EXTRACTABILITY PROFILING AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS IN SORGHUM GRAIN AND NON-GRAIN MATERIALS

A Dissertation

by

NENGE LYNDA AZEFOR NJONGMETA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Food Science and Technology

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ABSTRACT

Extractability Profiling and Antioxidant Activity of Flavonoids in Sorghum Grain and Non-grain Materials. (May 2009)

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Chair of Advisory Committee: Dr. Lloyd W. Rooney

Grains, leaves, sheaths, glumes and stalks of sorghum varieties were analyzed for total phenols, condensed tannins, flavan-4-ols, anthocyanins and *in vitro* antioxidant activity. Black sorghum bran was used to evaluate the effectiveness of organic acids and enzymes on extractability of phenols. Flavonoid profiles of grains and non-grain tissues were determined and characterized using HPLC-PDA and HPLC-ESI-MS^n.

The presence of a pigmented testa and spreader genes (B_1B_2S) is a predictor for polymeric flavonoids (tannins) but not for simple phenols such as flavan-4-ols, 3-deoxyanthocyanins, flavones and flavanones. Simple flavonoids increased antioxidant capacity of sorghum, and were present in all sorghum except for the white pericarp sorghums that did not have flavanones. The “red turning into black” gene increased phenols in Type I sorghum.

The leaves, sheath and glumes of sorghum had higher levels of phenols

(78-600 times more), with *in vitro* antioxidant properties than commonly seen in grains. Pigmentation of plant components increased levels of 3-deoxyanthocyanins but not flavones nor flavanones. The leaves of biomass sorghum, *Collier* variety, had 3.4 times more 3-deoxyanthocyanins than the leaves of Tx430 Black x Sumac which had the highest levels (1810 µg/g) of 3-deoxyanthocyanins among the leaves.

The use of 1% HCl/ethanol provides a possible food grade substitute solvent for 1%HCl/methanol in the extraction of phenolic compounds from sorghum. All enzymes evaluated broke down bran particles forming a gel-like material which had increased phenols and antioxidant activities but not 3-deoxyanthocyanins as revealed by HPLC analysis. Microscopy examination showed the gel matrix rich in fiber and can possibly be used for nutraceutical applications. Careful understanding of enzyme activities is necessary for effective extraction of 3-deoxyanthocyanins from sorghum.

Sorghum leaves, sheaths and glumes are excellent sources of bioactive compounds, up to 600 times more than the grains of some varieties. Sorghum with the “red turning to black genes” is a potential source of 3-deoxyanthocyanins and flavan-4-ols. With the trend towards sorghum as biomass for ethanol production, plant breeders must select special traits aimed at developing enhanced desired functionality such as antioxidant potential and other healthy attributes with application in food, pharmaceutical/nutraceutical and cosmetic industries.

DEDICATION

To my children:

Ndallah Ntunibu Njongmeta

and

Maema Shiminyie Njongmeta

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my supervisor Dr. Lloyd Rooney, for the opportunity to study under his guidance and most especially for his support in making it possible for me to achieve this milestone in my career. I am indebted to Dr. Jimmy Keeton for his keen interest in my career and for serving on my committee. I am also grateful to my other committee members: Dr. Luis Cisneros-Zevallos for his valuable feedback throughout my research and Dr. Steve Talcott for continuous guidance and for granting me unconditional access to his LS-MS and ORAC equipment.

Special thanks to Dr. William Rooney for providing the samples for this work and for helping me understand the agronomics of sorghum. I appreciate the suggestions and comments from Dr. Joseph Awika and in whose lab part of the ORAC analysis was done. I wish to thank Dr. Linda Dykes for helping me understand HPLC analyses and for her comments and suggestions throughout my research; Lisbeth Pacheco-Palencia for taking me through Mass Spectrophotometry and Cassandra McDonough for her help with microscopy and for her friendship. My gratitude also goes to Victor Taleon for his help in sample collection from the field and for all his support. Thanks to Pamela Littlejohn for all she does. I am very appreciative of the team spirit, friendship and support of the members of the Cereal Quality Laboratory that I have worked with over the years.

I wish to thank the Fulbright program for sponsorship to study at Texas A&M University and the University of Ngaoundere for granting me permission to pursue my studies.

I owe many thanks to my Mum Emmerentia Azefor and Dad James Azefor (RIP) who instilled in me the gift of perseverance. I am particularly indebted to my brothers: Jude and Ephraim and my sisters: Mabi, Ngembong, Ngenwie and Ida for their outstanding love, support and encouragement. You all stand out in your unique nature. Special thanks to George, Atsi and Fred for their prayers and encouragement. Thanks to my nieces for all their prayers and their little giggles in very dark moments of my Ph.D program. Special thanks to Dr. Awahmukalah Dominic for his encouragement throughout my education. Special thanks to all my family and friends. I am indebted to Mr. Aloysius Chi and Mrs. Jackie Chi for their friendship and support.

My most loving thanks are due my beloved husband and friend, Dr. Leo Moh Njongmeta; you held me up through the most challenging times in my life and through the toughest moments of my education. Thank you for believing in me more than I do in myself. Finally, I want to thank my kids, Mambo, Maema and Ndallah for their endurance, understanding, unflinching love and support; you are the reason I did this. Thank you for your ultimate cooperation. I want you to know you mean so much to me and I love you the whole wide world and back again.

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CHAPTER I

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop in the world and a dietary staple of more than 500 million people in more than 30 countries (ICRISAT 2009) in tropical Africa, South America, central and north India, and China. It plays an important role in food security as well as a source of income (Dendy 1995; Anglani 1998; Taylor & Dewar 2001; Belton & Taylor 2004; Dicko et al 2006). It is one of the most drought tolerant cereal crops currently under cultivation (Elkin et al 1996) and thrives in semi-arid regions.

Sorghum contains a wide variety of phenolic compounds which affect the classification, color, appearance, nutritional quality and functionality of the grain. The levels and profiles of phenolic compounds present in each sorghum cultivar are unique and influenced by both genetics and the environmental conditions under which the grain matures (Awika and Rooney 2004; Dykes and Rooney 2006; Krueger et al 2003).

Phenolic compounds are synthesized by plants during normal development (Harborne 1991; Pridham 1959) as phytoalexins, contributing to resistance against microorganisms/mold, insects/predation and against stress

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This dissertation follows the style of Cereal Chemistry.

from UV-light among others (Lo et al 1999; Seitz 2004; Serna-Saldivar and Rooney 1995; Waniska and Rooney 2000; Waniska et al 2001). Sorghum phytochemicals also act as antioxidants as demonstrated by *in vitro* assays (Awika et al 2003b; Hagerman et al 1998) and some are known to be more potent than vitamins found in other plants (Rhodes and Price 1997).

The chemo-protective properties associated with phytochemicals and the restrictions on the use of synthetic antioxidants due to possible toxic/carcinogenic effects (Branen 1975; Frankel et al 1995; Ito et al 1983) have triggered worldwide interest in phytochemicals as dietary sources of antioxidants and food/beverage colorants. Anthocyanins and other flavonoids are receiving renewed attention for potential health benefits associated with their antioxidant properties. Many studies also suggest that flavonoids exhibit biological activity that includes being antiallergenic (Ueda et al 2002), anti-inflammatory (Mazza and Miniati 1993; Ueda et al 2002; Wang et al 1997) and vasodilating (Brignall and Lamson 2000; Rice-Evans et al 1997), neuroprotective (Sharmaa et al 2007) and having anti-cancer properties (Cherng et al 2007; Gates et al 2007).

Phenolic compounds in sorghum grains are concentrated in the bran layer as a protective mechanism against insects and diseases (Awika et al 2005; Hahn and Rooney 1986). The extraction of sorghum bioactive compounds is difficult because in most whole grains, 75 to 85% are present in the bound form (Hahn 1984; Waniska et al 1989) compared to fruits and vegetables which have more free than bound phenolic compounds (Liu 2007).

Extraction of phenolic compounds from sorghum is a challenge because the cell wall consists mainly of insoluble polysaccharides, protein and lignin in which the cell wall matrix restricts solubilization. The structural complexity of the sorghum grain cell wall matrix makes extraction of phenolic compounds not only difficult but also leads to underestimation of the phenolic compounds present in sorghum.

The commonly used solvents for extraction of phenolic compounds in sorghum include 1% HCl/methanol. The residues obtained after extraction with 1% HCl/methanol still remain dark indicating incomplete extraction. Other solvents have been used for the extraction of phenolic compounds from fruits and vegetables as well as cereals with varying degrees of success. For example, aqueous acetone has been considered a good solvent for the extraction of procyanidins, anthocyanins and other phenolic compounds from fruits and vegetables (Garcia-Viguera et al 1998; Kallithraka et al 1995). Preliminary work in our laboratory using different combinations of acetic, citric and tartaric acids in aqueous ethanol resulted in the same profiles of 3-deoxanthocyanins when compared with the commonly used extraction solvent, 1% HCl in methanol (Njongmeta et al 2007b).

Given the new enzyme technology developed for biomass and ethanol production, enzyme-assisted extraction of phenolic compounds from sorghum should be evaluated. Enzyme extraction of sorghum polyphenols is advantageous in that the economics of enzymatic hydrolysis and its impact on

the environment could outweigh any benefits other extraction solvents provide. Additionally, the use of enzymes could provide an eco-friendly process for the extraction of phenolic compounds from sorghum.

Studies of sorghum phenolics, until now, focused on grains of various sorghum cultivars shown to possess a wide range and proportion of major flavonoids (Dykes 2008). Limited studies have suggested that other parts of the sorghum plant such as the leaves and stems contain phenolic compounds like 3-deoxyanthocyanins, flavones and flavanone (Doherty et al 1987; Kwon and Kim 2003; Rey et al 1993; Seitz 2004). Sereme et al (1993) reported higher levels of anthocyanins in the leaves and sheaths of *Sorghum caudatum* variety Monema kaya compared to the grains and the roots. Thus, our interest was to identify and quantify phenolic compounds from glumes, leaves, stalks and sheaths as well as sorghums grains.

Overall, the project goals were to determine the phenolic profile of intensely pigmented sorghum, including their leaves, glumes and stalks. The project intended to optimize the extractability of phytochemicals from sorghum by the use of enzyme-assisted extractions and evaluate food-friendly extraction solvents to replace 1% HCl/methanol. Phenol profile and distribution depends on plant genetics, thus the results of this study will provide sorghum breeders with appropriate information for the selection of sorghum varieties with special traits to enhance desired functionality and health attributes. Based on the outcome of this research, we intend to propose a more food friendly and effective solvent for

the extraction of phenolic compounds from sorghum. This study will provide fundamental information that will enable decisions about the validity of the whole sorghum plant as a viable source of bioactive compounds.

The objectives of this research were to:

1. Determine the effect of pigmented testa on flavonoid profiles and antioxidant activities of sorghum varieties.
2. Determine the effect of pericarp color on flavanones.
3. Determine the phenolic profiles in leaves, sheaths, glumes, grains and stalks of sorghum varieties with varying secondary plant colors.
4. Determine the most effective food-friendly solvent(s) for the extraction of 3-deoxyanthocyanins from black sorghum.
5. Determine feasibility of enzyme extraction of phenolic compounds from black sorghum.

CHAPTER II

LITERATURE REVIEW

Sorghum Kernel Structure

The characteristics of sorghum grain have been documented by Rooney and Miller (1982). Sorghum caryopses comprise discrete anatomical constituents namely: the pericarp (outer layer), the testa or seed coat, which occurs just under the pericarp layer; the endosperm tissue, which is divided into the aleurone layer, floury and corneous endosperms and the germ “embryo” (Waniska 2000).

The Pericarp

The outer layer of sorghum caryopses originates from the ovary wall and is divided into three histological tissues, the epicarp, mesocarp, and the endocarp (Earp and Rooney 1982). The outermost layer, the epicarp, in most cases is covered with a thin layer of wax. The epicarp is two or three cell layers thick consisting of rectangular shaped cells and may often contain pigmented material. The sorghum mesocarp unlike in most cereals, contains starch granules (Waniska and Rooney 2000). A thick pericarp usually contains three or four mesocarp cell layers filled with small starch granules. The endocarp and the inner pericarp tissue are composed of cross and tube cells.

Many interacting factors control to the color and overall appearance of sorghum caryopses. Appearance is mainly affected by pericarp color and thickness, the presence of pigmented testa and endosperm color (Rooney and Miller 1982). It is postulated that the pericarp color of sorghum results from a combination of the anthocyanin and anthocyanidin pigments in addition to other flavonoid compounds present in sorghum (Hahn and Rooney 1986; Hahn et al 1984).

The appearance and quality of sorghum is controlled by genetics and the environment under which the sorghum grain matures. Numerous studies report that the *R* and *Y* genes interact epistatically to produce red, yellow and white pericarp colors (Dykes and Rooney 2006; Rooney 2000; Waniska and Rooney 2000). A combination of these genes can produce: white or colorless pericarp when the Y locus is homozygous recessive (R_yy or rryy), lemon yellow when the R locus is recessive, with at least one dominant allele at the *Y* locus (*rrY_*), or red when both R and Y loci posses a dominant allele (*R_Y_*) (Hahn et al 1984). The *Y* gene is thought to be the basic gene for the synthesis of the flavonoid skeleton from phenolic acids, while the *R* gene appears to control the reduction of flavanone to its corresponding flavan (Hahn et al 1984).

The intensifier (I) gene controls the intensity of the pericarp color and is most obvious when the pericarp is red (*R_Y_*). The *Z* gene influences pericarp thickness, with thin pericarp dominant over thick pericarp. A pericarp is thick when the gene is homozygous recessive (*zz*) and thin when it is dominant (*ZZ*).

Secondary plant color is controlled by the *P* and *Q* genes. Plants with homozygous dominant *PQ* genes are purple or red pigmented plants, while plants with recessive *pq* genes produce tan-pigmented plants (Dykes and Rooney 2006).

The Testa

The seed coat of sorghum may either be pigmented or non-pigmented and this is genetically controlled by B_1 and B_2 genes (Waniska and Rooney 2000). For a pigmented testa to be present, both genes must be dominant ($B_1_B_2_$) and thus the sorghum is known to contain tannins. If at least one of the genes is recessive then the pericarp is non-pigmented and hence the sorghum does not contain tannins (Fig. 1).

According to Hahn et al (1984), the dominant B_1 and B_2 genes appear to control the polymerization of flavans (anthocyanidins) to flavan-3-ol polymers (tannins). The spreader gene (*S)* controls the amount of tannins and other phenolic compounds present in the pericarp (Gous 1989). When *S* is dominant, more brown pigments are present in the pericarp and testa and the tannin content is higher (Doherty et al 1987). The testa color is controlled by *Tp* genes, and can be either brown or purple. A testa is purple when *Tp* is homozygous recessive (*tptp*) and brown when it is dominant (*Tp_*).

The Endosperm

The endosperm is a triploid, resulting from the fusion of a male gamete with two female polar cells. It is composed of the aleurone layer, peripheral, corneous and floury areas. The aleurone is the outer layer and consists of a single layer of rectangular cells adjacent to the testa or tube cells (Waniska and Rooney 2000). The cells possess a thick cell wall, large amounts of proteins (aleurone grains, enzymes), ash (phytin bodies) and oil (spherosomes).

The peripheral starchy endosperm is composed of several layers of dense cells containing more protein bodies and smaller starch granules. The corneous and floury endosperm cells are composed of starch granules, a protein matrix, protein bodies and cell walls rich in glucuronoarabinoxylans. The starch granules and protein bodies are embedded in the continuous protein matrix in the peripheral and corneous areas.

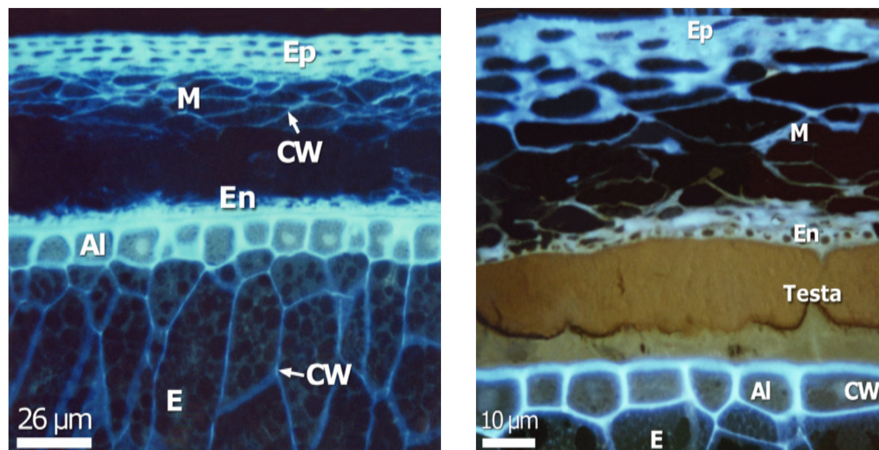


Fig. 1. Fluorescence photomicrograph of cross-sections of sorghum. A non-tannin sorghum (left) and a tannin sorghum kernel (right). Al, aleurone; CW, cell wall; E, endosperm; En, endocarp; Ep, epicarp; M, mesocrap; Testa, pigmented testa. Adapted from Earp et al 2004a.

Germ

The germ consists of two major parts: the embryonic axis and the scutellum. The embryonic axis contains the new plant and is divided into a radicle which develops into the primary roots and plumule which forms the leaves and stems. The scutellum is a single cotyledon and contains reserve nutrients which include moderate amounts of oil, protein, enzymes and minerals and serves as the bridge or connection between the endosperm and germ.

Classification of Sorghum Varieties Based on Tannin Content

Sorghum varieties are sub-categorized into three major categories based on genetics and chemical analyses (Hahn and Rooney 1986; Rooney and Miller 1982). Genetics control the presence or absence of a pigmented testa, and subsequently tannins. Sorghum containing tannin is called tannin or brown sorghum even though the pericarp color may be white, yellow, or red. Grain appearance is not always related to the presence of tannin. Most cultivated sorghums however do not contain condensed tannins even though non-tannin, phenolic compounds are sometimes erroneously reported as tannins.

Type I sorghums ($b_1b_1B_{2}_,B_{1}_b_2b_2,b_1b_1b_2b_2$) do not have a pigmented testa, have no tannins and contain low levels of phenols. Meanwhile Types II and III both have a pigmented testa and contain tannins. Types II and III sorghums however differ from each other in that type II sorghums have a pigmented testa and a recessive spreader gene ($B_1_B_2_ss$), while type III

sorghums have both a pigmented testa and a dominant spreader gene "B_1_B_2_S_" (Earp et al 1981).

Earp et al (2004b) showed that the tannins in type II sorghums are deposited in the vesicle within the testa layer, whereas the tannins in type III are deposited along the cell walls of the testa with some present in the pericarp. The tannins in type II sorghum is more difficult to extract than those in type III with the tannins in Type II sorghums being extracted with acidified methanol (1% HCl methanol) while the tannins in Type III sorghums extracted with either methanol or acidified methanol when performing the vanillin/HCl assay.

Phenolic Compounds in Sorghum

Phenolic compounds constitute a large diverse group of secondary plant metabolites commonly found in the plant kingdom and posses a common characteristic of an aromatic ring bearing one or more hydroxyl substituents. The phenolic units are encountered in proteins, alkaloids and among terpenoids (Harborne 1991). Polyphenols are derived from the Shikimate and acetate-malonate pathways. Shikimate pathways continues with the production of phenylalanine, which is subsequently deaminated by the enzyme phenylalanine lyase into cinnamate derivatives (Dicko et al 2006). On the other hand, acetate-malonate pathways contributes to flavonoid biosynthesis which continues with the conversion of acetyl CoA to malonyl CoA by acetyl CoA carboxylase (ACC) (Fig. 2). All sorghum contain phenolic compounds and among the major cereals,

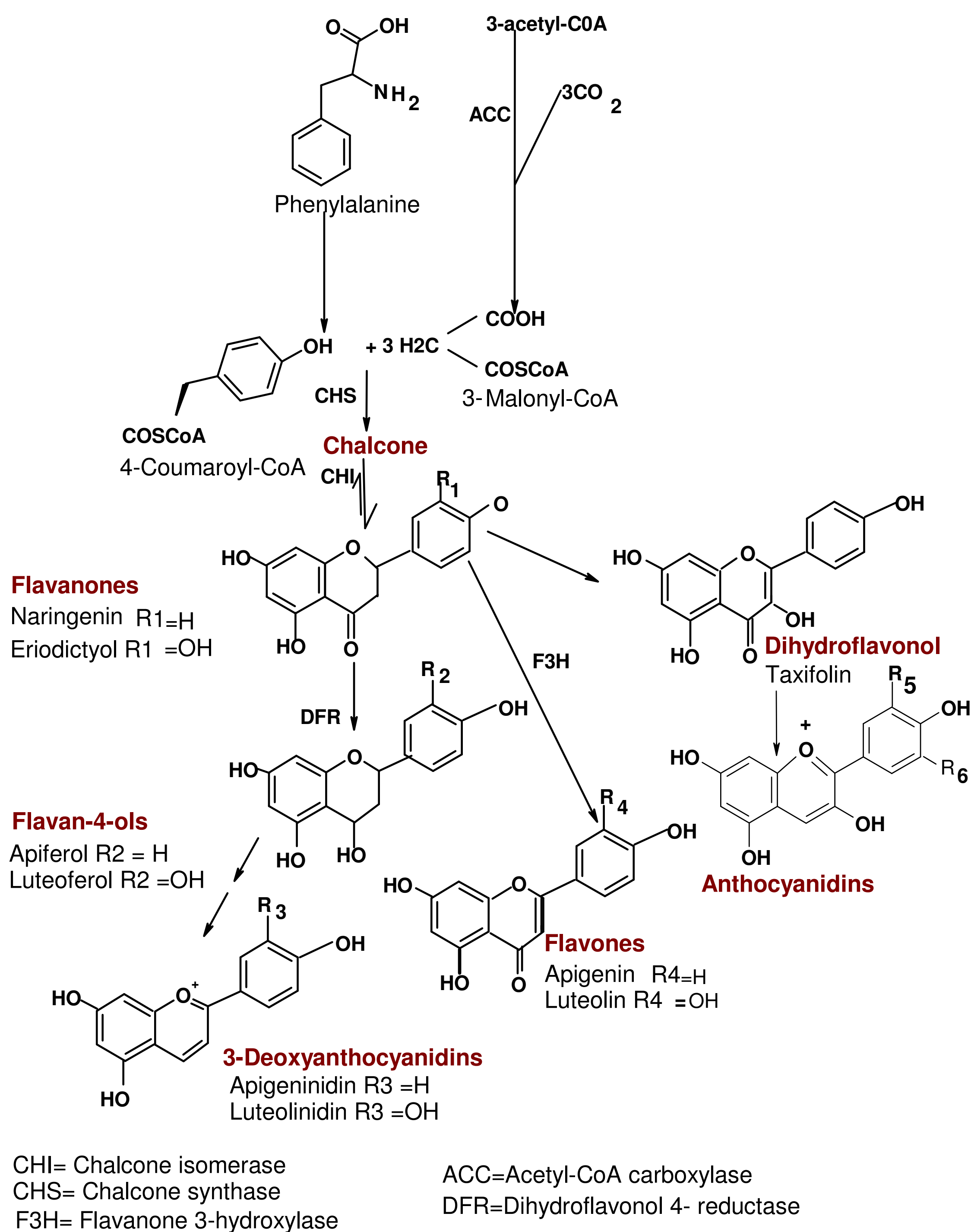


Fig. 2. The biosynthetic pathway of major sorghum flavonoids. Note how the biosynthetic route of 3-deoxyanthocyanidins and anthocyanidins are proposed to diverge from one another after the synthesis of naringenin. Modified from Wharton & Nicholson 2000, Boddu et al 2005.

sorghum remains unique because of the quality and quantity of phenolic compounds, including the condensed tannins in some varieties (Butler 1990). Phenolic compounds most frequently encountered in nature are linked to a sugar moiety (glycosides) and they are usually water-soluble (Harborne 1991).

In cereal, phenolic compounds are found in both free and bound form. Free phenolic compounds are proanthocyanidins or flavonoids, while the bound phenolic compounds are ester-linked to cell-wall polymers with ferulic acid and its dehydrodimer derivatives being the major bound phenolic compounds present (Bonolia et al 2004).

