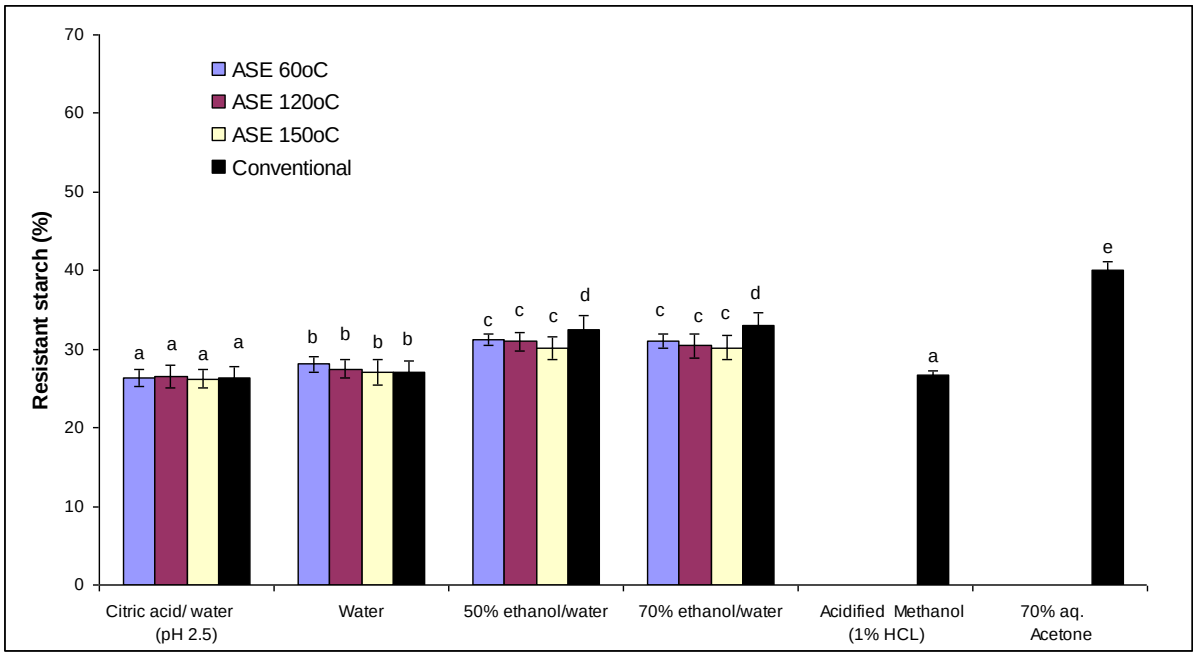


Fig. 29. Effect of tannin sorghum phenolic extracts (10% starch basis) from ASE at different temperatures and by conventional extraction (25^{o}C/ 1 atm) on resistant starch content of high amylose starch cooked in an autoclave (121^{o}C/30min) and cooled (4^{o}C) overnight (3 heating/cooling cycles).

Errors bars indicate ± standard deviation. Means followed by the same letter within treatment are not significantly different ($P \leq 0.05$).

CHAPTER VI

SUMMARY AND CONCLUSIONS

This is the first study that demonstrates specific interactions between condensed tannins and starch molecules (amylose and amylopectin). Sorghum condensed tannins are more effective in interacting with amylose possibly through hydrophobic and hydrogen bonding, significantly increasing RS content of normal and high amylose starches compared to monomeric sorghum polyphenols such as phenolic acids and 3-deoxyanthocyanins. Thus high molecular weight polyphenols may provide new opportunities to produce functional food ingredients that reduce caloric density of starch-containing products while providing added health benefits.

ASE at temperatures above 100^{o}C using water and ethanol/water significantly improved extraction of polyphenols from black sorghum compared to conventional extractions using the same solvents. The same amount of phenols and antioxidants were obtained when ethanol/water was used compared to extractions using aqueous acetone and acidified methanol. This opens opportunities for use of these aqueous extracts from black sorghum in colorant, foods and beverages with potential health benefits.

SUGGESTIONS FOR FURTHER RESEARCH

Our work demonstrated that there are strong interactions between starch and sorghum tannins which increase resistant starch formation. More studies aiming to understand specific mechanisms of interactions between condensed tannins and starch should be done. Analyses using NMR, X-ray, FT-NIR and DSC will help to investigate

presence of hydrogen, hydrophobic bonding or any other interaction present. This information will be used to promote the utilization of tannin sorghum grains, their phenolic extracts and purified proanthocyanidins.

More work should be done on the ASE. Acidified ethanol/water should be used in order to extract more anthocyanins from black sorghum. Alkaline treatment and enzyme (break down cell wall components) pre-treatment should be done in order to improve efficiency of polyphenols using ASE.

A material from cooking/cooling high amylose starch with different levels of sorghum tannin bran could be tested in-vivo and in humans to understand the effects of interactions between tannins/starch on postprandial glycemic response.