Phenolic compounds identified in sorghum belong to three major groups: phenolic acids, polymeric flavonoids and simple flavonoids (Awika and Rooney 2004, Dykes and Rooney 2006, Serna-Saldivar and Rooney 1995).

Phenolic Acids

Phenolic acids constitute two groups; hydroxybenzoic acid and hydroxycinnamic acids which are the common phenolic acids in plants (Wrolstad et al 2005). All sorghum contain phenolic acids located in the pericarp, testa, aleurone layer and endosperm (Hahn et al 1984), although the bound phenolic acids are associated with the cells walls. Many phenolic acids have been identified in sorghum (Table 1).

Table 1
Phenolic Acids and Substitutions Identified in Sorghum

Phenolic Acid	[a]R_1	R_2	R_3	References
Hydroxybenzoic Acids				
Gallic	OH	OH	OH	Hahn et al (1983)
Protocatechuic	H	OH	OH	Hahn et al (1983); McDonough et al (1986)
p-Hydroxybenzoic	H	OH	H	Hahn et al (1983); McDonough et al (1986)
Gentisic	OH	H	OH	McDonough et al(1986); Waniska et al (1989)
Salicylic	OH	H	H	McDonough et al(1986); Waniska et al (1989)
Vanillic	CH_3O	OH	H	McDonough et al(1986); Hahn et al (1983)
Syringic	CH_3O	OH	CH_3O	McDonough et al(1986); Waniska et al (1989)
Hydroxycinnamic Acids				
Ferulic	CH_3O	OH	H	Hahn et al (1983); McDonough et al(1986)
Caffeic	OH	OH	H	Hahn et al (1983); McDonough et al(1986)
p-Coumaric	H	OH	H	Hahn et al (1983); McDonough et al(1986)
Cinnamic	H	H	H	Hahn et al (1989); McDonough et al(1986)
Sinapic	CH_3O	OH	CH_3O	Waniska et al1989); (McDonough et al(1986)

[a] :Parent skeletons are shown on Fig. 3.

Hydroxybenzoic and hydroxycinnamic acids are benzoic and cinnamic acid derivatives, respectively (Fig. 3). Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acids, among others. Hydroxycinnamic acids include coumaric, caffeic, ferulic, and sinapic acids and have a C6-C3 structure. Phenolic acids in cereal grains generally exist as free phenolic acids, and as soluble and insoluble esters.

Hahn et al (1984) identified free and bound phenolic acids in sorghum, although the bound forms were predominant. Ferulic acid is the most dominant (24-47%) of the phenolic acids. In sorghum, gallic acid is found in bound form, whereas cinnamic acid exists in the free form (Hahn et al 1984). Free and bound phenolic acids are extracted with methanol and boiling 2*N* HCl, respectively.

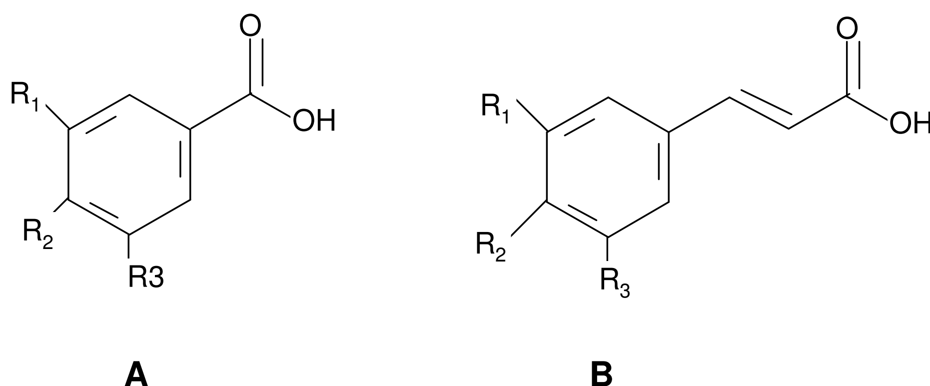


Fig. 3. Basic structure of phenolic acids: Benzoic acids (A) and Cinnamic acids (B).

Tannins (Polymeric Flavonoids)

Tannins are high molecular weight polymeric flavonoids with molecular weights between 500 and 3,000 (Fennema 1996). Tannins are classified as hydrolyzable or condensed tannins and are capable of tanning leather or precipitating gelatin in solution. Hydrolyzable and condensed tannins can easily be differentiated by their structure and reactivity towards hydrolytic agents.

Structurally, hydrolyzable tannins (e.g, tannic acid) contain a central core of polyhydric alcohol such as glucose and hydroxyl groups which are fully or partially esterified by gallic acid or hexahydroxydiphenic acid. Hydrolyzable tannins break down into sugars and a phenolic acid (gallic or ellagic) when treated with acid, alkali or some hydrolytic enzymes such as tannase (Harborne 1991). Sorghum does not contain tannic acid and hydrolysable tannins (Awika and Rooney 2004; Waniska 2000).

Sorghum varieties with a pigmented testa contain condensed tannins, mainly polymerized products of flavan-3-ols and/or flavan 3, 4-diol subunits (Fig. 4), that are deposited in the pigmented testa layer of sorghum kernels (Kaufman et al 2006). Only sorghum cultivars with a pigmented testa ($B_1_B_2_$genes), produce condensed tannins or proanthocyanidins (Waniska 2000). According to Hahn and Rooney (1986), tannins apparently occur only in the pericarp and pigmented testa layers although tannins have also been reported in the glumes and leaves of sorghum (Ring 1984). Tannins have *in vitro* antioxidant properties (Hagerman et al 1998) and over the last decade are considered as nutraceuticals (Zhang et al 1997; Lui et al 2005).

Condensed tannins are formed by the biosynthetic condensation of catechin or gallocatechin units to form dimmers and higher oligomers, with carbon-carbon linking one flavan unit to the next by a 4-8 or 6-8 links (Harborne 1991; Haslam 1996). Condensed tannins are also referred to as proanthocyanidins because they yield anthocyanidins when treated with mineral

acids (Hahn et al 1984). Most proanthocyanidins are procyanidins, which means that they yield cyanidins or catechin upon treatment with acid (Harborne 1991). Condensed tannins are primarily procyanidins (Gu et al 2004).

Fig. 4. Structure of proanthocyanidin (condensed tannin) polymer; n >10.

Simple Flavonoids in Sorghum

Flavonoids are polyphenolic compounds ubiquitously present in the plant kingdom. They are the largest class of phenolic compounds studied and more than 8,150 have been identified (Anderson and Markham 2006; Sweeny and Iacobucci 1983). Flavonoids constitute important natural pigments with diverse biochemical and antioxidant benefits.

The basic flavonoid structure is composed of the flavan nucleus, which consists of 15 carbon atoms derived from two phenyl groups (A and B- rings), connected by a three-carbon bridge (C-ring) to form a C_6-C_3-C_6 skeleton (Fig. 5). Flavonoids are biosynthetically derived from phenylalanine (Fig. 2).

Three molecules of glucose from malonyl-coenzyme A (CoA) from the acetate-malonyl pathway of glucose metabolism condenses to form ring A, catalyzed by chalcone synthetase. Ring B and C also come from glucose metabolism, but via the shikimate pathway through phenylalanine, which is converted to cinnamic acid and then to coumaric acid. Coumaric acid CoA and three malonyl CoAs are condensed in a single enzymatic step to form naringenin chalcone.

The C-ring closes and becomes hydrated to form 3-hydroxyflavonoids (e.g catechins), 3, 4-diol flavonoids (e.g quercetin), and procyanidins (Formica and Regelson 1995). Flavonoids can undergo modifications of their aromatic cycles, including hydroxylations, methylations and glycosylations.

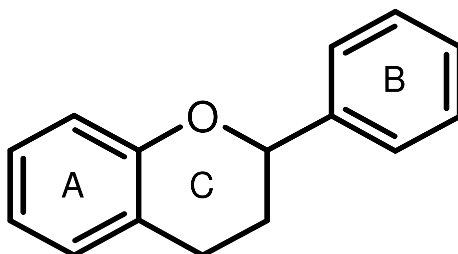
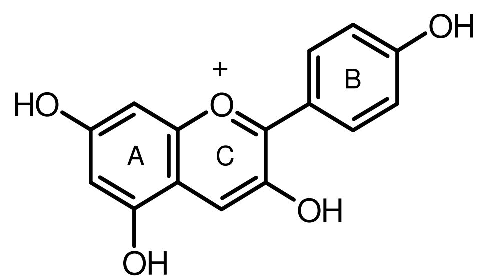


Fig. 5. Basic diphenylpropane C_6-C_3-C_6 skeleton.

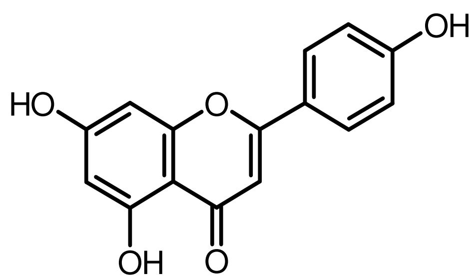
Only plants have the biosynthetic capabilities to synthesis flavonoids. Flavonoids occur in various forms in plants such as aglycones, glycosides or hydroxyl, methyl and methoxyl derivatives (Pourcel et al 2006). Polyphenols are divided into various classes on the basis of their molecular structure (Fig. 6) with the major classes differing in the level of oxidation and substitution pattern on the C ring, while individual compounds within a class differ in the substitution pattern on the A and B rings (Fig. 5).

Many classes of flavonoids have been isolated and identified in sorghum (Table 2) including flavanols (e.g flavan-3-ols, flavan-4-ols), flavanones, flavones and anthocyanins (Fig. 5) (Awika et al 2004b; Awika et al 2004a; Dykes 2008).

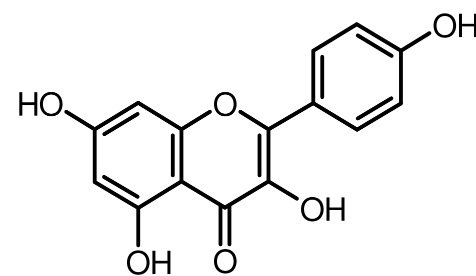
The six common anthocyanins (Fig. 7A) in nature are cyanindin, delphinidin, malvinidin, pelargonidin, petunidin, and peonidin. However, the 3-deoxyanthocyanins are a rare form of anthocyanins commonly found in ferns and mosses (Timberlake and Bridle 1975; 1980) with sorghum being the only known dietary source (Wu et al 2005).



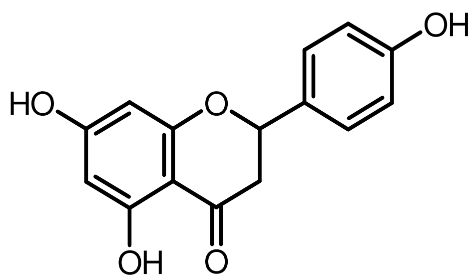
Anthocyanidins



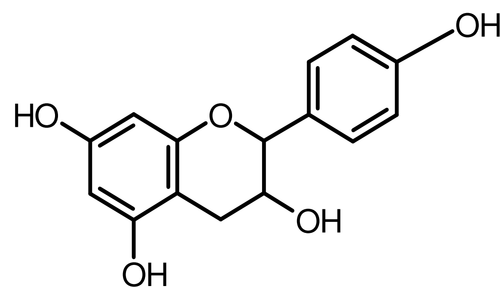
Flavones



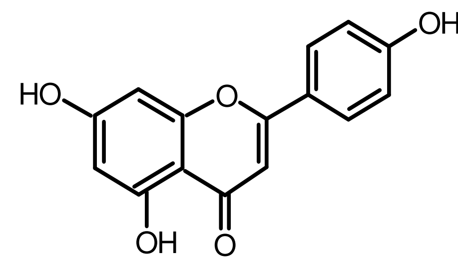
Flavonols



Flavanones



Flavanols



Isoflavones

Fig. 6. Basic chemical structure of various classes of flavonoids.

Table 2
Flavonoids Identified in Sorghums

Compound	References
Anthocyanins:	
Apigeninidin	Nip & Burns (1971); Gous (1989); [a]Rey et al (1993)
Apigeninidin 5-glucoside	Nip & Burns (1969,1971); Wu and Prior (2005)
Luteolinidin	Nip & Burns (1971); Gous (1989)
5-Methoxyluteolinidin	Seitz (2004); Wu and Prior (2005)
5-Methoxyluteolinidin 7-glucoside	Wu & Prior (2005)
7-Methoxyapigeninidin	Pale et al (1997); Seitz (2004); Wu and Prior (2005)
7-Methoxyapigeninidin 5-glucoside	Wu and Prior (2005)
Luteolinidin 5-glucoside	Nip and Burns (1971); Wu and Prior (2005)
5-Methoxyapigeninidin	Seitz (2004)
7-Methoxyluteolinidin	Seitz (2004)
Flavan-4-ols:	
Luteoforol	Bate-Smith (1969)
Apiforol	Watterson and Butler (1983)
Flavones:	
Apigenin	Gujer et al 1986; [a]Rey et al (1993); Seitz (2004)
Luteolin	[a]Rey et al (1993); Seitz (2004)
Tricin	[a] Kwon and Kim (2003)
Flavanones:	
Eriodictyol	Yasumatsu et al (1965); Kambal and Bate-Smith (1976)
Eriodictyol 5-glucoside	Gujer et al (1986)
Naringenin	Gujer et al (1986)
Quercetin 3,4'-dimethyl ether	[a] Kwon and Kim (2003)
Flavonols:	
Kaempferol 3-rutinoside-7-glucuronide	Nip and Burns (1969)
Dihydroflavonols:	
Taxifolin	Gujer et al (1986)
Taxifolin 7-glucoside	Gujer et al (1986)

Source: Modified from Dykes et al 2006.
[a] Flavonoid identified in stem

Sorghum anthocyanins are called 3-deoxyanthocyanins and are the major class of flavonoids studied in sorghum (Awika et al 2005; Dykes 2008; Dykes and Rooney 2006; Njongmeta et al 2007b; Njongmeta et al 2007a). Sorghum 3-deoxyanthocyanins are similar to the common anthocyanins, (Fig. 7A), however, they do not contain the hydroxyl group in the 3-position of the C-ring (Fig. 7B). This unique structural feature gives sorghum 3-deoxyanthocyanins very different

chemical and biochemical properties. For example, 3-deoxyanthocyanins are more stable to pH changes and other conditions compared to the common anthocyanins (Awika et al 2004b; Awika and Rooney 2004; Gous 1989; Mazza and Brouillard 1987; Njongmeta et al 2007a; Sweeny and Iacobucci 1983), giving these compounds a competitive advantage as potential natural food colorants compared to the common anthocyanins.

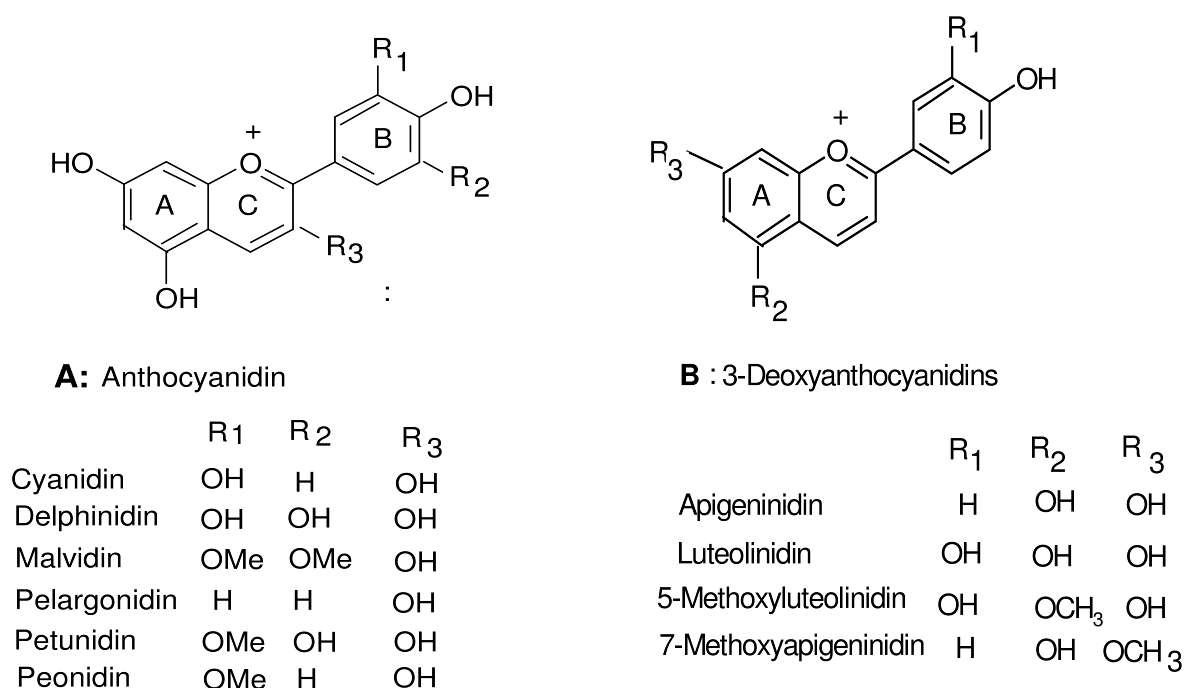


Fig. 7. Structures of the six common anthocyanidins (A) versus the sorghum 3-deoxyanthocyanidins (B).

3-Deoxyanthocyanins are produced as phytoalexins in plants as a response to mold invasion or other stress factors in sorghum (Lo et al1999; Seitz 2004, Waniska and Rooney 2000). They have *in vitro* antioxidant activity (Awika et al 2004a). The yellow apigeninidin and the bright orange luteolinidin are the two most prominent sorghum 3-deoxyanthocyanins (Awika et al 2004b; Gous 1989; Nip and Burns 1971; Wu and Prior 2005).

As reviewed in Dykes and Rooney (2006), various substituted forms of 3-deoxyanthocyanins have also been identified in sorghum grains which include apigeninidin 5-glucoside, luteolinidin 5-glucoside, 5-methoxyluteolinidin, 5-methoxyluteolinidin 7-glucoside (Wu and Prior 2005), 7-methoxyapigeninidin, 7-methoxyapigeninidin 5-glucoside (Wu and Prior 2005), 5-methoxyapigeninidin (Seitz 2004), and 7-methoxyluteolinidin (Table 2).

Structural Features Relevant to Functions of Phenolic Compounds

Generally, flavonoids are the principal components of phenolic compounds responsible for antioxidant capacity and flavonoids with multiple hydroxyl substitutions have very potent antioxidant activity against peroxyl radicals (Cao et al 1997). Other attributed biological features of polyphenols include their potential cytotoxicity (Pourcel et al 2006).

Phenolic Compounds as Colorants

Anthocyanins and 3-deoxyanthocyanins can be used as colorants due to their ability to reflect or emit different quantities of energy at wavelengths able to stimulate the retina in the eye. Anthocyanins and 3-deoxyanthocyanins have double bonds in abundance and these are easily excited. The ease with which a molecule is excited depends on the relative electron mobility in the structure.

The color of these compounds results from excitation of the molecule by visible light. Increasing substitution on these molecules results in a deeper hue, due to bathochromic change (longer wave length), with a visible light absorption band from violet through red to blue.

The bathochromic effects are caused by auxochrome groups. Auxochrome groups are electron-donating groups and in the case of anthocyanins are the hydroxyl and methoxy groups. The methoxy groups contribute to deeper hues than the hydroxyl group because their electron donating capacity is greater than that of the hydroxyl group. These structural features make anthocyanins and 3-deoxyanthocyanins suitable for use as colorants.

In terms of stability, sorghum 3-deoxyanthocyanins are superior to other common anthocyanins as they are more stable, over a wide range of pH change, because of the lack of the hydroxyl group in position 3 of the C-ring, a future lacking in the common anthocyanins. Preliminary work in our laboratory showed that the stability of the 3-deoxyanthocyanins from sorghum compares

favorably with commercial food colorants such as FC & D Red # 3 and 40 (Cardenas-Hinojosa et al 2007; Njongmeta et al 2007a; Cardenas-Hinojaso 2008).

Phenolic Compounds as Antioxidants

Oxidizing events initiated by free radicals are responsible for many human pathological conditions such as stroke, cancer and diabetes (Lam et al 2007). Increased consumption of plant foods rich in antioxidants such as phenolic phytochemicals are recommended for the prevention of human diseases caused by free radicals. Non-radical compounds such as Reactive Oxygen Species (ROS) are generated through normal physiological processes in biological systems and become increased during pathological conditions (Mathew and Abraham 2006). Studies on free radical scavenging capacity of antioxidants have led to increased interest in the use of plant phytochemicals for the treatment of pathological conditions caused by ROS (Aboul-Enein et al 2007).

Phenolic compounds act as scavengers of various oxidizing species (Awika 2003; Awika et al 2003b), and act as antioxidants by donating a proton to a free radical, thus stabilizing it while the antioxidant free radical generated is stabilized by resonance due to the presence of the benzene ring which allows for the existence of many resonant structures (Coultate 1996).

The ring orientation of the compound determines the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical and the ability of the compound to support an unpaired electron. The antioxidant activity of anthocyanins has been associated with a variety of properties including free radical scavenging, chelation of trace metals, and inhibition of lipid peroxidation and DNA oxidation. The structural characteristics responsible for antioxidant effect of anthocyanins are generally associated with the number of free hydroxyls around the pyrone ring (greater number of hydroxyls= greater antioxidant capacity).

The conjugation of the anthocyanin ring structure with the C2-C3 double bond is consistently associated with a higher antioxidant capacity and a stabilizing effect on the phenoxy radical. The positioning of the hydroxyl group in relation to one another is also a very important determinant of the antioxidant capacity of anthocyanins. Hydroxyl groups in close proximity, such as the ortho-hydroxyls of the B-ring appear to greatly enhance the antioxidant capacity of the anthocyanin in experimental models (Lien et al 1999; Zheng and Wang 2003), but information on this in biological systems is however limited.

The antioxidant property of some phenolic compounds, for example tannins, is attributed to the proximity of many aromatic rings and hydroxyl groups and the fact that tannin cannot act as prooxidants (Hagerman et al 1998). It has been suggested that the free radical scavenging in human systems by antioxidants is a possible protective mechanism for reducing the pathological

damages of free radicals and ROS on human genetic materials and other biological molecules such as lipids (Wu et al 2004).

Antimutagenic and Anticarcinogenic Properties of Phenolic Compounds

Natural foods and food-derived antioxidants including vitamins and polyphenols continue to receive great attention as these bioactive phytochemicals are known to affect many biological processes such as antimutagenic and anticarcinogenic activities. The antimutagenic and anticarcinogenic properties of phenolic compounds are directly related to their antioxidant capacity (Potter 1997; Rafter 2002; Roy et al 2003). This is associated with their redox properties and the number of hydroxyl groups and the ability to donate proton which plays a role in adsorbing and neutralizing free radicals.

Phenolic compounds possess ortho-hydroxyls in the B-ring of some flavonoids (e.g, catechin, quercetin and luteolin) which have antimutagenic and anticarcinogenic properties (Lee et al 2002; Mouria et al 2002; RoyChowdhury et al 2002; Yamashita & Kawanishi 2000). *In vitro* and *in vivo* studies suggest that phenolic compounds exhibit antimutagenic and anticarcinogenic activities by inducing cell cycle arrest, apoptosis and inhibiting proliferation of cancer cell lines and tumor development in rats (Mertens-Talcott and Percival 2005; Laurent et al 2007).

Antimicrobial Properties of Phenolic Compounds

Growing interest in phenolic compounds and other natural antimicrobials from foods for treating human diseases has been fueled by the emergence of antimicrobial resistance to current antibiotics. Keen interest in polyphenols as antimicrobials is based on their protective role in plants against biotic and abiotic stress since they are produced as phytoalexins in response to microbial attack (Seitz 2004; Waniska et al 2001).

The efficacy of polyphenols as antimicrobial agents has been demonstrated *in vitro* against a wide array of microorganisms (Lin et al 2004). Polyphenols such as isoflavonoids, flavans and flavanones are recognized as antifungal agents in plants and have been proposed for use against fungal pathogens in humans (Harborne and Williams 2000).

The presence of hydroxyl group on the phenolic compound is the main structural feature contributing to their antimicrobial properties (Harborne and Williams 2000). The site(s) and number of hydroxyl groups on the phenols are related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Giron et al 1988). Some authors reported that more highly oxidized phenols are more inhibitory (Scalbert 1991).

The extent of antioxidant potential of phenolic compounds in humans and other observed positive health effects studied both *in vitro* and in *vivo* is dependent on absorption, metabolism, distribution and excretion of these compounds within the body after ingestion (Rice-Evans 2003).

Phenolic Compounds in Non-Grain Materials of Sorghum

In Benin and other West African countries, red pigments extracted from the leaves of local sorghum cultivars are used as food colorants and dyes for leather and other art work (National Research Council. 1996). In Ancient China, sorghum glume pigments used as dyes in local textile making were very stable under variable light and heating conditions (Zou and Shi 1999). Presently, this Chinese "Red Sorghum Pigment" extract has been patented. It is commercially available and used as additives/colorant in foods (meat and candy) and as coating in tablets and capsules. This suggests that the leaves, glumes, sheaths and stalks of these cultivars contain high levels of phenolic compounds such as 3-deoxyanthocyanin which are stable to light and heat (Cardenas-Hinojosa et al 2007) and other antioxidant compounds.

Limited studies have been done on the identification and quantification of phenolic compounds from non-grain materials of sorghum such as the stems, leaves, stalks and glumes (Doherty et al 1987; Kwon and Kim 2003; Rey et al 1993). Doherty et al (1987) identified free phenolic compounds and tannins in the glumes of sorghum cultivars. Kwon and Kim (2003) and Rey et al (1993) identified flavonoids and antioxidant compounds such as apigeninidin, luteolin and apigenin in the stems of some other sorghum cultivars. Seitz (2004) reported 3-deoxyanthocyanidins and flavones in glumes of purple and tan-plant hybrid sorghums with varying degrees of moldiness; however the sorghum varieties were not reported.

In other plant species for example, in Brazil, sugarcane (*Saccharum officinarum* L., Gramineae), a close relative of sorghum, has a promising potential as a source of flavonoid compounds, mainly the form of *C*-glycosides (Colombo et al 2006). This shows that evaluating the whole sorghum plant will offer added benefit to the utilization of sorghum.

Extraction of Sorghum Phenolic Compounds

The principal goal in extraction of compounds from any plant material is usually to maximize removal of most of the compounds from the sample into solution (Waterman and Mole 1994b). Extraction is a critical step for accurate determination of phenol profiles in any given food material and the process is dependent on factors such as the extraction solvents, the number of extraction cycles and the concentration in the sample relative to the solvent.

The physical process involves solvation or dissolution and diffusion into solution; hence, phenolic compounds are extracted faster if they are not saturated in the extraction solvent. This process also depends on temperature. Generally, the dissolution process takes longer from dry material compared to fresh material (Waterman and Mole 1994b).

Several methods have been used in the extraction and isolation of phenolic compounds from plant materials. In sorghum, Hahn (1984) and Gous (1989) found 1%HCl in methanol was the most efficient solvent, whereas 70%

aqueous acetone was reported as the most efficient solvent for extracting polyphenols from fruits (Kallithraka et al 1995).

The extraction of sorghum bioactive compounds is difficult because of the way these compounds are located in the cell wall matrix which restricts solubilization. In whole grains, 75 to 85% of the phenols are present in the bound form (Hahn 1984; Waniska et al 1989) while in fruits and vegetables there are more free than bound phenolic compounds (Liu 2007).

Extraction of phenolic compounds from sorghum is also a challenge because the cell wall consists mainly of insoluble polysaccharides, protein and lignin. Acidified 1% HCl/methanol is the commonly used solvent for the extraction of phenolic compounds from sorghum. The residues obtained after extraction with 1% methanol/HCl still remains dark indicating incomplete extraction.

Other solvents have been used for the extraction of phenolic compounds from fruits and vegetables as well as cereals with varying degrees of success. For example, aqueous acetone has been considered a good solvent for the extraction of procyanindins, anthocyanins and other phenolic compounds in fruits and vegetables (Garcia-Viguera et al 1998; Kallithraka et al 1995). Lu and Foo (2001) observed significant anthocyanin interaction when aqueous acetone was used as extraction solvent for fruits and vegetables. Awika et al (2004b) reported modification of the HPLC-spectral characteristic of 3-deoxyanthocyanins associated with formation of pyranoluteolinidin and

pyranoapigeninidin, which resulted in significantly lower levels of detectable anthocyanins.

Different combinations of acetic, citric and tartaric acids in aqueous ethanol gave the same profiles of 3-deoxanthocyanins as the commonly used 1% HCl in methanol extractions although with about 50% reduction (Njongmeta et al 2007b).

Enzyme assisted extraction of phenolic compounds from fruits is successfully practiced. For example, in the extraction of antioxidant compounds from black currant press residues (Ribes nigrum), enzymes increased phenol yields (Landbo and Meyer 2001). In another study, cell wall degrading enzyme preparations increased anthocyanin yield and phenol profiles in bilberry and black currant juices (Buchert et al 2005). Kim et al (2005) reported increased yields in the extraction of phenolic compounds from apple peel when cellulases from *Thermobifida fusca* were used. Enzyme-assisted extraction is un-exploited for sorghum phenolics and offers an opportunity to optimize the extractability of sorghum phenolics.

Analysis of Phenolic Compounds from Sorghum

Several methods are used for determination of phenolic compounds (Hagerman et al 1997; Shahidi and Naczk 1995). Most methods use spectrophotometric assays because the techniques are easy to master and are very valuable in the evaluation of phenolic compounds (Waterman and Mole

1994b). Total phenols are measured using the Folin-Ciocalteu assay (Singleton and Rossi 1965; Kaluza et al 1980) or the Prussian Blue assay (Price and Butler 1977). Total phenol assays measure the ability of phenolic groups to reduce the folin-reagent (Huang et al 2005).

Folin-Ciocalteau Assay

This is the most commonly used method as reported by (Waterman and Mole 1994b). The principle is based on a reduction-oxidation reaction in which the phenolate ion undergoes oxidization under alkaline conditions while reducing the phosphotungstic-phosphomolybdic complex in the reagent. Since this is credited to the reducing power of phenolic hydroxyl groups (Hahn et al 1984), the reactions are not specific to a class of phenols. For example, the assay measures the amino acid tyrosine (Hahn and Rooney 1986) and non-phenolics such as ascorbic acid (Hagerman et al 1997; Waterman and Mole 1994b).

Bleach Test

The presence of condensed tannin is determined using rapid methods of identifying tannin sorghums. One such method is the bleach test which qualitatively identifies sorghum with tannins (Waniska et al 1992). In the bleach test, the pericarp is dissolved by the bleach reagent, to expose the testa layer, which is black in tannin sorghum, and white to yellow in non-tannin sorghum. The bleach test provides an estimate of the amount of tannin sorghum present

which is calculated from the percentage of kernels containing the pigmented testa. It is a good method to determine whether a sample contains a mixture of tannin and non-tannin sorghum. The bleach test is inexpensive and relatively quick to perform. It is also effective when used with the appropriate standards. It does not measure tannin content or differentiate between Type II and Type III sorghums (Waniska et al 1992). There are more specific tests which can be done to confirm the presence or absence of tannins.

Vanillin-Hydrochloric Acid (Vanillin/HCl) Method

Condensed tannins are measured using the vanillin/HCl or the butanol/HCl assays. The modified vanillin/HCl method of (Price et al 1978) involves the condensation of the aromatic aldehyde vanillin (4-hydroxy-3-methoxy benzaldehyde) with monomeric flavanols and their oligomers in the presence of mineral acids to form a red adduct or colored complex, which absorbs at 500 nm.

Type I sorghums give low tannin values due to the interference of other non-tannin phenolics (Waniska and Rooney 2000). Sorghums that do not have a pigmented testa contain non-tannin phenolics that react with the reagent and give some “tannin values” that are artifacts (Earp et al 1981; Hahn and Rooney 1986). These values are generally reported as tannin content in the literature and give rise to the false assumption that all sorghums have tannins (Rooney 2005). Because of the lack of an appropriate standard for condensed tannins,

the vanillin/HCl assay does not measure tannin content accurately; a major limitation based on the heterogeneous nature of these compounds (Schofield et al 2001). The preferred standard for the vanillin/HCl assay is catechin but it gives values that are unrealistically high (Schofield et al 2001). Attempts to obtain pure tannin standards over the years have proven challenging.

In the vanillin-HCl assay, the flavonoid A-ring at the C-6 position reacts with vanillin forming a red chromophore, thus the assay detects any monomeric or polymeric flavanols using catechin as a standard (Beta et al 2000). In most cases, if this assay is used alone, it leads to a wrong assertion about tannin content of a given sample. For example, in sorghum, the tannins reside mainly in the pigmented testa, and the pigmented testa is about 5-6% dry weight of the kernel. Consequently, high catechin equivalent values are unrealistic in some varieties. Hence, tannin values from this assay have generally been viewed as relative indices of tannin content among samples only and the values are combined with information on the genetic background of the sorghum to make meaningful conclusions on the tannin content.

Butanol/HCl Assay Method for Condensed Tannins

The butanol/HCl assay also measures tannin content and involves the depolymerization of condensed tannins in boiling acidic butanol to yield anthocyanidins (Porter et al 1986). The butanol-HCl method is specific for proanthocyanidins. As upon treatment with mineral acid solutions,

proanthocyanindins depolymerize to colored anthocyanidins that absorb at maximum wavelength of around 550nm. The cleavage of the inter-flavanoid bond results in the formation of carbocations, which undergo autooxidation to yield anthocyanidins (Porter et al 1986).

Modified Butanol/HCl Method for Flavan-4-ol

Flavan-4-ols are measured by a modified method of the butanol/HCl (Govindarajan and Mathew 1965). Flavan-4-ols are reported as absorbance readings due to the absence of an appropriate standard curve for quantification. The instability of the pure compounds such as luteoferol and apiferol standards makes it difficult to obtain a standard curve.

pH Differential Method for Total Anthocyanins

Total anthocyanin content is commonly determined using the pH differential method of Fuleki and Francis (1968). Absorbance is read spectrophotometrically at two separate wavelengths; 485 nm (luteolinidin) and 465 nm (apigeninidin). It is possible to compare the absorbance readings with a standard curve of either luteolinidin or apigeniniden to obtain values that are universally acceptable.

Separation and Identification of Phenolic Compounds Using HPLC

Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV-Vis or photodiode array (PDA) detection, using C18 columns has been successfully used to separate, identify, and quantify sorghum phenolic acids and flavonoids (Awika et al 2004a, Chen et al 2001, Gujer et al 1986, Hahn et al 1983, Lopez et al 2001). The main chromatographical separation principle involved in reversed-phase HPLC is partition of solutes between the polar mobile phase and the non-polar stationary phase. The overall polarity and stereochemistry of the flavonoids are the key factors for the separation (Anderson and Francis 2004, Strack and Wary 1994). The elution of the flavonoids in reversed-phase HPLC columns depends on the pattern of hydroxylation/methoxylation of aglycone, the degree of glycosylation, acyl substitution as well as the mobile phase composition and solvent gradient steepness. For quantification, standard calibration curves are prepared by plotting the area of peaks against different concentrations of phenolic compound standards (Lopez et al 2001).

Condensed tannins or procyanidin profile in tannin sorghum is determined using normal phase chromatography. However, the sample extracts are usually cleaned of sugars and phenols by gel filtration methods (Waterman and Mole 1994a). Sephadex LH-20 columns have been successfully used in the separation of condensed tannins from sorghum (Awika et al 2003a; Nomusa 2007). Separation of procyanidins from other phenolic compounds is possible

because in 95% ethanol, tannins are absorbed by Sephadex LH- 20 and then they are eluted with aqueous acetone (Strumeyer and Malin 1975).

Determining Antioxidant Activity of Phenolic Compounds in Sorghum

Three *in-vitro* methods have been commonly used in determining antioxidant activity of phenolic compounds in sorghum and sorghum products. The 2, 2'–azinobis (3-ethylbenzothiozoline-6-sulphonic acid) (ABTS**ÿ**$^{+}$) and 2, 2-Dipheny-1-picrylhydrazyl (DPPH**ÿ)** are the two commonly used free radicals (Awika et al 2003b).

ABTS

The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS**ÿ**$^{+}$ generated in aqueous phase as compared with Trolox standard (a water soluble vitamin E analogue). The ABTS**ÿ**$^{+}$ is generated by reacting a strong oxidizing agent (e.g potassium permanganate or potassium persulfate) with the ABTS salt (Awika et al 2003b). The reduction of the blue-green ABTSÿ$^{+}$ by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum (Miller and Evans 1997).

The reaction is based on a single electron transfer mechanism. The ABTS method can be used over a wide range of pH values (Arnao et al 1999; Lemanska et al 2001), and the radical is soluble in both aqueous and organic

solvents (Awika et al 2003b). Results are expressed as Trolox equivalent antioxidant capacity (TEAC).

The method is simple, inexpensive to perform and is highly repeatable. The ABTS radical however, does not exist in humans and has not been correlated with biological systems and as such the actual relevance to *in vivo* antioxidant efficacy is unknown.

DPPH

The DPPH assay determines the capacity of a sample to donate hydrogen and/or electrons to quench DPPH˙ radicals. The assay mimics quenching and prevention of radical expansion through hydrogen atom transfer (HAT). As the free radical is quenched, the free radical changes the color of DPPH from deep purple to light yellow and the absorbance at 515nm decreases. The DPPH method is widely used to determine antioxidant activity of purified as well as crude natural plant extracts (Brand-Williams et al 1995). The DPPHÿ is a stable free radical that absorbs at 515 nm, but loses its absorption when reduced by an antioxidant or free radical species. Basically the method measures the decrease of DPPH**ÿ** absorbance in the presence of antioxidants. From the reaction kinetics, Brand-Williams et al (1995) observed that most phenolic antioxidants react slowly with DPPH**ÿ** and most phenols react with DPPH**ÿ** in a third order kinetics taking 1- 6 hours to reach a steady state. The slow reaction rate suggests a complex reaction mechanism to reach a steady

state (Bondet et al 1997). Consequently, antioxidant activity using DPPH**ÿ** must be evaluated over time (Awika et al 2003b).

The reaction of DPPH like the ABTS assay is based on a single electron transfer mechanism. The DPPH method has good repeatability and is cheap to perform but also has limited applicability in biological systems since DPPH**ÿ** is extraneous to biological systems. One serious drawback of the DPPH assay is the fact that in systems with anthocyanins, color interference of the DPPH**ÿ** with anthocyanins leads to underestimation of antioxidant activity (Arnao 2000). In addition, the DPPH method is sensitive to low pH. The results are commonly expressed as Trolox equivalent antioxidant capacity (TEAC).

ORAC

Oxygen radical absorbance capacity (ORAC) method developed by Cao et al (1993) measures the ability of antioxidants to protect protein from damage by free radicals and has been used to evaluate the antioxidant capacity of sorghum (Awika et al 2003b; Kamath et al 2004; Wu et al 2004).

ORAC generally employs different generators to produce three radicals; peroxyl (ROOÿ), hydroxyl (OHÿ) and Cu^{2+}, a transition metal. Measured antioxidant activity of biological samples depends on which free radical or antioxidant is used in the assay (Cao et al 1996). Recently, the methods adopted peroxyl radical (ROOÿ) as standard radical because of its common place and relevance to biological systems (Cao and Prior 2001). Since this radical is present in biological systems, it gives ORAC values more credibility as

an indication of antioxidant activity of phenols in biological systems. The ROOÿ reacts with a fluorescent probe, fluorescein (a synthetic protein) to produce a non-fluorescent product that is measured by fluorescence (Ou et al 2001).

The method has an advantage of being automated and standardized for comparison of data. However it requires expensive equipment, limiting its wide usage. Moreover, the reaction is sensitive to variations in temperature among other factors making the results highly variable from day to day.

CHAPTER III

FLAVONOID PROFILE AND ANTIOXIDANT ACTIVITY OF SORGHUMS WITH AND WITHOUT PIGMENTED TESTA

Introduction

Sorghum has unique and diverse bioactive phenolic compounds that could provide health benefits beyond standard nutrition. High levels of various phenolic compounds have been reported in sorghum. Hahn et al (1984) reported high levels of polyflavanols (970 mg CE/g) in sorghum, while Awika (2004) showed that both brown sorghum grains and brans had high proanthocyanidin contents (21-58 mg/g) compared to blueberry (20 mg/g).

The genetics affect the presence or absence of a pigmented testa and consequently tannins. Most cultivated sorghums do not contain condensed tannins even though non-tannin phenolic compounds are sometimes reported as tannins when vanillin/HCl or butanol/HCl methods are used to measure tannin.

As a source of flavonoids, sorghum has numerous competitive advantages over other natural sources such as ease of storage for long periods of time because of its dry nature at harvest and the ease of processing into shelf-stable concentrates (Awika and Rooney 2004). This suggests that sorghum may not only be a source of uniquely stable compounds but also contain high antioxidant compounds with potential health benefits that may complement those of fruits and vegetables.

This work focuses on different sorghum varieties selected based on types and will provide potentially useful information for the selection of sorghum varieties with special traits to enhance flavonoid composition, thus improving functionality and health attributes of the sorghum grain. The specific objective of this chapter was to determine how the presence or absence of a pigmented testa affects the level and profile of flavonoids in sorghum.

Materials and Methods

Sources of Materials

Twenty four sorghum varieties grown in a sorghum breeding nursery in College Station, TX in 2005 were analyzed. They comprised 4 non-tannin and 20 tannin sorghums grouped as Type I, Type II, Type III red pericarp and Type III white pericarp (Figs. 8-11). For ORAC assay ATX631 x RTX436 grown in College Station, TX in 2001 was used as a control.

Reagents

Gallic acid, catechin hydrate, 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), naringenin and potassium persulfate, were purchased from Sigma-Aldrich (St. Louis, MO). The 2-2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Acros Organics (Morris Plains, NJ), and Trolox (6-hydroxy-2, 5, 7, 8 tetramethylchroman-2-carboxylic acid) was obtained from Aldrich (Milwaukee, WI). Apigenin and luteolin were obtained from Indofine Chemical Co., Inc.

(Hillsborough, NJ). Eriodictyol, luteolinidin chloride, and apigeninidin chloride were obtained from ALSACHIM (Strasbourg, France) and 7-methoxyapigeninidin chloride was obtained from ChromaDex (Santa Ana, CA). *Sec*-butanol was reagent grade, while all other solvents were HPLC grade.

Extraction for Colorimetric Assays

All samples were ground for 2 min using a Cuisinart DCG-20 coffee grinder (East Windsor, NJ) prior to analysis. For all assays with the exception of the DPPH and ORAC assays, samples (0.1-0.5 g) were weighed into centrifuge tubes in which 25 mL 1% HCl/methanol (v/v) was added, and rocked for 2 hours at low speed on an Eberbach shaker (Eberbach Cor, MI). For the butanol assay, samples were extracted in 10 mL of 1% HCl in methanol (v/v). For the DPPH and ORAC assays, samples (0.15-0.3 g), depending on tannin content, were extracted in 25 ml (for DPPH) or 20 ml (for ORAC) 70% aqueous acetone (v/v). After shaking in each instance, all extracts were centrifuged at 2790 x g for 15 minutes in a Sorvall SS-34 centrifuge (DuPont Instruments, Wilmington, DE) and then decanted. To avoid oxidation, extracts were stored in the dark at 0℃ and analyzed within 24 hours.

Fig. 8. Type I sorghum varieties grown in College Station TX, 2005.

Fig. 9. Type II sorghum varieties grown in College Station TX, 2005.

Shawaya Brown

Shawaya Red with Black

Shawaya Red

SC1321

SC124

SC1318

Hi Tannin

SC 103

Fig. 10. Type III red pericarp sorghum varieties grown in College Station, TX 2005.

Fig. 11. Type III white pericarp sorghum varieties grown in College Station TX, 2005.

Extraction for HPLC -PDA Analysis

Extraction and analyses were done according to the method of Dykes (2008). Briefly, 1 g ground samples were extracted in 10 mL of 1% HCl/methanol (v/v) for two hours in a shaker. The extracts were centrifuged at 2790 x *g* for 15 minutes, and were then decanted. A second set of extracts were prepared in the same manner for flavanone analysis, but after decanting, each supernatant was transferred to glass tubes, sealed, and placed in a water bath at 80ºC for 90 minutes to hydrolyze flavanone glycosides to their aglycones prior to flavanone analysis. All extracts were immediately filtered using a 0.45µm nylon membrane filter (Whatman Inc., Maidstone, UK) prior to HPLC analysis.

Colorimetric Assays

Determination of Total Phenol Content

The modified Folin-Ciocalteu method of Kaluza et al (1980) was used for the analysis of total phenols. One aliquot (0.1 mL) of HCl acidified methanolic extract was combined with 1.1 mL of water, to which 0.4 mL of Folin reagent and then 0.9 mL of 0.5 M ethanolamine were added. The reaction was allowed to proceed at room temperature for 20 minutes. Gallic acid was used to prepare a calibration curve and the absorbance readings of standards and samples were taken at 600 nm using a UV/Vis spectrophotometer. Measurements were expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of dry weight sample.

Determination of Tannin Content

Tannin content was determined using the vanillin-HCl method as described by Price et al (1978). The ground samples were extracted at 30°C for 20 min using acidified methanol. The extracts were centrifuged at 2790 x *g* for 15 minutes, and then decanted. One aliquot (1 mL) of the supernatant was mixed with 5 mL vanillin reagent, allowed to react at room temperature for 20 min and the absorbance readings taken at 500 nm. Catechin was used as the standard and tannin content was expressed as milligrams catechin equivalents per g (mg CE/g).

Antioxidant Assay

Antioxidant activity was determined using the 2, 2'-Azinobis (3-ethylbenzothioline-6-sulfonic acid) (ABTS), 1, 1-Diphenyl-2-picry-hydrazyl (DPPH) and Oxygen radical absorbance capacity (ORAC) assays.

ABTS

The ABTS analysis was performed as described by Awika et al (2003b). First, the ABTS radical was generated overnight (12 hours) in the dark, by reacting equal volumes of 8 mM ABTS solution in distilled/deionized water with 3 mM potassium persulfate. Then a working solution was prepared by diluting the 5mL ABTS free radical mixture with 145 mL of phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS), to obtain an initial absorbance of 1.5 at

wavelength of 734 nm. One aliquot (0.1mL) of sample extract and standard were reacted separately, with 2.9 mL of ABTS solution for 30 min at room temperature. Trolox was used as the standard. The absorbance was measured at 734 nm and results expressed as micromolar Trolox Equivalent Antioxidant Capacity per g (µmol TE/g) dry weight sample.

DPPH

The DPPH analysis was done based on the method of Brand-Williams et al (1995) as modified by Awika et al (2003b) for grain products. The DPPH reagent was dissolved in methanol and kept in the dark prior to use. One aliquot (0.15 mL) of sample extract was reacted with 2.85 mL DPPH mixture. The reaction was left for 6 hours in the dark and the absorbance measured at 515 nm. Trolox was used as the standard and the results expressed as micromolar Trolox Equivalent Antioxidant Capacity per g (µmol TE/g) of dry weigh sample.

ORAC Analysis

The ORAC procedure was adapted from Cao et al (1993) and Prior et al (2003) to determine the free radical scavenging activity of sorghum samples, using an automated Fluostar Optima plate reader (BMG technologies, Offenburg, Germany). All analyses were conducted in phosphate buffer pH 7.4 and at 37°C. Peroxyl radical was generated using 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (Wako chemicals, Richmond, VA), and

fluorescein was used as probe. The AAPH was added to individual wells just prior to assaying. A microplate reader with excitation at 485 nm and emission at 520 nm was used to read samples. The final ORAC values were calculated using a quadratic regression equation between Trolox concentration or sample and net area under the fluorescein decay of sample extracts. The results were expressed as micromoles of Trolox Equivalent Antioxidant Capacity per g (µmol TE/g) of dry weight sample.