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APPENDIX A

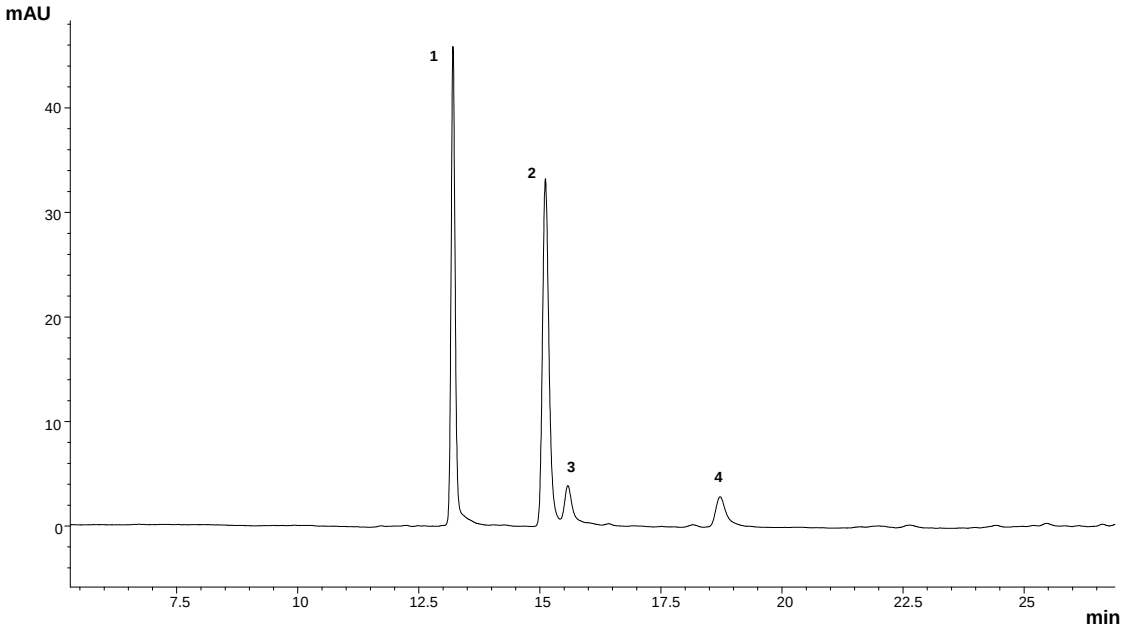
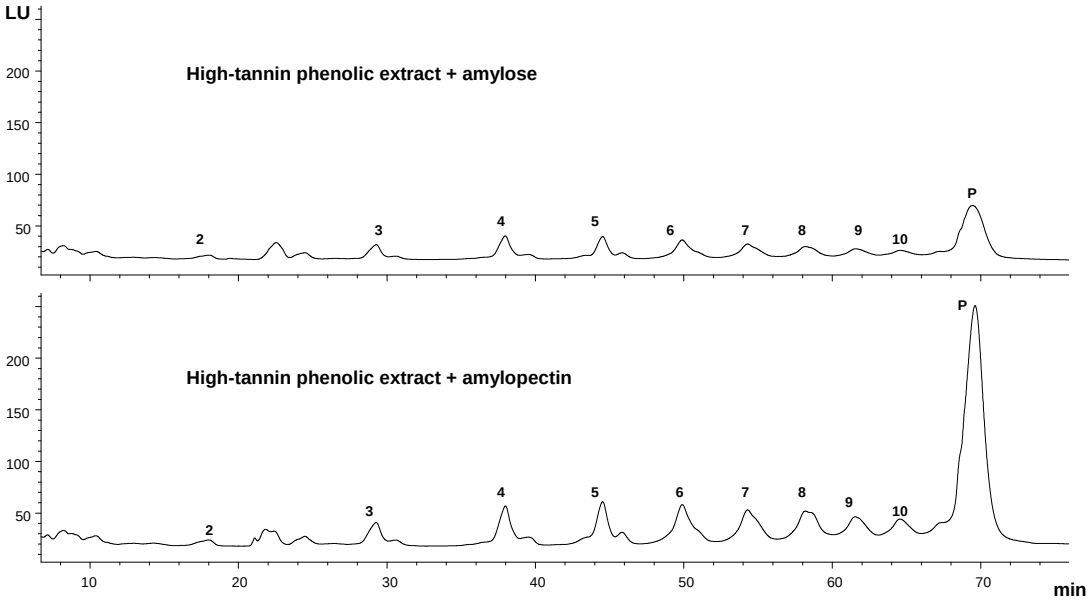


Fig. A1. Deoxyanthocyanin levels (mg/g) in the freeze-dried black sorghum phenolic extract [a] Analytical HPLC chromatogram monitored at 480 nm.

1- Luteolinidin (3.95 ± 0.33)
2- Apigeninidin (3.81 ± 0.21)
3- 5-Methoxyluteolinidin (1.10 ± 0.07)
4- 7-Methoxyapigeninidin (1.66 ± 0.13)

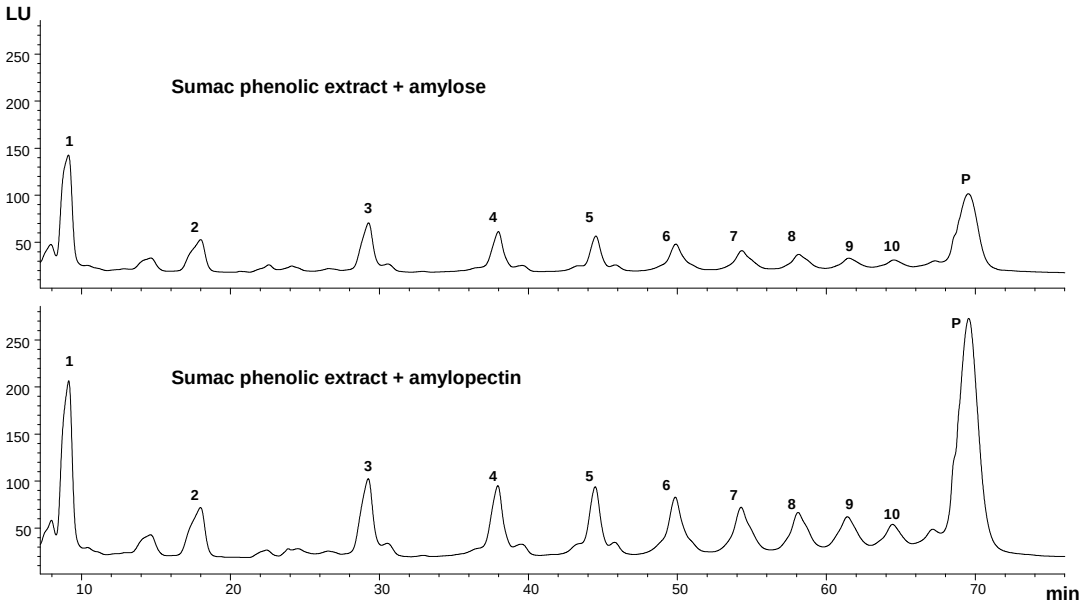
[a] Values are mean of triplicates ± standard deviation.



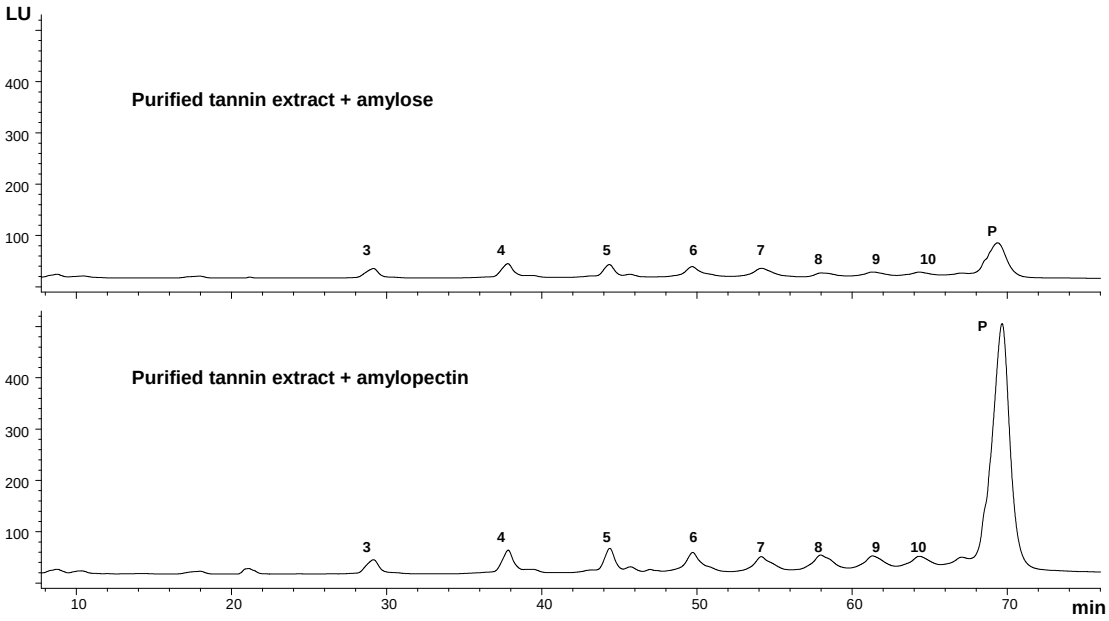
:d

with amylose and amylopectin.

Numbers on peaks denote degree of polymerization. P = polymers with DP >10.



Numbers on peaks denote degree of polymerization. P = polymers with DP >10.



Numbers on peaks denote degree of polymerization. P = polymers with DP >10.