Determination of Flavan-4-ol Content

Flavan-4-ol content was determined using the modified method of Govindarajan and Mathew (1965) as described by Gous (1989). Briefly, one aliquot of extract (1 mL) was reacted with 5 mL of HCl-butanol reagent, prepared by dissolving 0.0616g of FeSO4.7H2O in 5% HCl in sec-butanol (V/V). The reaction was allowed to proceed for 1 hour at room temperature and the absorbance measured at 550 nm and expressed as absorbance per milliliter (abs/mL) per gram of dry weight sample.

Determination of Anthocyanin Content

Anthocyanin content was measured using the method of Fuleki and Francis (1968) and Wrolstad (1976) with some modifications. One aliquot of each sample was diluted 2-fold using the extraction solvent and allowed to

equilibrate for 2 hours at room temperature and in the dark. Absorbance readings were taken at 465 nm for apigeninidin and 485 nm for luteolinidin.

The concentrations of anthocyanins were calculated using the molar extinction coefficient (ε) of luteolinidin (29,157). The molar extinction coefficient (ε) of luteolinidin was determined from the formula based on Lambert Beer's Law ($A = \varepsilon CL$) as described by Wrolstald (1976): $C_{(mg/l)} = A/\varepsilon L \times 10^3 \times MW \times$ Dilution Factor, where MW is molecular weight which was 306.7 because luteolinidin standard was in the chloride form, ε is molar absorbance or molar extinction coefficient, A is absorbance, C is molar concentration. Rearranging the Lambert-Beer's Law equation, $C = A/\varepsilon L$ (L, the pathlength is commonly 1); concentration in milligrams per liter was obtained by multiplying by the molecular weight of luteolinidin.

From rearranging the equation, we obtained $\varepsilon L = A/C_{(mg/l)} \times 10^3 \times MW \times$ Dilution Factor. MW in this equation is the MW of the pigment, in this case luteolinidin (270.0), because that was the predominant pigment in the sorghum extracts. The results were expressed as mg luteolinidin equivalent (LE) per gram sample dry weight.

HPLC -PDA Analysis

HPLC analyses of extracts were done using an Alliance 2695 system (Waters Corp., Milford, MA) connected to a Waters 996 photodiode array detector (PDA) as described by Dykes (2008). Sorghum phenolics were separated using a Luna C18 column (150 mm x 4.6 mm i.d., 5 µm) from Phenomenex (Torrance, CA). Column temperature was maintained at 35ºC, injection volume was 20 µL. The mobile phase consisted of 4% formic acid in water (v/v) (Solvent A) and acetonitrile (Solvent B). The solvent flow rate was 1.0 mL/min. Different conditions were used for the separation of the various phenolic compounds in the extracts. 3-Deoxyanthocyanins were separated using the following gradient: 0-20 min., 12-20% B; 20-40 min., 20-50% B; 40-50 min., 50% B. Flavones and flavanones were separated using the following gradient: 0-45 min., 15-41% B; 45-50 min., 41% B. Detection wavelengths for the 3-deoxyanthocyanins, flavones, and flavanones were 485 nm, 340 nm, and 280 nm respectively.

Identification of sorghum flavonoids was determined based on commercial standards' retention times, and UV-Vis spectra. Quantification of each compound was accomplished by comparing peak areas with that of a standard curve of each respective standard. Molecular weight correction factors (Chandra et al 2001; Wu et al 2006) were used to quantify 5-methoxyluteolinidin and 7-methoxyapigeninidin. The molecular weight correction factors for the specific calculation of individual 3-deoxyanthocyanins were determined by

dividing the molecular weight of the 3-deooxyanthocyanin to be quantified by that of the standard 3-deoxyanthocyanin (Chandra et al2001). Data was collected and processed using the Empower software version 1.0 (Waters Corp., Milford, MA).

Statistical Analysis

All values are expressed as means ± standard deviation for three replicates. One-way ANOVA was used to determine significant differences in total phenols, antioxidant activities, tannin contents, anthocyanin levels and flavan-4-ols among sorghum types. Least square means (LSMeans) were used to compare the means. Separation of means was achieved using Turkey's Minimum Significant Difference (MSD). Pearson correlations were used to determine correlation between total phenol, tannins ABTS antioxidant activity, DPPH antioxidant activity, ORAC antioxidant activity anthocyanins and flavan-4-ols. P values were considered significant when less than 0.05. All statistical analyses were done using the statistical software SAS version 9.1 (SAS Institute Inc. Cary, NC) and SPSS version 16.0 (SPSS Inc. Chicago, IL).

Results and Discussion

Evaluating Sorghum Total Phenols

Figure 12 compares the total phenols for the sorghum varieties grouped by types. Total phenols for Type III red pericarp and Type III white pericarp sorghums ranged from 4.3-14 mg GAE/g and 4.6-9.4 mg GAE/g, respectively. Meanwhile, Type II and Type I sorghum varieties had total phenols ranging from 3.4-6.7 mg GAE/g and 2.3-5.6 mg GAE/g, respectively. Three of the 4 Type I sorghums had higher total phenols than some of the Type II and III sorghums. For example, Shawaya mostly black had 6 mg GAE/g while Hegari had 4 mg GAE/g total phenols. Thus, total phenols can be high in sorghums without a testa, especially the black varieties. These results agreed with Gous (1989).

Statistical differences ($p<0.05$) were observed in the mean total phenols for Type III red (9.4mg GAE/g) and Type III white (7.2 mg GAE/g) pericarp sorghum varieties. There were no differences in mean total phenols between Type II (4.8 mg GAE/g) and Type I (3.9 mg GAE/g) sorghums.

The presence of a pigmented testa, the dominant spreader gene and the red turning black genes also had an effect on total phenols. The presence of a pigmented testa and “B1_B2_S_” increased total phenols among Type III sorghums (Fig. 12). Among the Type I sorghums, the presence of the red turning into black genetics in the Shawaya black sorghum increases the levels

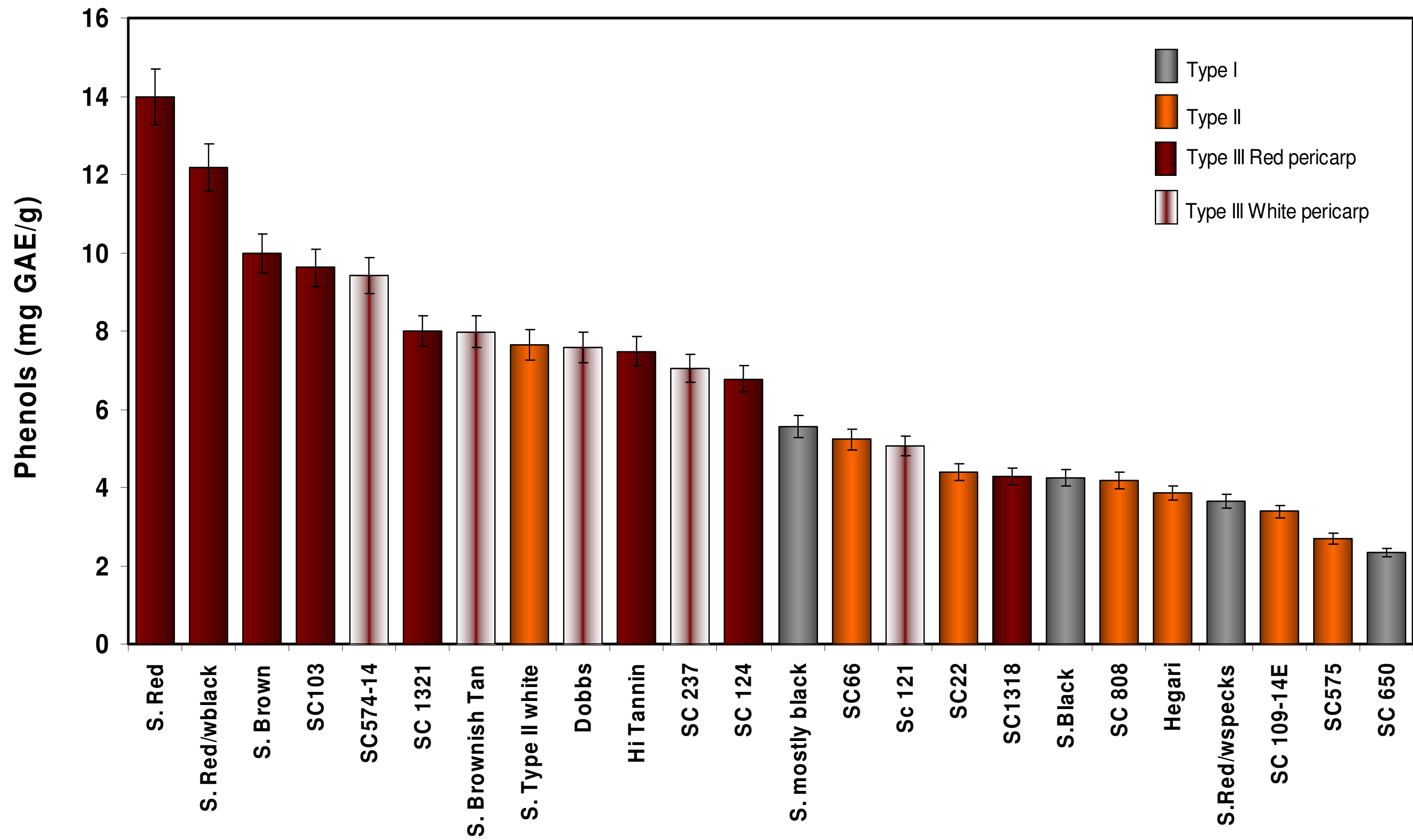


Fig. 12. Total phenols in sorghums grown in College Station, TX 2005. S = Shawaya.

of total phenol compared to the levels in SC650. Shawaya mostly black had higher total phenols than SC1318, a Type III red pericarp sorghum (Fig. 12).

The effect of pigmented testa and pericarp color on total phenol content was consistent with Dykes et al (2005); Dicko et al (2005) and Nomusa et al (2007). Other attributes that affect total phenol distribution among sorghum varieties include pericarp thickness and plant color. Beta et al (1999) and Dykes et al (2005) reported a positive correlation between pericarp thickness and total phenol. Sorghum from red and purple plants had higher levels of total phenol.

The sorghum germplasm remains a viable source of phenolic compounds because it competes favorably with other known sources. Berries, for example are known to be good sources of phenolic compounds. Total phenol of blue berry fruits for example is 1.8 mg GAE/ g dry weight (Ehlenfeldt and Prior 2001), while it is between 3- 5 GAE/ g dry weight for raspberries (Liu et al 2003). The levels of total phenols in 64 % of the sorghums varieties used in this study were 1.2-2.8 times higher than in fruits and berries.

Evaluating Condensed Tannins in Sorghum

Figure 13 shows the tannin levels among the different types of sorghum that ranged from 3.3-30 mg CE/g and were within the range reported in other studies (Hahn and Rooney 1986; Awika 2000). The presence of pigmented testa and a dominant spreader gene affected tannin content. Type III red and white pericarp sorghums had the highest levels of tannin ranging from 5.8-30 and

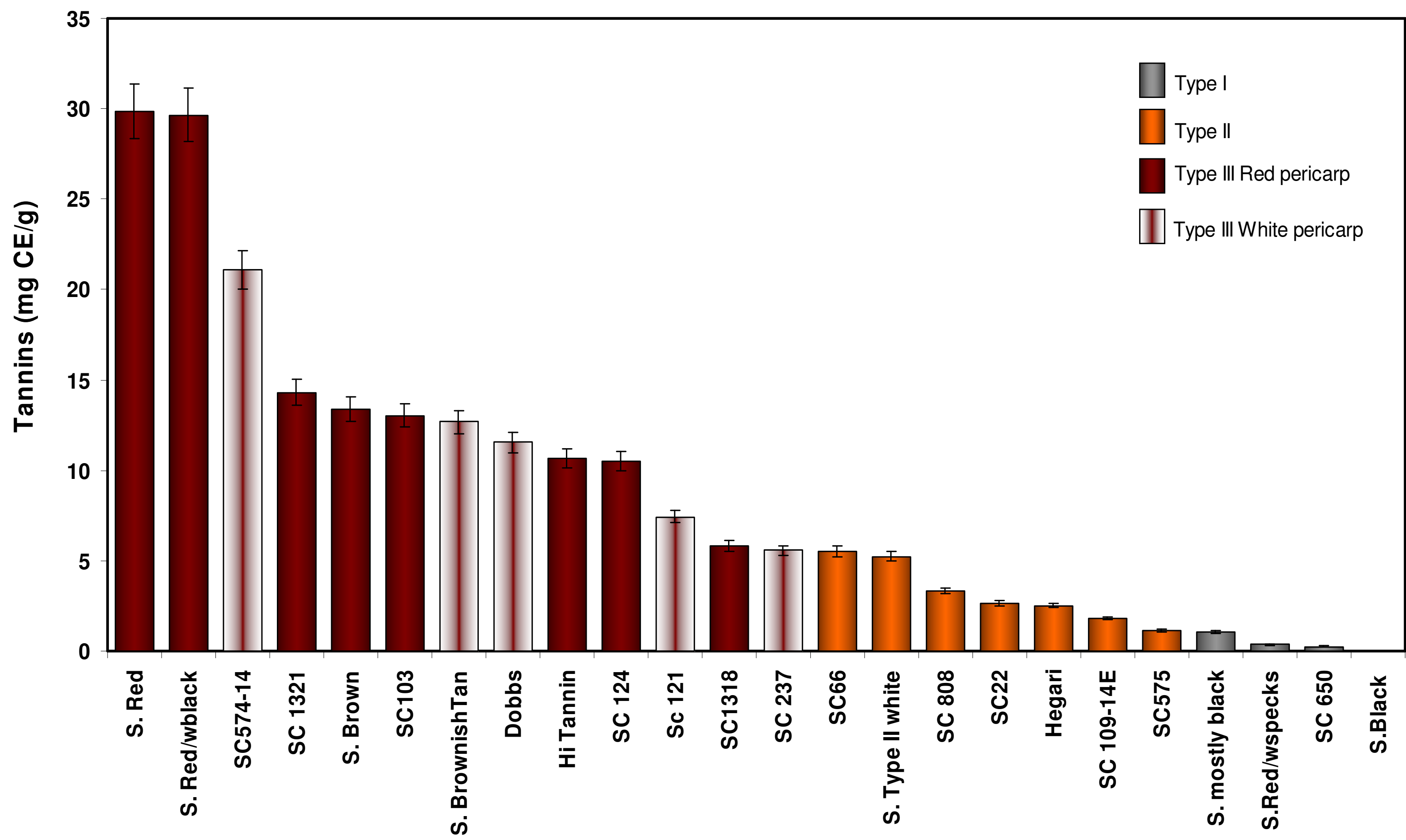


Fig. 13. Condensed tannins in sorghums grown in College Station, TX 2005. S = Shawaya.

7.4-21.1 mg CE/g respectively. Shawaya Red and Shawaya Red with Black had the highest levels of tannins (30 mg CE/g). SC103 had 13 mg CE/g tannins, lower than 18.7 mg CE/g reported by Awika (2003). The tannin content of Type II sorghums ranged from 1.1-5.7 mg CE/g, while Type I sorghum had undetectable levels of tannins.

These values were lower than those reported for some of the sorghum varieties. For example, Boren & Waniska (1992) reported 6-16 mg CE/g for Type II sorghums, Hegari had 7 mg CE/g compared to 3 mg CE/g, while SC109-14E had 6 mg CE/g versus 2 mg CE/g reported in this study. They found higher values for Type III sorghums with Dobbs at 20 mg CE/g compared to 12 mg CE/g found in this study. Overall, the authors reported about twice the levels of tannin reported in this study. The low levels found in this study may be attributed to the weathered condition of the 2005 grains used. Levels of phenolic compounds in sorghum are greatly affected by genetic and the environment under which the grains mature.

The mean tannin content for Type III red and Type III white pericarp sorghums were 16 and 12 mg CE/g respectively and both means were different from Types I (0.6 mg CE/g) and Types II (4 mg CE/g) varieties ($p<0.05$). Extracted tannins often may react with other components in the extraction medium to form products that do not react with vanillin, thus giving false low absorbance and consequently low to non-detectable tannin levels even in tannin sorghum.

Tannins in type II sorghum are generally more difficult to extract than those in type III and this could be the reason for higher tannin content in Type III sorghums compared to Type II varieties. The difficulty in extraction of tannins from Type II sorghums might be due to their location in the sorghum grain. The tannins in type II sorghums are deposited in vesicles within the testa layer, whereas the tannins in type III are deposited along the cell walls of the testa with some present in the pericarp as observed by Earp et al (2004a).

Evaluating Flavan-4-ols in Sorghum

Flavan-4-ol levels were in the range of 1.2-8.6 abs/mL/g (Fig.14). Type III red pericarp varieties had flavan-4-ols levels ranging from 4.0-8.6 Abs/mL/g except for SC1318 with a value of 1.4 abs/mL/g, while levels for Type III white pericarp varieties ranged from 1.4-3.3 abs/mL/g. Type I sorghum had values in the range of 3.3-7.1 abs/mL/g while Type II values ranged from 1.2-2.1 abs/mL/g.

In general, the red and black pericarp sorghums had higher levels of flavan-4-ols than the white pericarp sorghum. These were consistent with the findings of Menkir et al (1996) and Dykes (2008) who reported higher flavan-4-ols in red sorghums.

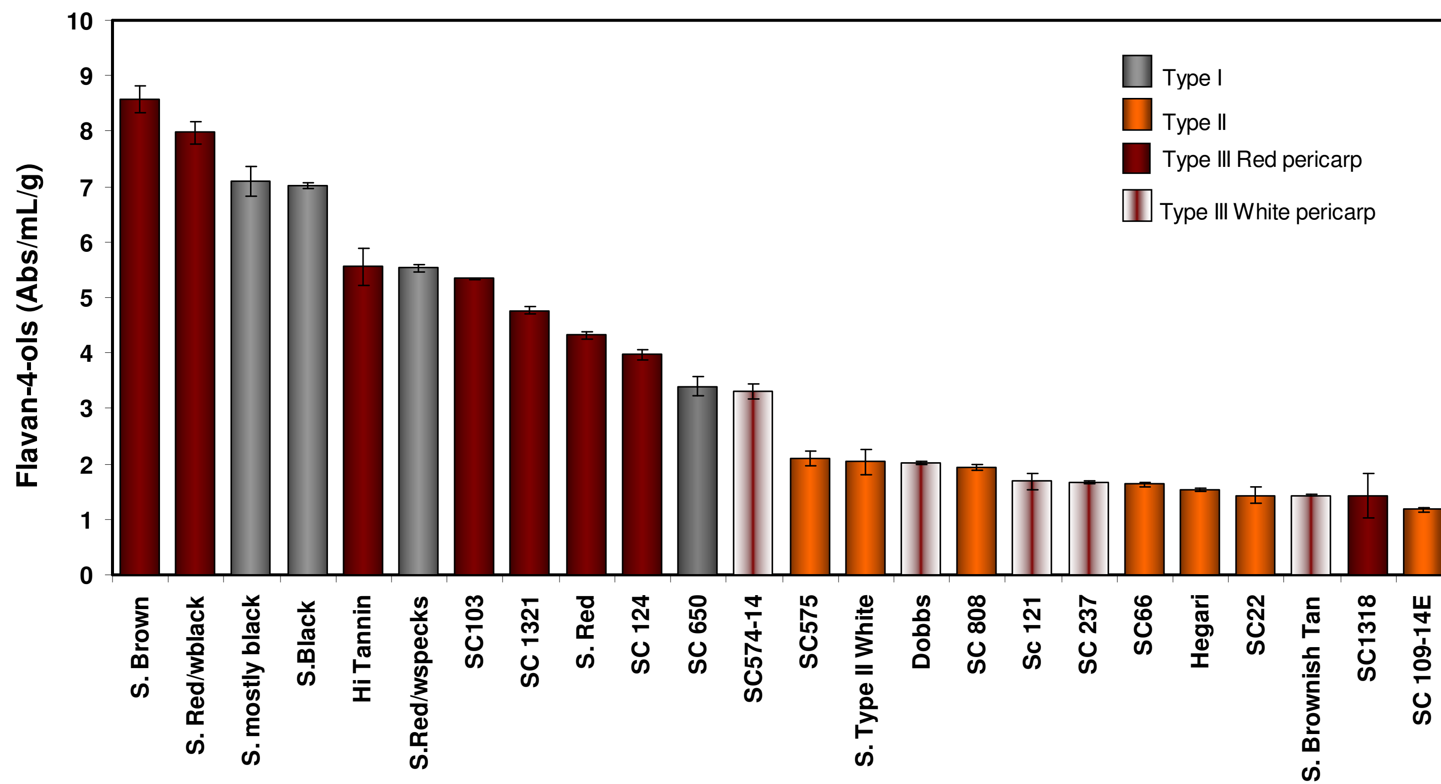


Fig. 14. Flavan-4-ol levels in sorghums grown in College Station, TX 2005. S = Shawaya.

Flavan-4-ol compounds such as luteoferol and apiferol are produced from flavanones and are considered precursors of 3-deoxyanthocyanins (Watterson and Butler 1983; Wharton and Nicholson 2000). It is not clear whether flavan-4-ols are produced in response to mold attack or as a byproduct of a defense mechanism by the plant (Jambunathan et al 1986), but both flavan-4-ols and 3-deoxyanthocyanins are produced in response to biotic and abiotic stresses. Flavan-4-ols and 3-deoxyanthocyanins may exist in a state of equilibrium or in about the same amount. Whatever is the case merits further investigation.

Evaluating Sorghum Anthocyanins

Anthocyanin levels ranged from 1.1-4.7 mg LE/ gram (Fig.15). Luteolinidin was identified as the main anthocyanin in 92 % of the sorghum varieties used. Its molar extinction coefficient was determined as 29,157 in 1%HCl/methanol as described earlier (p. 52).

Black pericarp sorghums had higher levels of anthocyanins (3.4-4.7 mg LE/g), followed by the red pericarp sorghums (2.9-3.4 mg LE/g). White pericarp sorghums had the lowest levels of anthocyanins with 1.1 mg LE/g in SC 121. Anthocyanins levels of 3.7 mg/g were reported for black sorghum bran (Gous 1989), which according to Awika (2003) could have been an underestimation in the amount of anthocyanins because the standard used was cyanidin which is not a major pigment in sorghum.

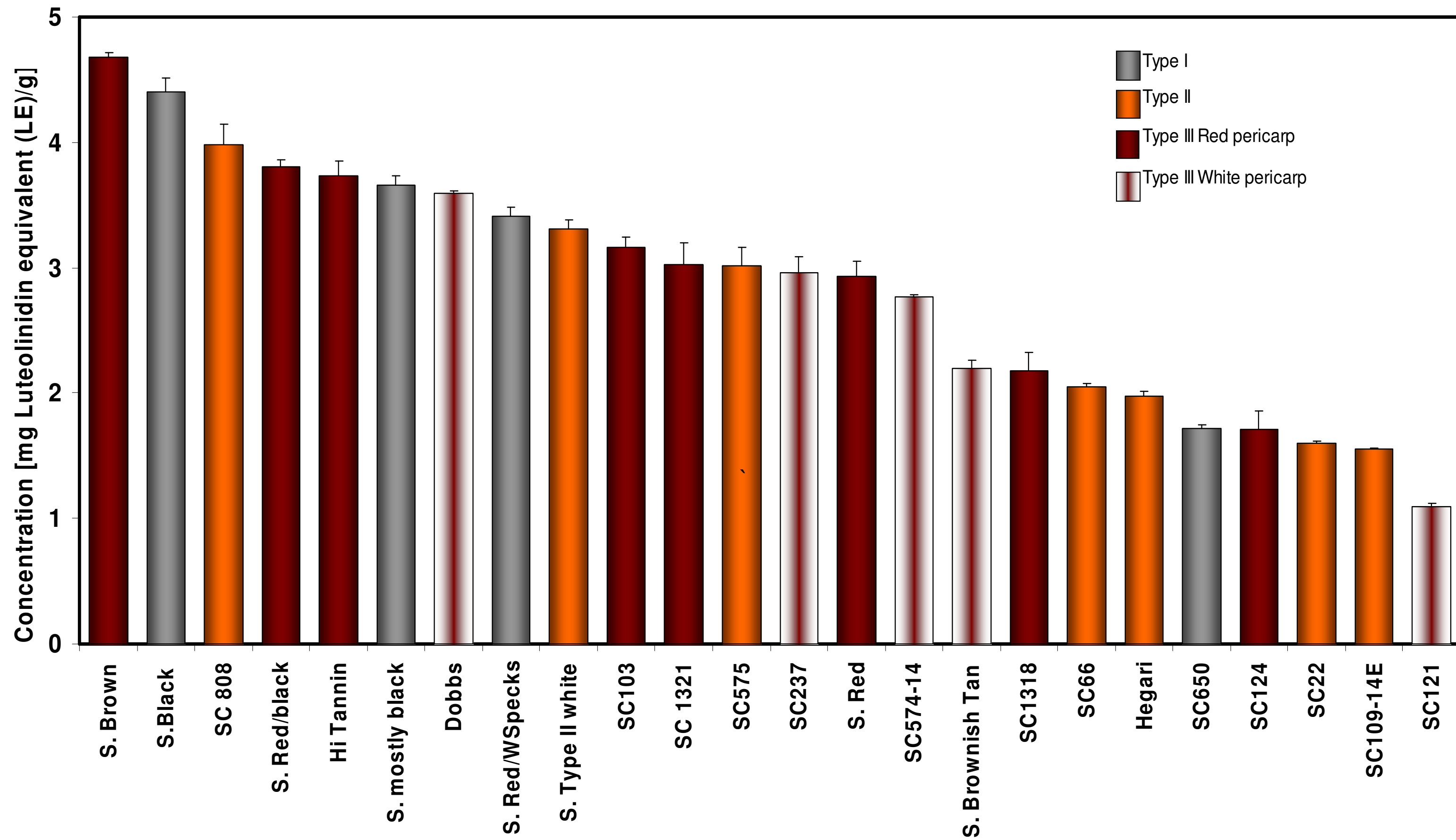


Fig. 15. Anthocyanin content in sorghums grown in College Station, TX 2005. S = Shawaya.

The levels of anthocyanins increased when grains were spotted irrespective of the pericarp color, for example in SC808 (4.0 mg LE/g). Seitz (2004) reported that 3-deoxyanthocyanins in sorghum were present mostly in the damaged spots on seeds depending on the pericarp color. 3-Deoxyanthocyanins are produced in response to biotic and abiotic stresses and are released and accumulated by the host plant as by-products of the defense mechanism.

Antioxidant Potential of Sorghum Varieties

Antioxidant Activities by ABTS

Antioxidant activities as measured by ABTS (Fig. 16) ranged from 21-175 µmol TE/g. Shawaya Red sorghum, a Type III red pericarp variety, had the highest antioxidant activity (175 µmol TE/g), while SC575, a Type II variety, had the lowest level (21 µmol TE/g). The antioxidant activity of Type III red and Type III white pericarp sorghums were higher (49-175 µmol TE/g) than those for Type II and I sorghums except for Shawaya Type II white with antioxidant activity higher than some Type III red and Type III white pericarp sorghums.

The Type I sorghum with black pericarp had higher antioxidant activities than Type II sorghums except for Shawaya Red with Specks (Fig. 16). The high levels of flavan-4-ols and the increased levels of 3-deoxyanthocyanins

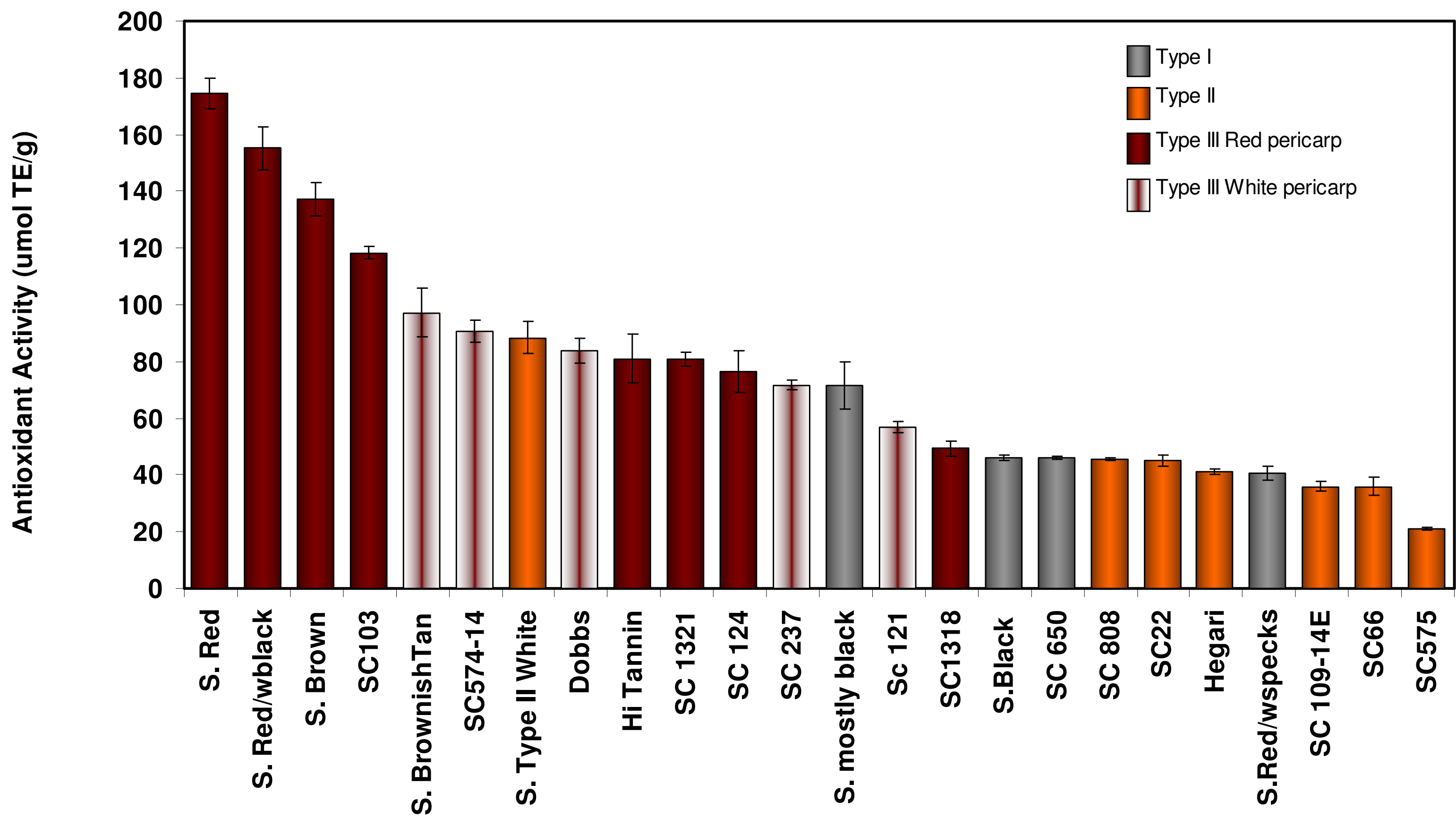


Fig. 16. ABTS antioxidant activity of sorghums grown in College Station, TX 2005. S = Shawaya.

contributed to the increased antioxidant activities in Type I sorghum with the red turning into black pericarp. Mean antioxidant activities of Type III red (109 µmol TE/g) and white (76 µmol TE/g) pericarp varieties were statistically different ($p<0.05$). The mean antioxidant activity of Type II and I sorghum varieties were 50 and 41 µmol TE/g, respectively, but were not statistical different.

The antioxidant activities as measured by ABTS showed Type III sorghums had higher antioxidant activity than Type II and I sorghums in that order. Tannins are more easily extracted from Type III than Type II sorghum. Type I sorghum do not contain tannins and the antioxidant activity observed was from flavan-4-ols, anthocyanins and other flavonoids in the Shawaya and SC650 sorghums. The antioxidant activity in tannin sorghum is contributed mainly by condensed tannins which have demonstrated higher free radical quenching ability *in vitro* than other phenolic compounds (Hagerman et al 1998; Awika et al 2003b).

Dykes (2008) reported twice the total phenol and antioxidant activity for Shawaya Black as reported in this study. Upon visual examination of the grains, the 2006 grains used by Dykes (2008) were bright black compared to the dull or grayish black color of the 2005 grains used in this study. The dull color was due to severe weathering suffered by the 2005 grain which likely altered the phenolic content of the grains.

Antioxidant Activities by DPPH

Type III red and white pericarp varieties had the highest DPPH antioxidant activity potential (Fig. 17*).* Type III red pericarp had values ranging from 42-134 µmol TE/g and 43-107 µmol TE/g for Type III white pericarp sorghums. Mean antioxidant activity levels were higher for Type III red pericarp (119 µmol TE/g), followed by Type III white pericarp (71 µmol TE/g) and were statistically different from each other ($p<0.05$). Type II varieties had antioxidant activity levels ranging from 5-13 µmol TE/g, while for Type I the range was 11-27 µmol TE/g. Type I varieties had higher antioxidant activity levels than Type II varieties except for Shawaya Type II white that had a value of 13 µmol TE/g.

Mean antioxidant activity levels from DPPH analysis for Type I and II varieties were 15.4 and 8.4 µmol TE/g respectively and were not different from each other statistically. Higher values observed in red and black pericarp sorghum may be attributed to the high levels of flavan-4-ols (Jambunathan et al 1991; Menkir et al 1996) and 3-deoxyanthocyanins contributing to the antioxidant activity.

Antioxidant Activities by ORAC

The presence of a pigmented testa did not affect ORAC values (Fig. 18). Type I sorghums are normally expected to have low antioxidant activity because they lack tannin, but the surprisingly high ORAC values observed for Type I sorghum varieties in this study is likely because of 3-deoxyanthocyanins and flavan-4-ols in the pericarp.

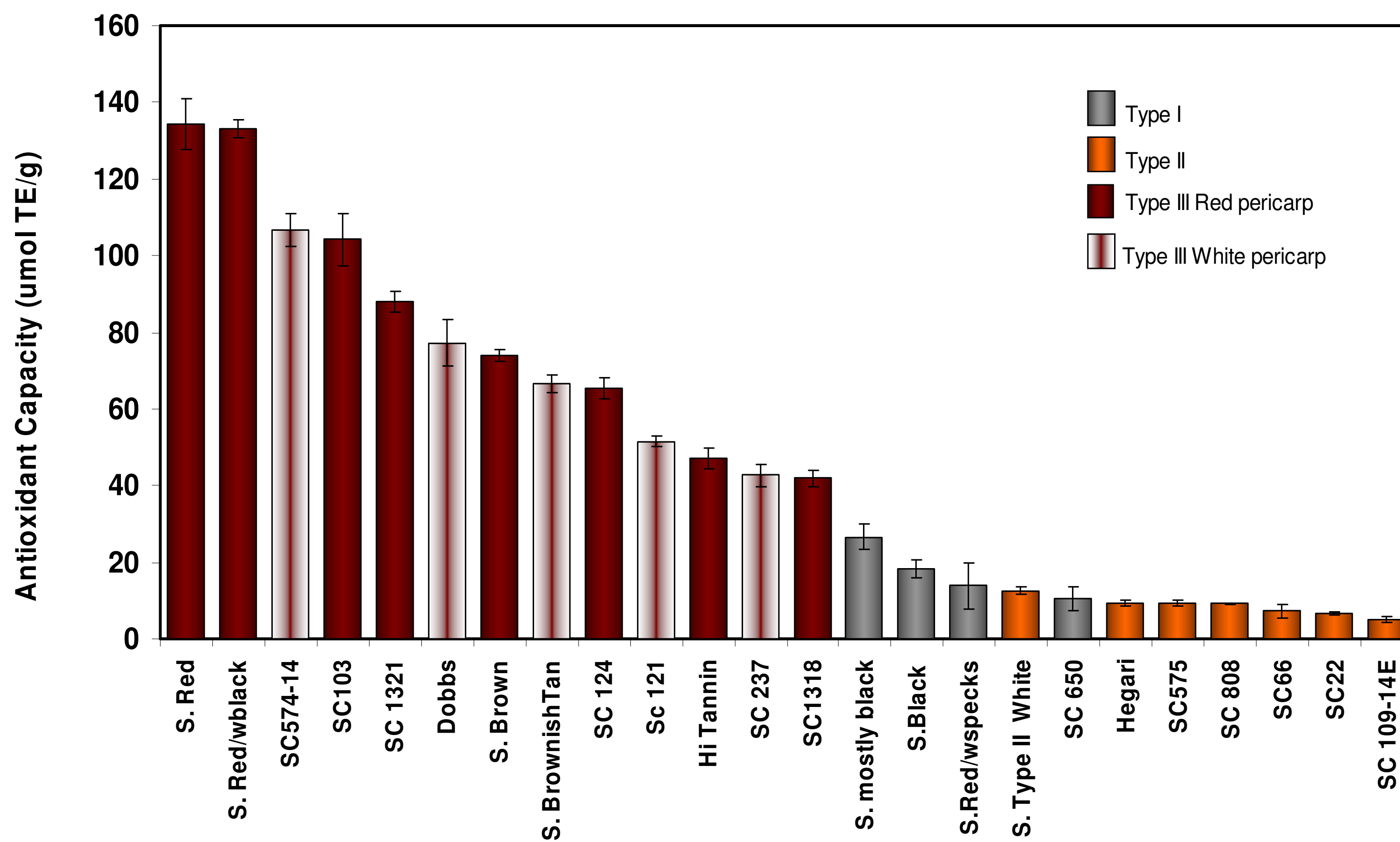


Fig. 17. DPPH antioxidant activity of sorghums grown in College Station, TX 2005. S = Shawaya.

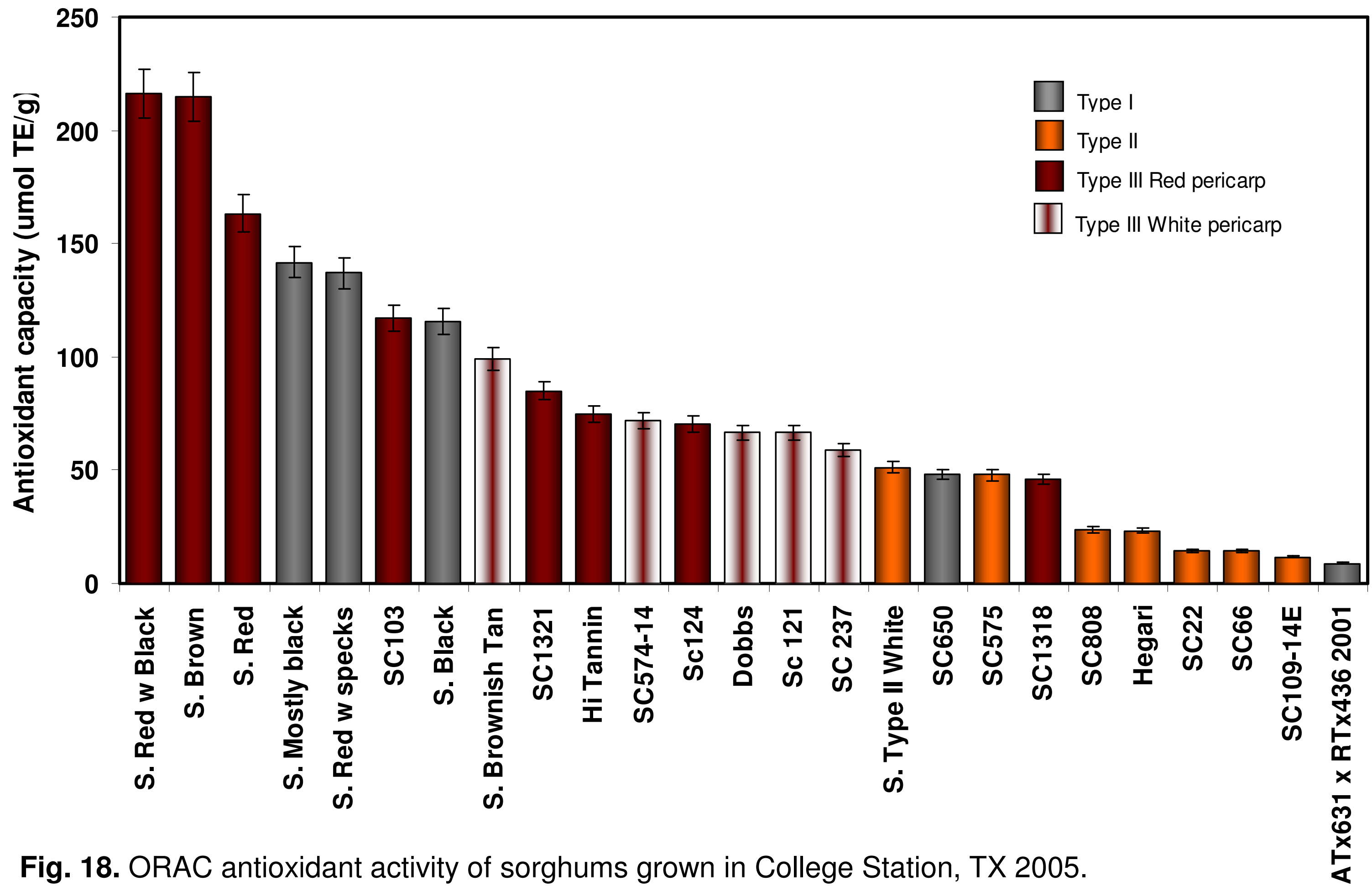


Fig. 18. ORAC antioxidant activity of sorghums grown in College Station, TX 2005. ATX 631 x RTX436 was from 2001 crop year. S = Shawaya.

Type I varieties without pigmented testa, having the special red turning black pericarp had higher ORAC values than most Type III and Type II varieties. Type I sorghum (non-black) had low ORAC values (Fig. 18). The special black sorghums had outstanding levels of 3-deoxyanthocyanins, contributing to the high antioxidant activity similar to Awika (2000).

Low values observed for DPPH might be due to color interference from colored pigment compounds such as 3-deoxyanthocyanins interfering with DPPH radical, leading to an underestimation of the antioxidant capacity (Arnao 2000; Awika 2003b; Dykes et al 2005).

Overall, pericarp color of sorghum did not affect antioxidant capacity. For example, Shawaya Brownish Tan (Type III white pericarp), had higher antioxidant capacity than Hi Tannin sorghum (Type III red pericarp). Awika (2003) reported black sorghum bran which has predominantly 3-deoxyanthocyanins giving low DPPH values because the DPPH chromogen absorption maxima (515nm) is close to the absorption range for anthocyanins (475-485nm).

Awika et al (2003b) reported ORAC values that were 2-3 times higher than ABTS and DPPH. A positive correlation was observed among the three assays. The antioxidant activity values are compared relative to that of trolox in all three assays, but because individual molecules are known to be more efficient in quenching particular radicals than others (Wang et al 1998; Lotito et al 2000), it is possible that high ORAC values (Table 3) are due to lower

reactivity of Trolox in the ORAC system compared to ABTS and DPPH systems (Awika 2003).

From the ORAC assay the red and black pericarp sorghums had higher antioxidant activity than the white pericarp sorghums. The high antioxidant activity may be due to the high levels of flavan-4-ols and 3-deoxyanthocyanins present in these varieties. The presence of pigmented testa in addition to red and black pericarp increased phenol levels and antioxidant activity as measured by DPPH and ABTS methods but when the ORAC method was used, the antioxidant activity levels were increased by the presence of the red turning to black pericarp.

ORAC is considered to be the most reliable method to assess *in vitro* antioxidant activity as it applies to biological systems (Cao et al 1996; Prior and Cao 2000; Cao and Prior 2001). ORAC values indicated Shawaya mostly black, Shawaya red with specks and Shawaya black had higher antioxidant activity than most Type III sorghums used in this study. The antioxidant capacities are contributed mainly by flavan-4-ols and 3-deoxyanthocyanins which were high in these varieties.

Correlations among Colorimetric Analyses

Correlation coefficients (Table 4) of total phenol with ABTS were 0.97 and 0.87 for total phenol with DPPH. The strong correlation between total phenol and

antioxidant activity indicates that phenolic compounds were responsible for antioxidant activity. The lower correlation coefficient of total phenol with DPPH could be attributed to the possible color interference from sorghum 3-deoxyanthocyanins.

Table 3
Antioxidant Activity of Sorghum Varieties Assessed by ORAC, ABTS and DPPH Methods

Sample ID	Types	ORAC	ABTS	DPPH
Shawaya Red with Black	Type III Red P	21600	15500	25800
Shawaya Brown	Type III Red P	21500	13700	7200
Shawaya Red	Type III Red P	16300	17500	26000
Shawaya Mostly Black	Type I	14200	7200	2600
Shawaya Red with specks	Type I	13700	4100	1300
SC103	Type III Red P	11700	11800	10900
Shawaya Black	Type I	11600	4600	1800
Shawaya Brownish Tan	Type III White P	9900	9700	6400
SC1321	Type III Red P	8500	8300	9200
Hi Tannin	Type III Red P	7500	8100	4800
SC574-14	Type III White P	7200	9300	11200
SC124	Type III Red P	7100	7600	6700
Dobbs	Type III White P	6700	8400	8100
SC 121	Type III White P	6700	5700	5400
SC 237	Type III White P	5900	5000	4500
Shawaya Type II White	Type II	5100	8800	1200
SC650	Type I	4800	2200	1000
SC575	Type II	4800	2700	1000
SC1318	Type III Red P	4600	4900	4300
SC808	Type II	2400	4600	900
Hegari	Type II	2300	4100	1000
SC22	Type II	1400	4500	700
SC66	Type II	1400	4600	700
SC109-14E	Type II	1200	3600	500
ATx631 x RTx436 2001	Type I	900	ND	ND

Values in µmol TE/100 g sample (Dry weight basis). Samples for ABTS were extracted in 1%HCl/Methanol, while samples for DPPH and ORAC were extracted in 70% aqueous acetone. P=Pericarp. ND=Not determined.

Table 4
Pearson's Correlation Coefficients of Sorghum Phenols and Antioxidant Activity

	TP	*ABTS*	*DPPH*	*ORAC*	*Tannins*	*Flavan-4-ols*
TP		0.97	0.87	0.78	0.97	-
ABTS			0.88	0.79	0.90	0.52
DPPH				0.88	0.95	.41
ORAC					0.71	0.86
Tannins						-
ANTH						

Correlation is significant at $p<0.01$
TP=Total phenols.

DPPH is measured at 515 nm while ABTS is at 734 nm, which is far beyond the absorption wavelength for other components that could interfere with the absorbance reading hence interference is not expected for ABTS (Awika et al 2003). Arnao (2000) associated lower DPPH values with other grain pigments including anthocyanins and carotenoids. The correlation coefficients of tannins with ABTS, tannins with DPPH, and tannins with ORAC were 0.90, 0.95 and 0.71 respectively ($p<0.01$). These correlations agreed with the results of Awika et al (2003a), Dykes et al (2005) and Nomusa et al (2007) for DPPH and ABTS.

Eighty-three percent of the samples had pigmented testa. Tannins are generally the most effective natural phenolic antioxidant *in vitro*, so a strong correlation between condensed tannins and antioxidant activity was expected. However, there was a low correlation between tannin and ORAC values. ORAC seems to be better at determining the antioxidant activities of non-tannin phenolic compounds such as 3-deoxyanthocyanins which were higher in the Type I sorghums with black pericarp.

ABTS and DPPH analysis are cheaper, easier and results are repeatable. They have consistently given repeatable results for sorghum antioxidant activity. ORAC is automated and more standardized across laboratories but it expensive and needs training with the technique. ORAC may however be a better method to assess antioxidant activity of non-tannin sorghums phenolics. There was a positive correlation (0.97) between total phenol and tannin ($p<0.01$).

Overall, the positive correlation between total phenol with both ABTS and DPPH suggest that total phenols can be used to predict antioxidant capacity in tannin sorghum but probably not in non-tannin sorghums. As noted by Awika et al (2003b), tannins are largely responsible for antioxidant activities in sorghum.

Evaluation of Sorghum 3-Deoxyanthocyanins

In all sorghum varieties, four main 3-deoxyathocyanidins were identified as luteolinidin (LUT), apigeninidin (AP), 5-methoxyluteolinidin (5-MeO-LUT) and 7-methoxyapigeninidin (7-MeO-AP) (Fig. 19). The other common anthocyanidins

such as cyanidin and delphinidin were not detected which agreed with the results of Awika (2003) and Dykes (2008). The presence of pigmented testa had no effect on 3-deoxyanthocyanins. 3-deoxyanthocyanin levels ranged from 15-234 ug/g (Fig. 20); SC121 had the lowest value. The presence of spots on the grains increased 3-deoxyanthocyanins levels among white pericarp sorghums. For example weathered grains of Dobbs, SC808 and Type II white sorghum had higher 3-deoxyanthocyanins than other Type III and Type II white sorghums (Figs. 9 & 11; p. 46 & p. 48). The spots on the grains were 3-deoxyanthocyanins produced in response to molds.

3-Deoxyanthocyanidins identified were of varying proportions among the sorghum varieties (Fig. 21). Shawaya black, for example had the highest levels of LUT (116 ug/g) accounting for 53% of the total 3-deoxyanthocyanin followed by Shawaya mostly black (95 ug/g) where LUT accounted for 41% of the total 3-deoxyanthocyanins. LUT and 5-MeO-LUT together contributed more than 50% of total 3-deoxyanthocyanin in 67% of the sorghum varieties and was more influenced by pericarp color than presence of a pigmented testa.

In general, the Shawaya sorghums had higher LUT and 5-MeO-LUT accounting for 55-78% of the total 3-deoxyanthocyanin with the exception of Shawaya Brownish Tan. In some sorghum varieties for example SC124, SC1321, SC22, Hegari, SC575, Hi Tannin and SC808, the methoxylated derivatives contributed higher levels to the total 3-deoxyanthocyanins than the

non- methoxylated components. In SC575 and SC808, 5-MeO-LUT accounted for 41 and 38 % respectively of the total 3-deoxyanthocyanins.

Pericarp color affected the levels of 3-deoxyanthocyanins, but not the profile of 3-deoxyanthocyanins. Shawaya sorghums with the red turning black pericarp had the highest levels of 3-deoxyanthocyanins ranging from 196-234 ug/g. These results provide vital information for the selection of sorghums for application in functional foods or dietary sources of colorants. The levels reported here were 20 % of those reported by Dykes (2008) due to weathering.

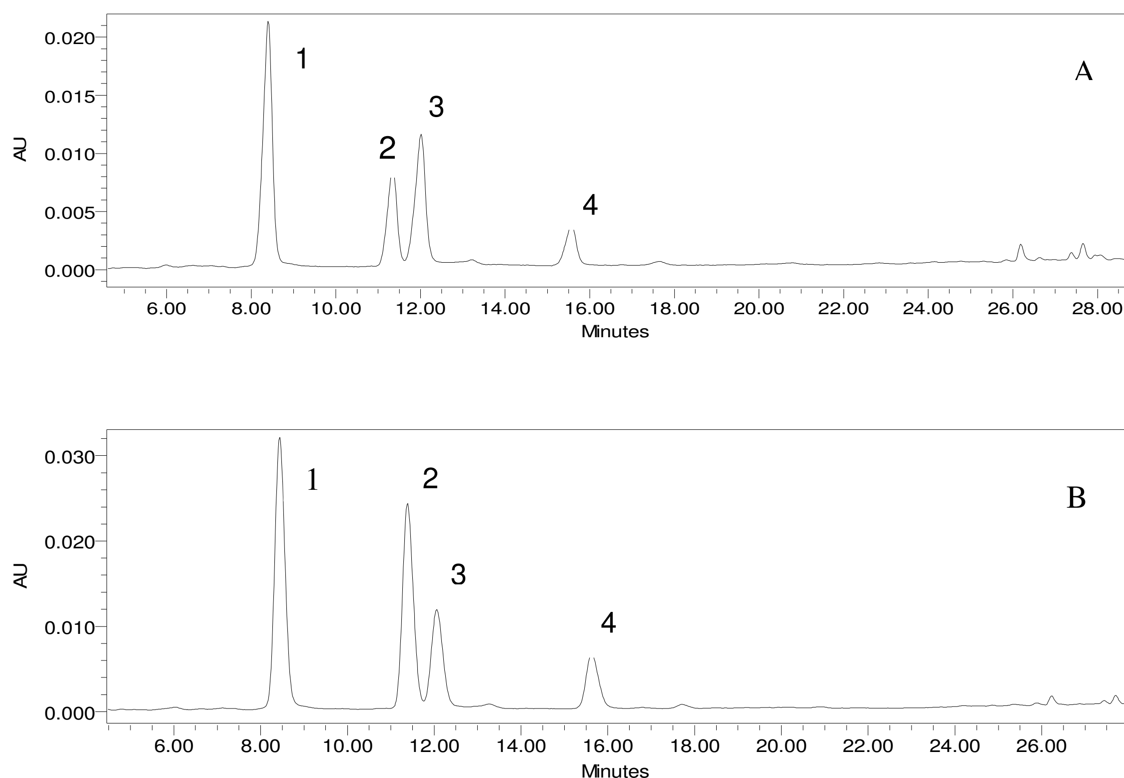


Fig 19. HPLC chromatograms for 3-deoxyanthocyanins of sorghum grown in College Station, TX 2005. A) Hageri; B) Dobbs. 1 = Luteolinidin; 2 = Apigeninidin; 3 = 5-Methoxyluteolinidin; 4 = 7-Methoxyapigeninidin.
PDA = 485 nm.

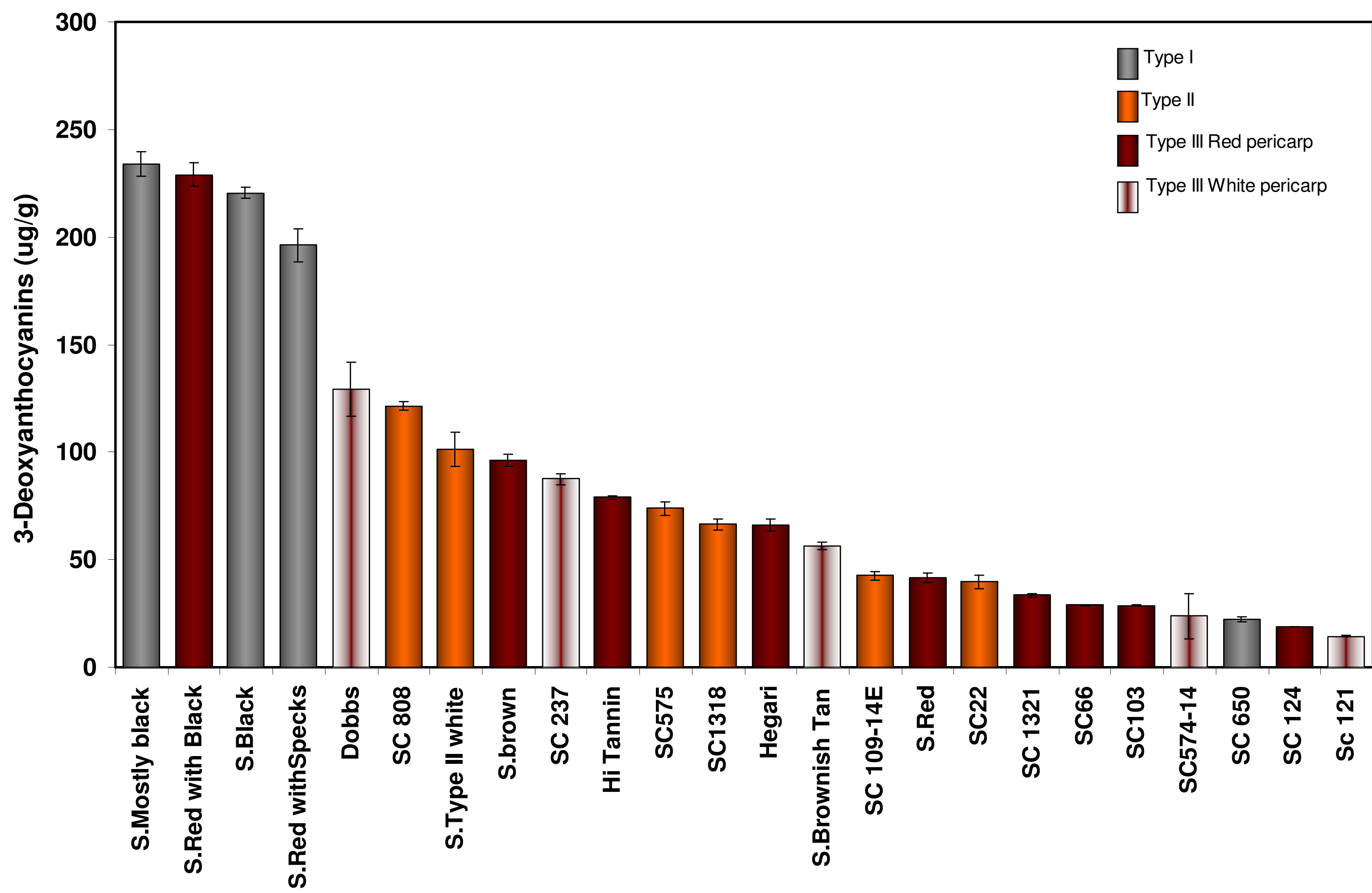


Fig. 20. 3-Deoxyanthocyanin levels in sorghums grown in College Station, TX 2005. S = Shawaya.

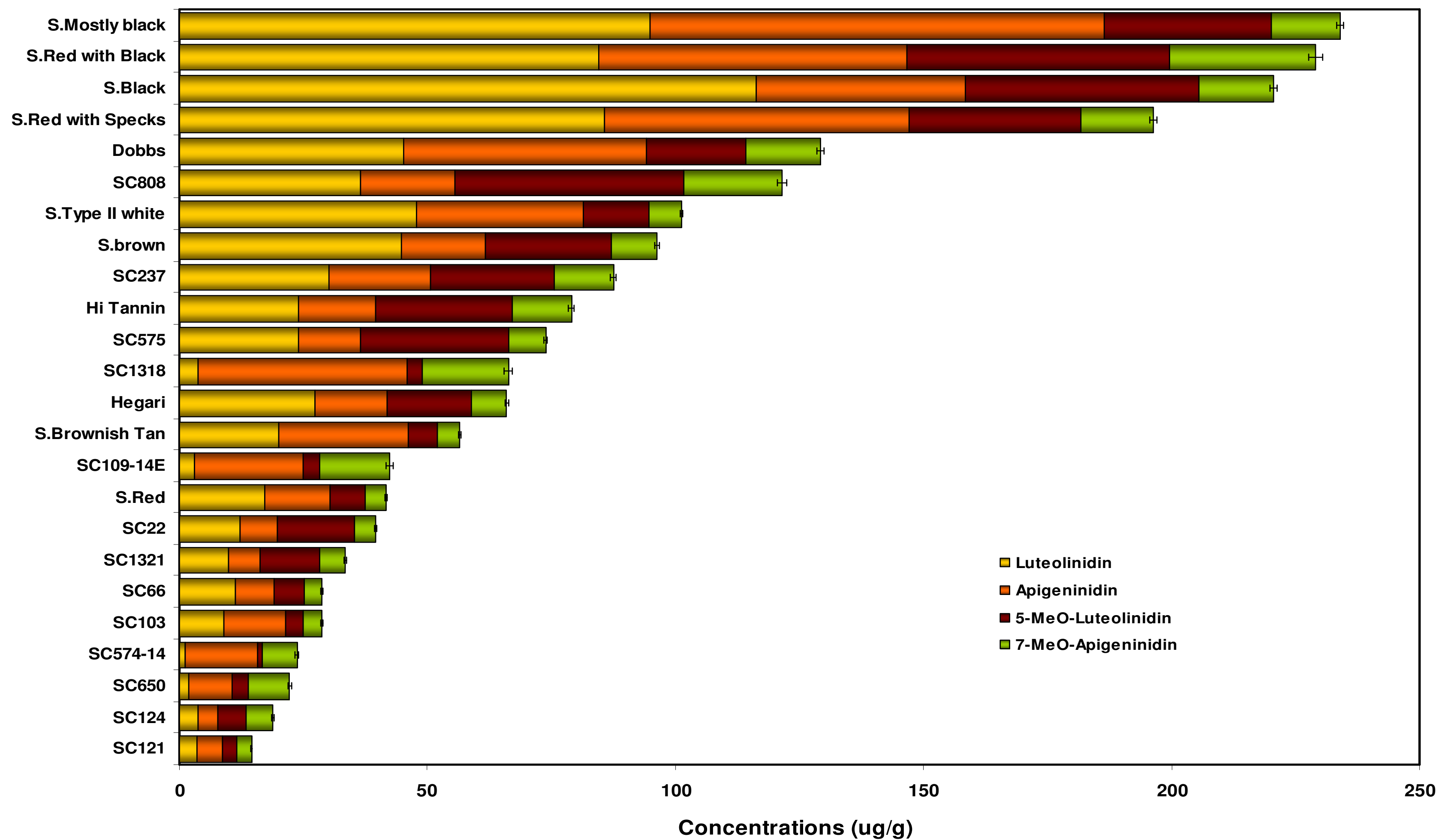


Fig. 21. 3-Deoxyanthocyanin profile of sorghums grown in College Station, TX 2005. S = Shawaya.

Visually, the 2005 Shawaya black sorghums had evidence of severe weather damage with grayish-brown kernels compared to the bright black appearance of the 2006 grains used by Dykes (2008). Awika (2003) using 2003 crops, identified 3-deoxyanthocyanins as the major flavonoids in black pericarp sorghums accounting for 50% of the total anthocyanins. Heavy rainfall reported in the summer of 2005, which was at the latter stage of sorghum development, was not observed for 2003 and 2004 (Dykes 2008). This variation both in weather and sorghum phenolic content over different crop years confirms that the environment under which a sorghum grain matures affects its phenolic profile.

Among the Type I sorghums, Shawaya mostly black had 11 times more 3-deoxyanthocyanins than SC650. This suggests that the genetics responsible for blackness are responsible for the synthesis of 3-deoxyanthocyanins in these varieties. SC650 (non-black) is a Type I red pericarp sorghum with low levels of 3-deoxyanthocyanins compared to the rest of the Type I sorghums with the red turning into black pericarp (Fig. 8; p. 45).

Among the Type III red pericarp sorghum, Shawaya Red with black sorghum had 10 times more 3-deoxyanthocyanins than SC574-14. Gous (1989) reported 8 times more 3-deoxyanthocyanins in Shawaya Black lines than in Type III control sorghum, ATx623xSC103. The presence of a pigmented testa did not affect the levels or the profiles of the 3-deoxyanthocyanins similar to studies by Dykes (2008). In addition, there was no significant difference between

the mean 3-deoxyanthocyanin levels of Type II sorghums (67 µg/g) and Type III white pericarp sorghum (62 µg/g).

Type II and Type III white pericarp sorghums with specks or dark spots had high levels of 3-deoxyanthocyanins (Shawaya Type II white, SC808, Dobbs) which were probably produced as phytoalexins. Dykes (2008) demonstrated that 3-deoxyanthocyanins may be produced in response to sunlight. However, weathering and other stress factors can also cause its synthesis (Lo et al 1999; Seitz 2004; Waniska and Rooney 2000). The bright black sorghum gave higher concentrations of 3-deoxyanthocyanins and other flavonoids. Genetic and environment play major roles in determining the 3-deoxyanthocyanin content of sorghum.

Evaluation of Flavones in Sorghum

The flavones detected were the yellow luteolin and the pale yellow apigenin with retention times of 7.3 and 9.4 minutes respectively (Fig. 22). Flavones were detected in all samples; levels ranged from 2-101 µg/g with SC650 and Shawaya Red the highest and lowest respectively (Fig. 23).

SC650 might be a viable source of flavones. Flavones in SC650 were distinctly higher (101 µg/g) than the other sorghum varieties which ranged from 2-29 µg/g.

This elevated flavone content might account for the high antioxidant activity obtained for SC650. Flavones are concentrated in the pericarp and reported as phytoalexins (Seitz 2004). The presence of a pigmented testa and

pericarp color did not increase flavone levels. Dykes (2008) also found pericarp color did not affect flavone levels.

Flavones were detected in varying proportions among the sorghum varieties (Fig. 24). Apigenin and luteolin were detected in all samples except SC574-14, SC66 and SC1321. SC650 had high levels of luteolin and apigenin accounting for 55 and 45% respectively of total flavones. In SC109-14E and SC1318, luteolin and apigenin were almost equally distributed. The Shawaya sorghums had lower levels of apigenin with luteolin accounting for most of the flavones detected. Shawaya black, Shawaya red with specks, Shawaya Brown and Shawaya Type II white, had very low levels of apigenin constituting only 4-6 % of the total flavone content.

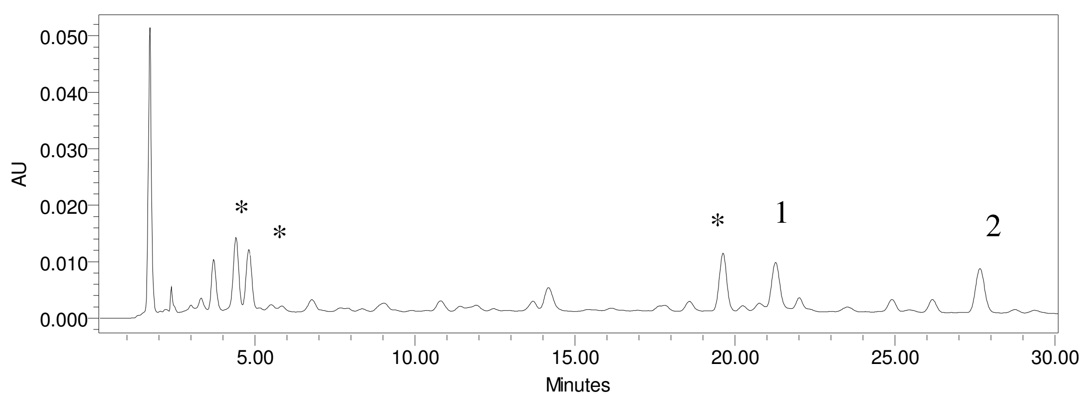


Fig 22. HPLC chromatograms for flavones of SC650 grown in College Station, TX 2005. 1 = Luteolin; 2 = Apigenin; * Identified as hydroxycinnamic acids (i.e. Caffeic and ferulic acids) on the basis of their UV spectra. PDA = 340 nm.

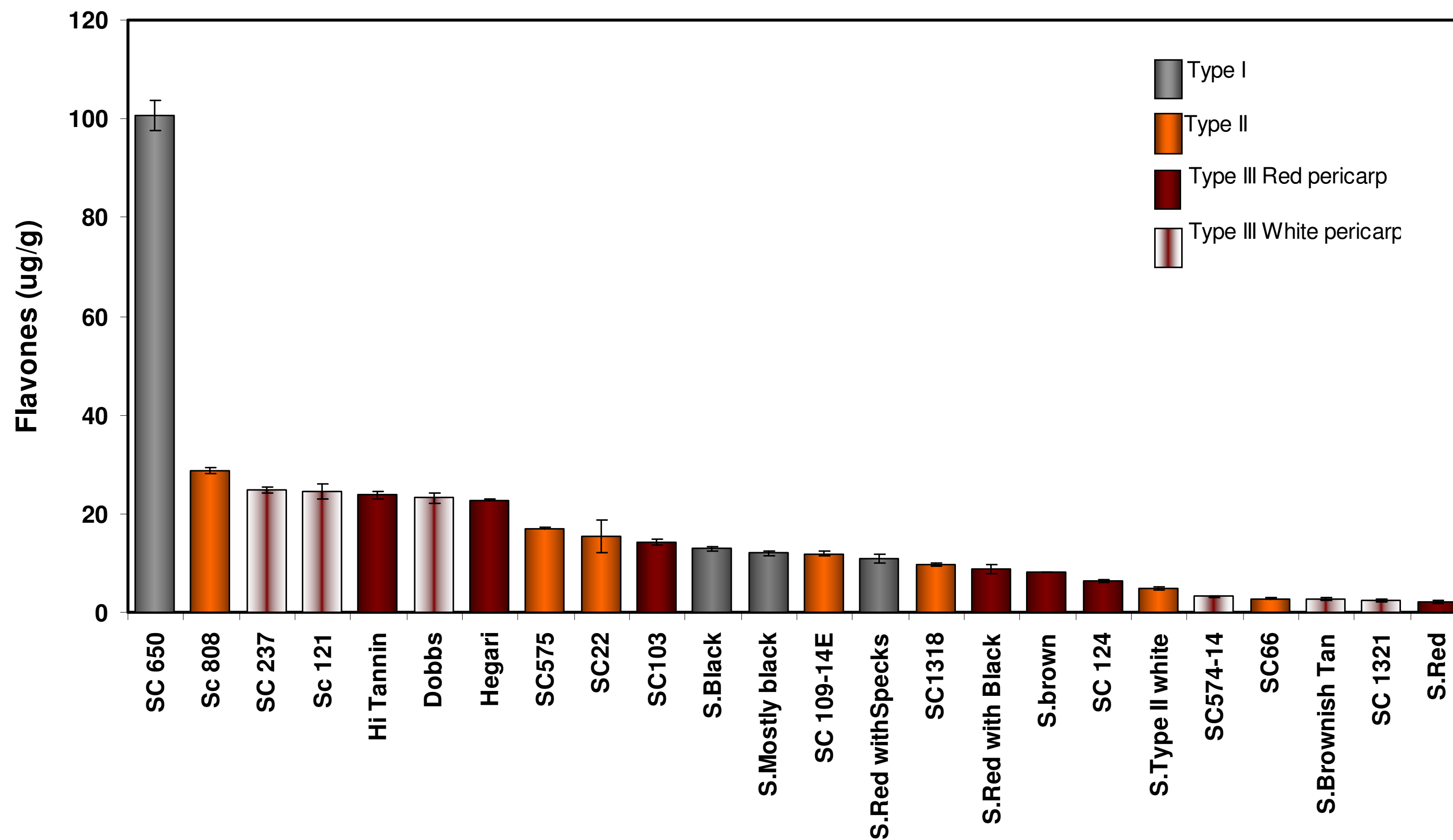


Fig. 23. Flavone levels in sorghums grown in College Station, TX 2005. S = Shawaya.

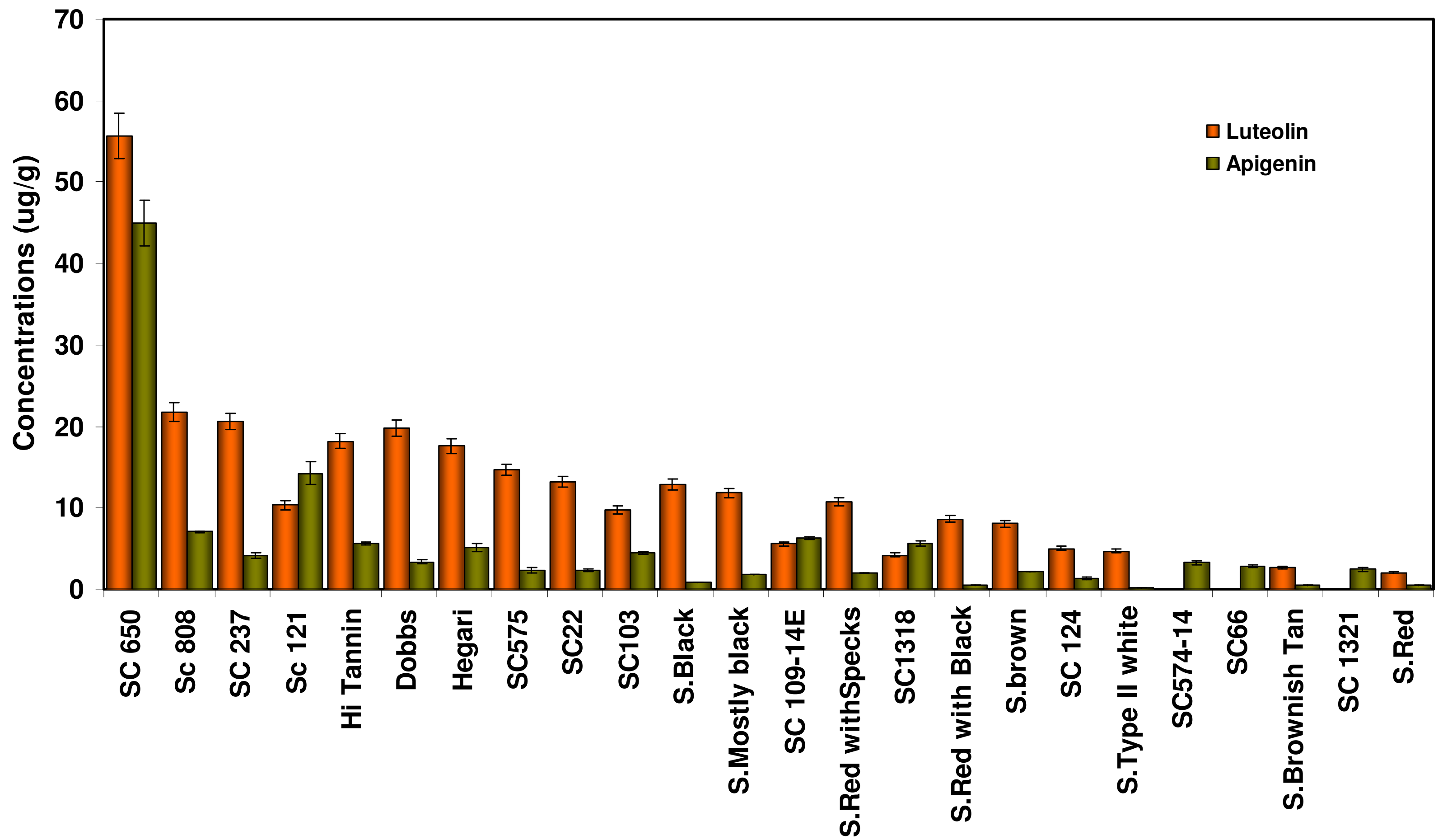


Fig. 24. Flavone profiles of sorghums grown in College Station, TX 2005. S = Shawaya.

Overall, luteolin accounted for between 79-95 % of total flavones in some varieties. Information on the source of flavones in sorghum is scarce but in this study, the presence of flavones in SC650 in such high levels suggests that plant breeders can develop sorghum varieties that are good sources of flavones. Flavones are synthesized to protect plants from UV-light and as phytoalexins with antimicrobial properties (Dixon 1986; Schmelzer et al 1988; Yu et al 2006). Flavones have anticarcinogenic, anti-inflammatory, antiallergenic, and analgesic properties (Hirano et al 2004; Matsui et al 2005; Cherng et al 2007; Ziyan et al 2007). They are used as vascular relaxation agents and for treating corneal neovascularization (Block et al 1998; Xu et al 2007).

Evaluation of Flavanones in Sorghum

Flavanones ranged from 28.1-229.1 ug/g (Fig. 25). The presence of pigmented testa did not affect flavanone content. However, pericarp color was associated with flavanones; the red pericarp sorghum had flavanones ranging from 74-229 ug/g while the white pericarp sorghum did not have flavanones.

The flavanone levels observed were in agreement with those obtained by Dykes (2008) who did not detect flavanones in white pericarp sorghum, but reported very high levels in lemon-yellow sorghums. The flavanone profiles varied among sorghum varieties (Fig. 26). Eriodictyol and naringenin were the two flavanones detected.

Eriodictyol predominated in all varieties with the exception of SC103 and Shawaya Brown, accounting for 53-86 % of the total flavanones. SC650 had almost equal distribution of eriodictyol and naringenin. In general, the red turning into black pericarp Shawaya had higher levels of eriodictyol than naringenin accounting for more than half the amounts of flavanones detected.

Total phenol in Type I sorghums were contributed mainly by flavan-4-ol and the flavones. Type I Black sorghum had elevated levels of 3-deoxyanthocyanins, while the red sorghum SC650 had very high levels of flavones. The presence of a pigmented testa did not influence levels of 3-deoxyanthocyanin; however, sorghums with a black pericarp had elevated levels of 3-deoxyanthocyanins.

In all samples, the four main sorghum 3-deoxyanthocyanins, the orange luteolinidin, the yellow apigeninidin, and their methoxylated derivatives, 5-methoxyluteolinidin and 7-methoxyapigeninidin were identified. 3-Deoxyanthocyanin levels ranged from 14-234 µg/g. Two flavones, apigenin and luteolin were detected in the range of 2-101 µg/g. Flavanones such as eriodictyol and naringenin, ranged from 28.1-229.1 µg/g. However, in white pericarp sorghums, flavanones were undetectable.

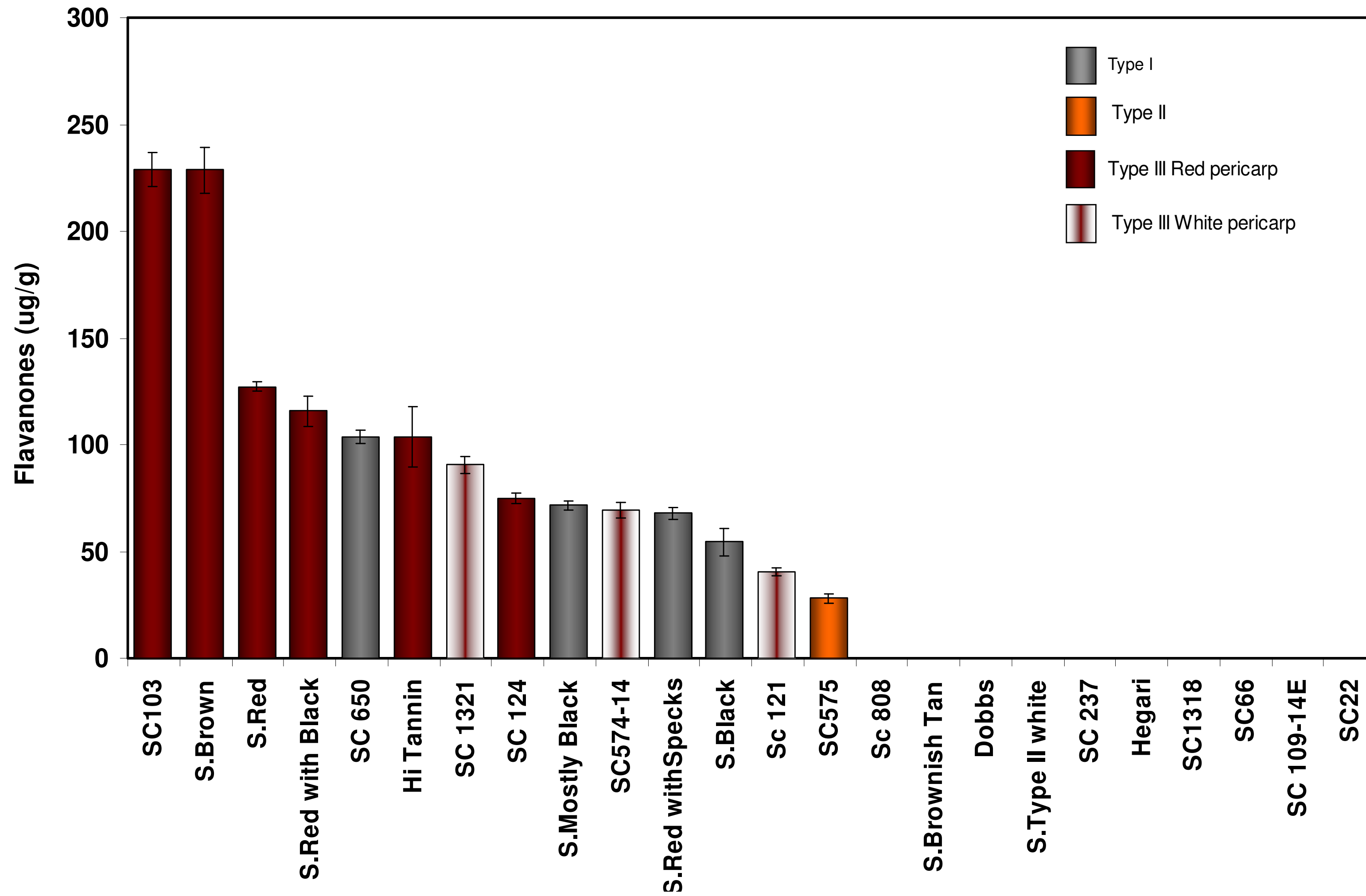


Fig. 25. Flavanone levels in sorghums grown in College Station, TX 2005. S = Shawaya.

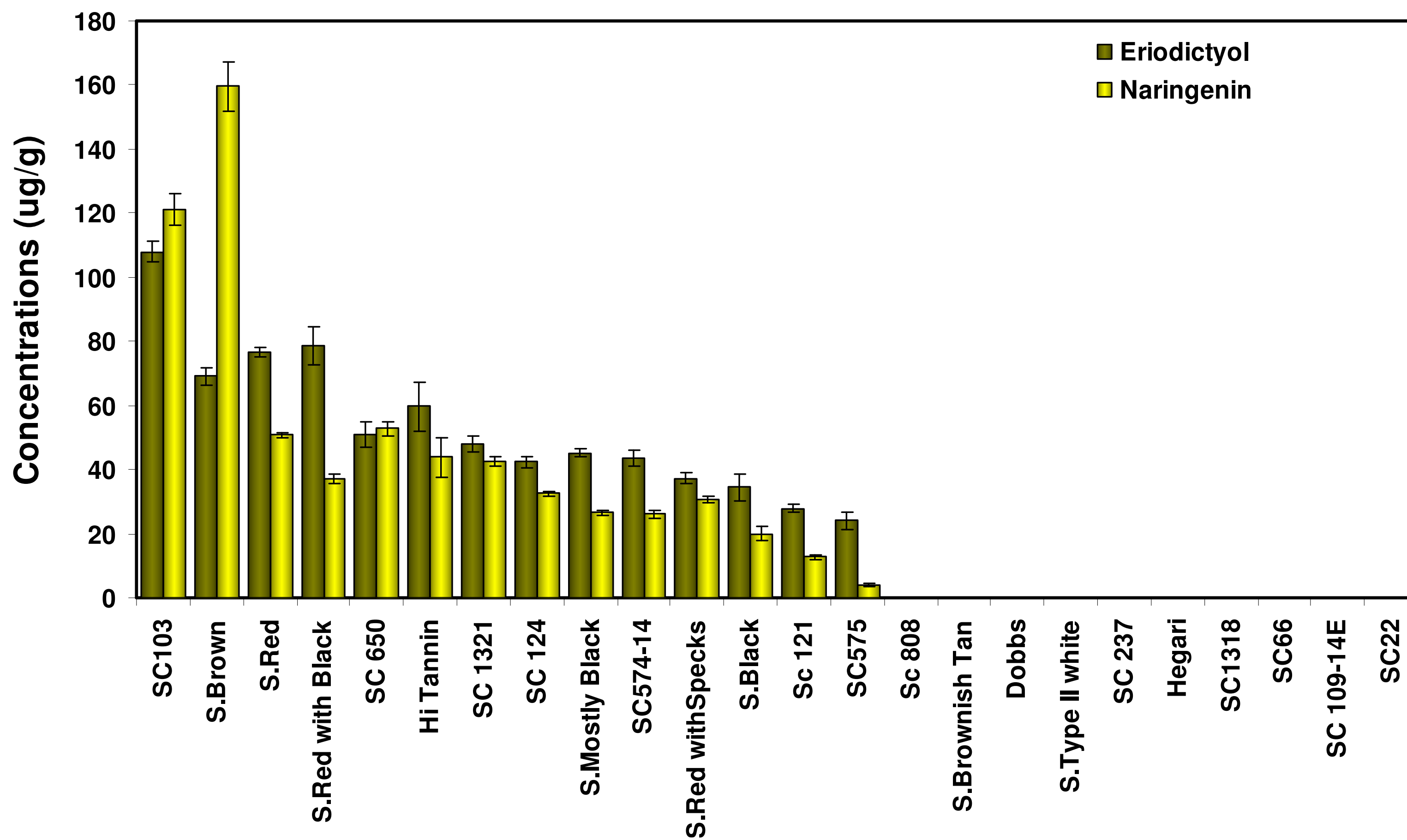


Fig. 26. Flavanone profile of sorghums grown in College Station, TX 2005. S = Shawaya.

Special black sorghums which are actually red pericarp sorghums which turn black upon exposure to UV light are very high in 3-deoxyanthocyanins and flavan-4-ols resulting in high antioxidant capacity. It is possible therefore to develop non-tannin sorghums with very high phenols and antioxidant potential. Sorghum breeders could also develop tannin sorghums containing the red turning to black pericarp to further enhance the antioxidant potential of sorghum.

Proper selection of sorghum and the right environmental conditions can enhance the availability of bioactive compounds from sorghum germplasm for variable beneficial applications. Developing special sorghums with antioxidant activity and other potential healthy attributes will increase the market potential of sorghum.

CHAPTER IV

FLAVONOID PROFILES AND ANTIOXIDANT ACTIVITY OF GRAINS, LEAVES, SHEATHS, GLUMES AND STALKS OF SOME SORGHUM

Introduction

The stalks, sheaths, glumes and leaves, constitute a greater portion of the total biomass of a sorghum plant than the grain. Typically for a US hybrid sorghum, the grain is 40-50% of dry weight, with 5-10% leaves and the rest are stalk and debris (Bill Rooney; personal communication) while biomass sorghum yields about 2.8-4.5% panicle and the rest are leaves, stalks and debris (Unpublised data). As the use of sorghum biomass for energy is developed, there is a possibility of tons of bioactive compounds synthesized as genetic tradeoffs and eventually left in by-products of ethanol production. The waste product might be a potential viable source of sorghum bioactive compounds.

Over the years, the grains of sorghum have been studied as a source of phenolic compounds. Other parts of the sorghum plant such as the leaves, glumes, sheaths and stalks probably contain 3-deoxyanthocyanins, flavones and flavanones that have not been characterized.

The phenolic profiles of leaves, glumes, sheaths and stalks of sorghums grown in College Station, TX in 2008 were identified and quantified. This will provide information on potential use of by-products from ethanol production as a viable source of phytochemicals.

Materials and Methods

Sorghum leaves, glumes, sheaths and grains of Tx2911, SC748-5, R-07007, Tx430 Black, Tx430 Black x Sumac, ATx631 x RTx436 (Figs. 27 & 28) and stalks from a purple plant (Tx430 black), a red plant (Tx2911) and a tan plant (ATx631 x RTx436) grown in College Station, TX, in 2008 were obtained from Texas AgriLife Research in College Station, TX.

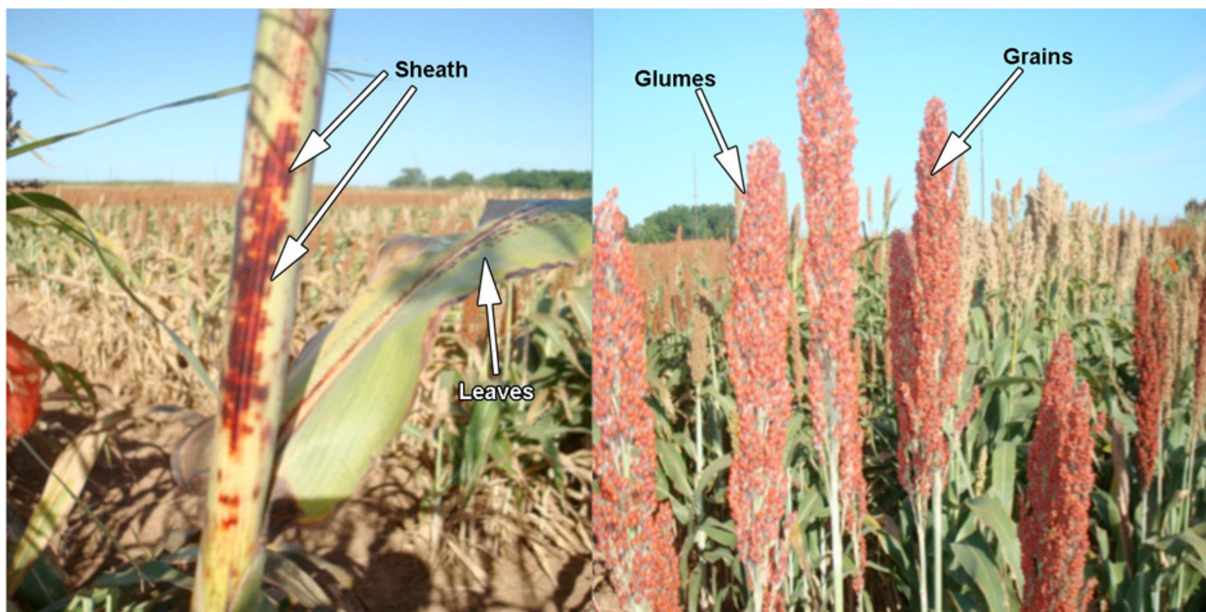


Fig. 27. Sorghum components. Sheaths are the thin covering surrounding the stalks. Glumes are the covering on the grains.

Tx2911 **SC748-5** **R-07007**

Tx430 **T x430 Black x Sumac** **ATX631 x RTX436**

Fig. 28. Sorghum varieties grown in College Station, TX 2008.

Reagents

The same reagents as described in Chapter III were used.

Sample Preparation

After harvesting, leaves, sheaths and stalks were placed in liquid nitrogen, and freeze-dried. The dried glumes and grains were stored in the refrigerator at 8°C. All samples were ground through a cyclotec mill (UDY Corp., Fort Collins, CO (0.5 mm mesh) prior to extraction.

Extraction for Colorimetric Assays

All extractions were carried out as previously described in Chapter III.

Extraction for HPLC and LC-MS Analyses

Ground samples (1 g) were extracted in 10 mL of 1% HCl/methanol (v/v) for two hours in a shaker, followed by centrifuging at 2790 x *g* for 10 min and then decanted. To remove the chlorophyll from the leaves and sheath extracts, 10 mL of petroleum ether was added to the supernatant and mixed by shaking for 1 min. To recover the petroleum ether, 10% water was added. The mixture was allowed to separate for 5 min. The 1%HCl/methanol fraction which settled at the bottom was carefully collected for HPLC and LC-MS analyses as previously described (p. 49). Grains and glumes were extracted in the same manner as previously described in Chapter III (p. 49).

Colorimetric Assays

All colorimetric assays were carried out as previously described in Chapter III (p. 49).

HPLC-PDA and LC-MS-ESI Analyses

Extracts were analyzed on an Alliance 2695 system (Waters Corp., Milford, MA) connected to a Waters 996 photodiode array detector (PDA). Sorghum phenolics were separated using a Luna C18 column (150 mm x 4.6 mm i.d., 5 µm) from Phenomenex (Torrance, CA) with a C18 guard column. Column temperature was maintained at 35°C with an injection volume of 20 µL. The mobile phase consisted of 4% formic acid in water (v/v) (Solvent A) and acetonitrile (Solvent B). The solvent flow rate was 1.0 mL/min. The 3-deoxyanthocyanins were separated using the gradient for sorghum grains as previously described in Chapter III (p. 52). Flavones and flavanones of glumes and grains were separated using the same gradient as described in Chapter III (p. 54). Flavones and flavanones in leaves and sheaths were separated using the following gradient: 0-5 min., 5-10% B; 5-41min., 10-40% B; 41-45 min., 40-65% B; 45-50 min., 65% B. The 3-deoxyanthocyanins, flavones, and flavanones were detected and identified as previously described in Chapter III (p. 54).

Structural information on flavonoids that could not be identified by HPLC-PDA was obtained by LC-MS analysis, performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source

(Thermo Fisher, San Jose, CA) adopted from the method of Pacheco et al 2008. Separations were conducted using the same Luna C18 column with a C18 guard column. Column temperature was maintained at 20ºC. The mobile phase consisted of 4% formic acid in water (v/v) (phase A) and acetonitrile (v/v) (phase B). The solvent flow rate was 0.4 mL/min and the sample injection volume as 50µL. The flavones were separated in the gradient elution program were phase B changed from 5 to 10% in 5 mins, continued at 10-40% B; 41-45 min., 40-65% B; 45-50 min., 65% B. Electrospray ionization was conducted in the negative mode for flavone glycosides under the following conditions: sheath gas (N2), 60 units/min; auxiliary gas (N2), 5 units/ min; spray voltage 3.3 kV; capillary temperature, 250ºC; capillary voltage, 1.5 V; tube lens offset, 0 V. The chromatograms were recorded at 340 nm for flavones.

Absorption Spectra of Chlorophyll

Chlorophyll constitutes a major component of sorghum leaves and is also present in the sheath. Based on the wavelength in which the colorimetric readings were measured, there were minimal concerns about interferences from chlorophyll. This was because the absorbance maxima of chlorophyll a and b (Fig. 29) were not within the maxima at which the phenolic compounds and antioxidant activities were measured. However, chlorophyll was removed before HPLC and MS analysis.

Results and Discussion

Total Phenols of Sorghum Plant Components

Total phenols in the different sorghum components ranged from 1.3-28.2 mg GAE/g (Fig. 30 & Table 5). Secondary plant color affected total phenols in sorghum leaves, sheaths, glumes and grains but did not for stalks. The red and purple sorghum plants (Tx2911, R-07007, Tx430 Black and Tx430 Black x Sumac) had higher total phenols than the tan plant (ATx631xRTx436) (Table 5), with the exception of the leaves of ATx631 x RTx436 (13.9 mg GAE/g) which had higher total phenol content than SC748-5 (10.5 mg GAE/g).

The sheaths, glumes and leaves of sorghum had higher phenol contents than their respective grains. These large differences in phenol levels between grains and other sorghum components could be because the phenolic compounds in the grains are not as easily extracted as those in the sheaths, leaves and glumes or simply that those components truly have higher levels than the grains. Other authors have reported similar patterns using other plant sources. For example, blueberry fruit was reported to have total phenol levels of 1.8 mg GAE/g fresh weight, while the leaves had 34% more (Ehlenfeldt and Prior 2001).

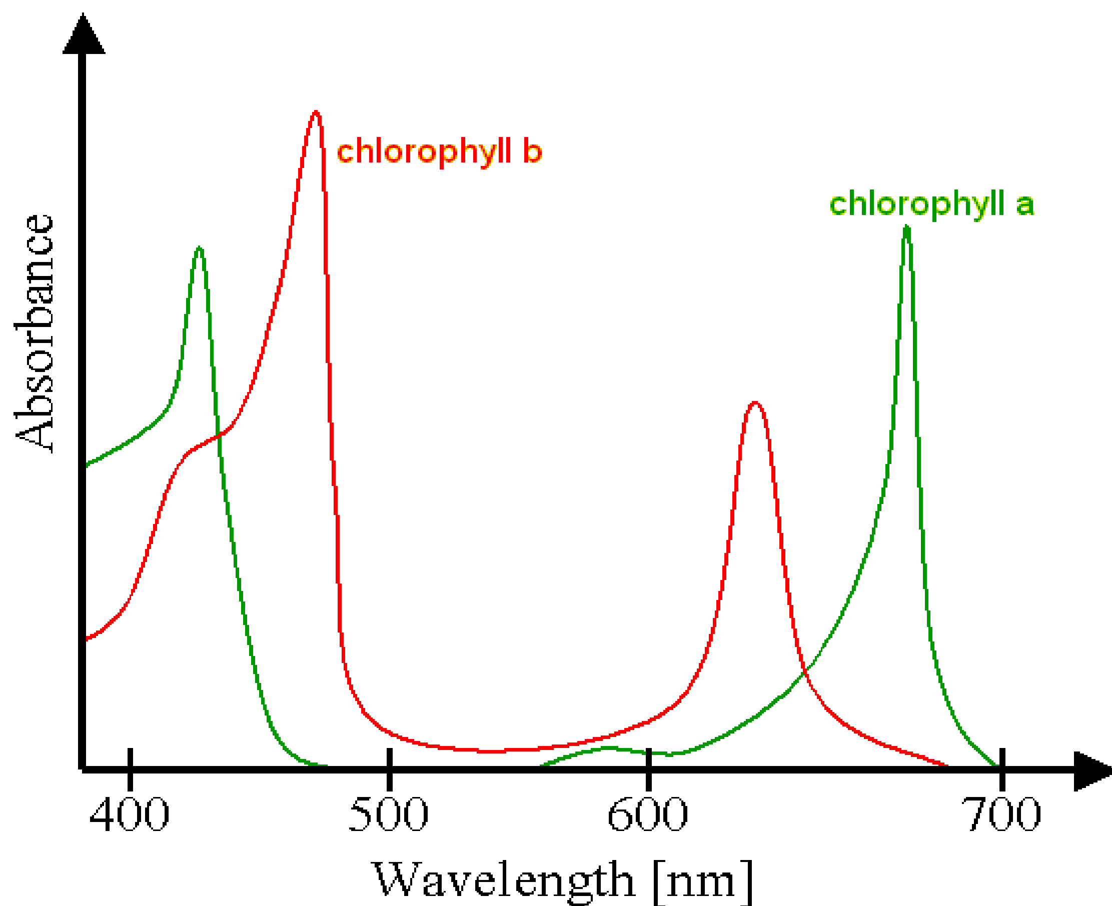


Fig. 29. Chlorophyll spectra. Chlorophyll a has approximate absorbance maxima of 430 nm and 662 nm, while chlorophyll b has approximate maxima of 453 nm and 642 nm. From Kurzon http://en.wikipedia.org/wiki/File:Chlorophyll_ab_spectra.png).

Simpson (2006) showed the leaves and roots of peanuts to have higher total phenols than the nuts on dry weight basis. Berries for example, are considered a high source of phenolic compounds. Liu et al (2003) reported total phenols of raspberries ranging from 3.59 to 5.13 mg GAE/g fresh weight.

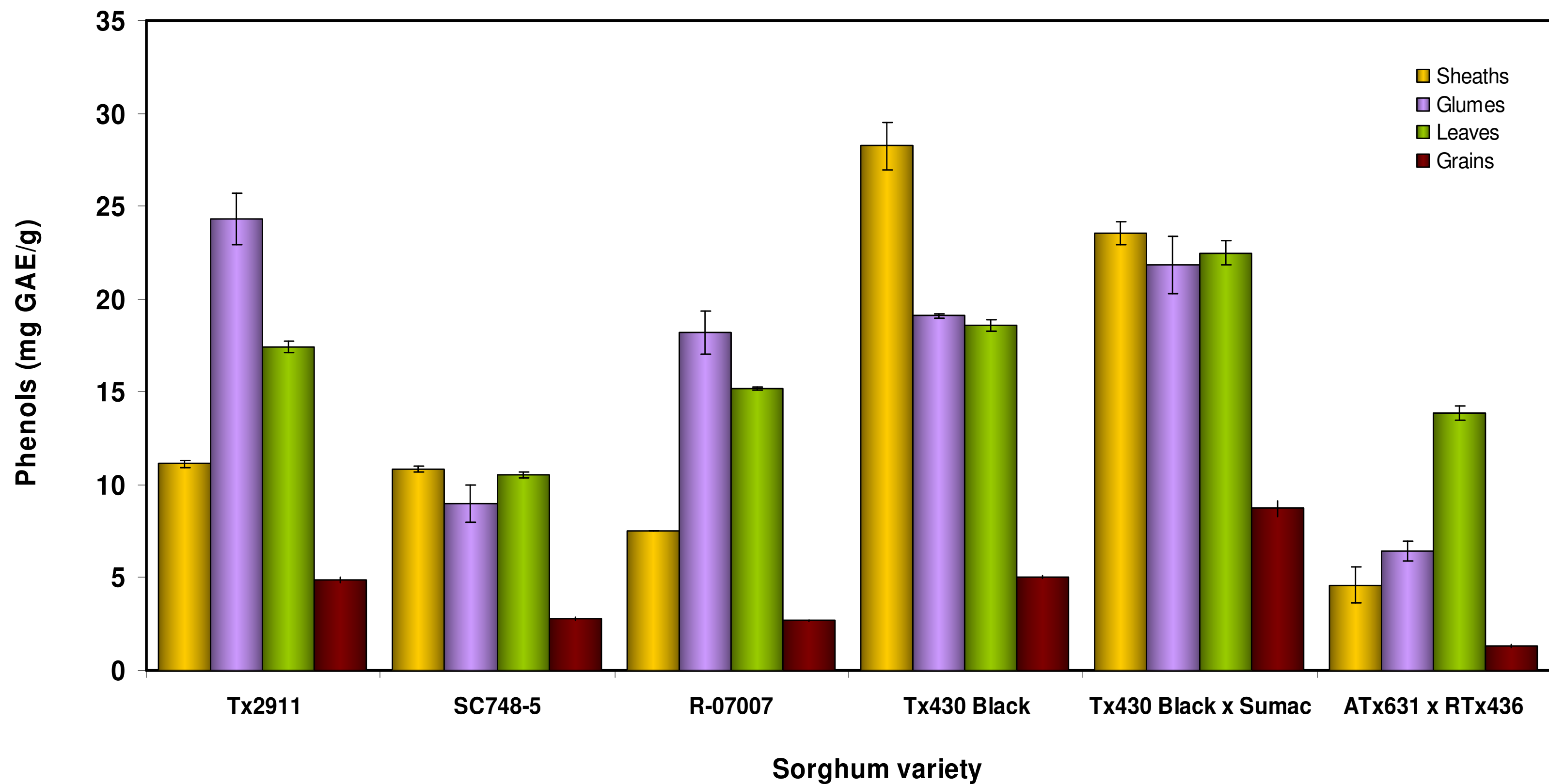


Fig. 30. Total phenols in sorghum components grown in College Station, TX 2008. Dry weight basis.

Table 5
Summary of Phenol and Antioxidant Levels in Sorghum Plant Components grown in College Station, TX 2008

Variety	Component	Total Phenols (mg GAE/g)[ab]	Tannins (mg CE/g)[cd]	Flavan-4-ols (Abs/mL/g)	Anthocyanins (mg LE/g)	ABTS (µmol TE/g)[e]	DPPH (µmol TE/g)
Tx2911	**Leaves**	17.43±0.34	0.46±0.01	16.8±0.51	13.50± 0.93	244.75±0.59	85.89±2.61
***Red**	**Glumes**	24.33±0.47	1.09±0.03	25.4±0.61	86.28±1.01	412.21±7.23	69.01±1.01
	Sheaths	11.13±0.17	1.02±0.01	19.8±0.61	20.35±1.19	207.85±2.61	48.49±1.43
	Grains	4.87±0.14	0.22±0.01	5.9±0.17	5.22±0.04	74.31±2.05	24.32±0.13
	Stalk	3.42±0.30	ND[f]	1.20±0.91	0.85±0.27	55.30±0.34	13.64±1.53
SC748-5	**Leaves**	10.52±0.16	0.56±0.01	8.2±0.11	4.17±0.20	164.77±0.97	41.21±1.81
Purple	**Glumes**	8.98±0.71	ND	13.6±0.39	24.56±1.05	150.04±3.46	56.32±3.23
	Sheaths	10.84±0.17	1.02±0.02	14.7±0.57	19.78±0.69	170.31±2.06	55.16±1.43
	Grains	2.82±0.08	ND	0.4±0.07	0.67±0.10	35.56±1.90	12.46±0.71
R-07007	**Leaves**	15.17±0.07	1.22±0.01	9.8±0.13	4.49±0.09	235.08±4.17	71.66±2.21
Red	**Glumes**	18.21±0.62	ND	19.1±0.71	69.69±1.10	337.36±7.14	47.74±2.11
	Sheaths	7.51±0.02	ND	3.1±0.07	6.89±0.19	136.19±1.68	36.32±0.63
	Grains	2.69±0.03	0.40±0.03	0.3±0.08	0.56±0.06	38.11±1.01	14.51±1.12
Tx430 Black	**Leaves**	18.59±0.37	ND	24.2±0.33	16.73±0.34	253.94±2.72	100.76±3.12
Purple	**Glumes**	19.09±0.09	ND	34.3±0.18	72.06±1.11	297.53±4.25	82.76±4.31
	Sheaths	28.24±0.69	1.53±0.01	55.7±0.12	101.89±2.61	364.43±8.78	167.55±5.12
	Grains	5.02±0.06	0.50±0.01	10.5±0.21	23.4±0.49	73.38±1.11	28.23±1.12
	Stalk	3.57±0.12	ND	0.84±0.11	2.17±0.04	61.77±0.17	11.68±0.11
Tx430 Black x Sumac	**Leaves**	22.47±0.66	1.58±0.02	17.7±0.91	97.15±0.58	387.85±3.41	134.35±3.00
	Glumes	21.83±0.56	ND	28.4±0.22	57.56±0.85	355.76±6.24	133.05±4.92
Red	**Sheaths**	23.52±0.61	ND	29.4±0.71	84.03±1.21	377.15±9.81	100.58±4.31
	Grains	8.72±0.44	18.37+0.11	5.6±0.23	6.69±0.06	122.79±4.37	37.56±0.55
ATx631xR Tx436	**Leaves**	13.87±0.67	0.71±0.03	15.6±0.31	3.90±0.43	190.20±2.36	57.69±2.50
Tan	**Glumes**	6.42±0.03	1.82±0.03	2.8±0.23	1.85±0.03	121.33±3.01	45.89±0.56
	Sheaths	4.59±0.96	1.20±0.01	2.0±0.83	0.77±0.07	84.33±2.61	16.43±0.62
	Grains	1.29±0.09	0.30±0.01	0.1±0.02	0.19±0.11	11.90±0.22	4.08±0.32
	Stalk	3.93±0.10	ND	2.31±0.08	0.75±0.30	58.75±0.47	16.11±0.80

[a]GAE = Gallic acid equivalents. [b] Dry weight basis. [c]CE = Catechin equivalents. [d]Components with values lower than 2.0 are considered tannin-free. [e]TE = Trolox equivalents. [f]ND = Not detected.* Secondary plant color.

The values obtained for total phenols from sorghum plant components compare favorably to those from other common food products and plant sources. These studies suggest that materials such as grains, nuts, leaves and roots can vary considerably in total phenol content even within the same plant.

Condensed Tannins of Sorghum Plant Components

Condensed tannins in all sorghum non-grain components ranged from 0-1.8 mg CE/g (Table 5). Contrary to Sereme et al (1993) who reported more than 8 mg/g of tannins in the sheaths, leaves, roots and stalks of *Sorghum caudataum* variety Moneme kaya grown in Burkina Faso in 1989, the leaves, sheaths, glumes and stalks in this study did not have tannins (Table 5). The tannin values reported by Sereme et al (1993) could not be an indication of tannin content because the standard used was tannic acid and results were expressed as tannic acid equivalents. This is misleading because sorghum does not contain tannic acid.

The grains of Tx430 Black x Sumac with a pigmented testa had the highest level of condensed tannin (18.4 mg CE/g). For this reason we expected the glumes, sheaths and leaves to have tannins, but this was not the case (Table 5).

Flavan-4-ols in Sorghum Plant Components

Flavan-4-ol levels ranged from 0.1-55.6 abs/mL/g (Table 5). The leaves, sheaths and glumes had higher levels of flavan-4-ols such as luteoforol and apiforol compared to the grains. Secondary plant color affected flavan-4-ols in all sorghum plant components except the stalk which all had low flavan-4-ols. Sorghum leaves, sheaths and glumes from red and purple-plant sorghums had higher levels of flavan-4-ols than the tan-plant sorghum. Tx430 Black had the highest flavan-4-ols for the leaves (24.2 abs/mL/g), glumes (34.3 abs/mL/g), sheaths (55.7 abs/mL/g) and grains (10.5 abs/mL/g) (Fig. 31 & Table 5).

Watterson and Butler (1983) reported apiforol in the leaves of 12 lines of sorghum [*Sorghum bicolor* (L.) Moench]. Jambunathan et al (1986) reported that leaves of mold-resistant sorghums have a much higher concentration of flavan-4-ols than mold susceptible varieties and concluded that higher concentrations of flavan-4-ols in leaves may give an indication of grain mold resistance.

Sorghum plant components from purple and red-plant with lemon-yellow pericarp grains had lower levels of flavan-4-ols than the plant components from purple and red-plant sorghum with red pericarp. The genetics for lemon-yellow pericarp might be responsible for reduced flavan-4-ols in sorghum components from the red and purple-plant producing yellow-lemon pericarp sorghums. The pigmented glumes had higher levels of flavan-4-ols than the non-pigmented or tan glumes (ATx631 x RTx436).

Dicko et al (2005) in a study using sorghum grains, reported higher levels of flavan-4-ols (0.20-0.42%, w/w, cyanidin, dry wt.) in red-plant sorghums with a red pericarp and pigmented glumes than the other varieties studied; Dykes (2008) reported a similar pattern for grains. The plant component of Tx430 Black x Sumac cross had the same flavan-4-ol pattern like the Tx430 Black. This could be due to the fact that the genetics of Tx430 Black was dominant over that of Sumac.

A positive correlation between flavan-4-ol and total phenols for sheaths and glumes (r^2=0.90 and 0.72 respectively, $p<0.01$) suggest that flavan-4-ol may contribute to total phenols in these components. A positive correlation between flavan-4-ol levels in grains with leaves, glumes and sheaths (r^2= 0.79, 0.77 and 0.89 respectively, $p<0.01$) suggest that the non grain components of sorghum with high levels of flavan-4-ols may equally be resistance to mold.

Anthocyanins in Sorghum Plant Components

Anthocyanin levels ranged from 0.2-101.9 mg LE/g in various plant tissues (Table 5). Anthocyanins followed a pattern similar to flavan-4-ols. The leaves, sheaths and glumes of red and purple-plant sorghums had higher levels of anthocyanins than the tan-plant sorghums (Fig. 32 & Table 5). The leaves of Tx430 Black x Sumac and sheaths of Tx430 Black had intense purple color with very high levels of anthocyanins (97 and 102 mg LE/g respectively) compared to leaves and sheaths from the other varieties.

The results are in agreement with Sereme et al (1993) who reported 108 mg Apigeninidin equivalent/g in the sheaths of *Sorghum caudatum vartiety* Moneme kaya. The sheaths and leaves of *Sorghum caudatum variety* Moneme kaya is used in Benin as dye for art work and colorant in food, cosmetics and folklore medicine probably because its intense color is due to high levels of anthocyanins (Sereme et al 1993; National Research council 1996).

High levels of anthocyanins from sheaths, leaves and glumes relative to grain, suggest that these compounds can be obtained in high concentrations from non-grain components. Thus high levels of anthocyanins can be obtained from non-grain components of the sorghum plant. The sheaths and glumes had 4-13 times higher levels of anthocyanins than the grains in some varieties (Tx430 Black: 4 and Tx430 Black x Sumac: 13). Sereme et al (1993) reported concentrated levels of anthocyanins in the sheaths of *Sorghum caudatum variety* Moneme kaya.

ATx631 x RTx436 (tan plant sorghum) was used as a control since they are typically low in phenolic compounds. All components had low levels of anthocyanins measured at 485 nm, supporting the fact that there was no interference with the peak maxima for chlorophyll, which are 430 and 662 nm for *chlorophyll a* and 453 and 642 nm for *chlorophyll b* (Fig. 29; p. 97).

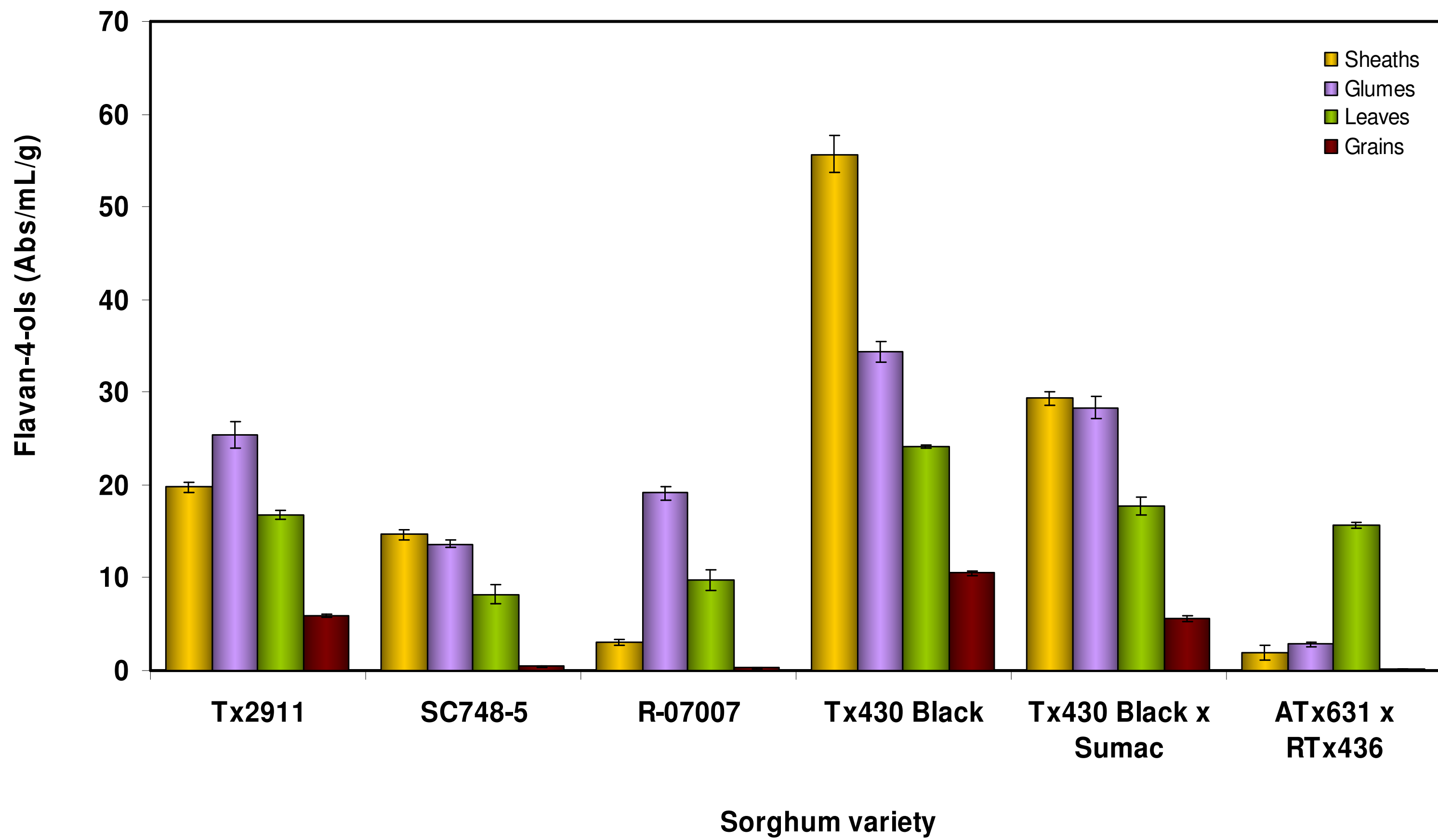


Fig. 31. Flavan-4-ol levels in sorghum components grown in College Station, TX 2008. Dry weight basis.

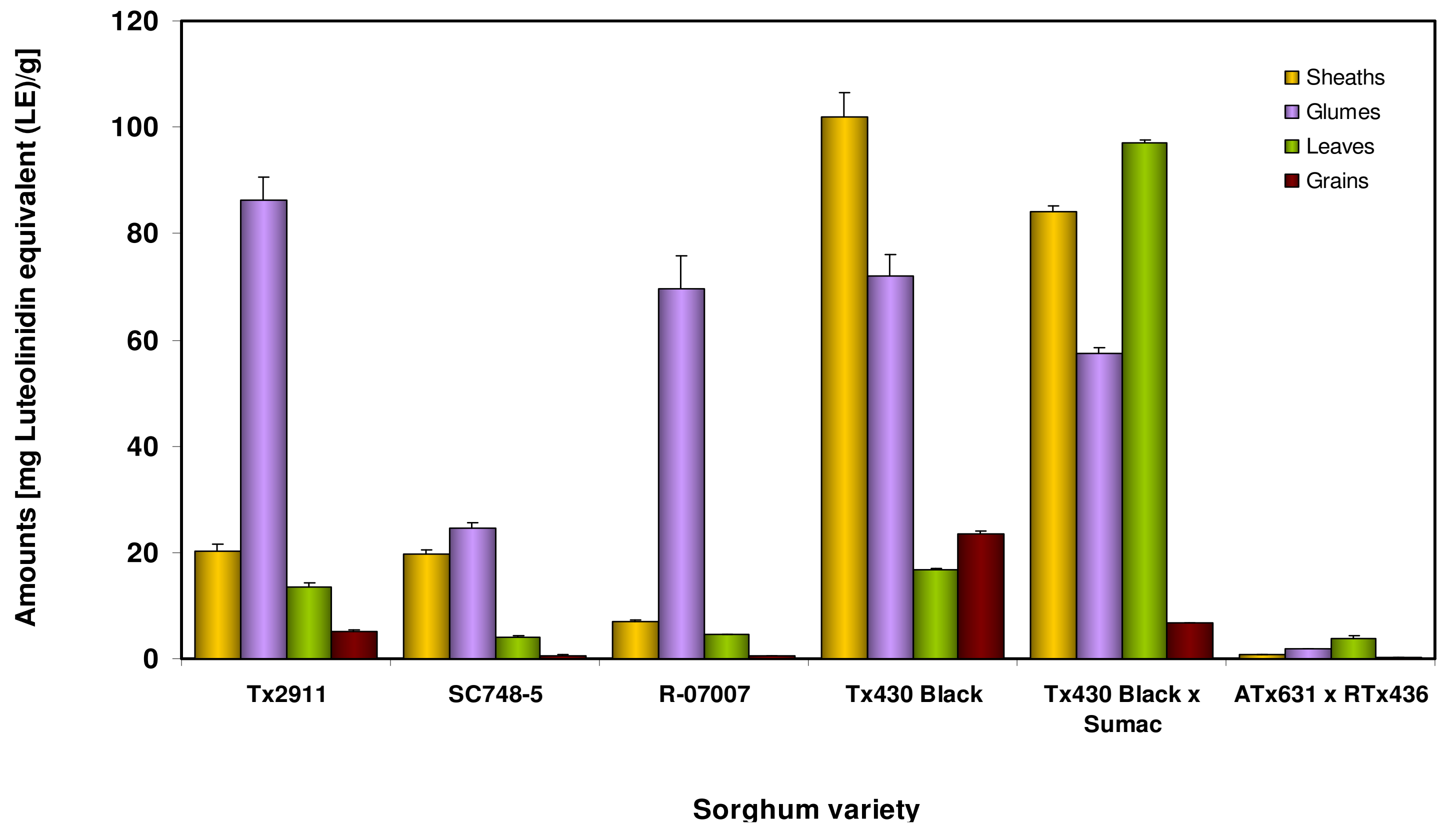


Fig. 32. Anthocyanin levels in sorghum components grown in College Station, TX 2008. Dry weight basis.

Anthocyanins and total phenols in sheaths, glumes and leaves were positively correlated (r^2=0.99, 0.89 and 0.65 respectively, $p<0.01$). This suggests that anthocyanins contribute greatly to the total phenol content of these plant components. Anthocyanins also positively correlated with flavan-4-ol in sheaths and glumes (r^2=0.87 and 0.72 respectively, $p<0.01$). Flavan-4-ols are precursors of 3-deoxyanthocyanins (Fig. 2; p. 12) (Wharton and Nicholson 2000).

Antioxidant Activities

Glumes, sheaths and leaves of all sorghum varieties had higher antioxidant activity than their respective grains (Table 5; p. 99). Secondary plant color affected antioxidant activity. The sheaths, glumes and leaves from red and purple-plant sorghums had higher antioxidant activity than those from tan plant sorghum with the exception of the leaves of SC748-5 (Fig. 33 & Table 5). Comparatively, the grains had relatively low antioxidant activity ranging from 12 to 123 µmol TE/g in Tx430 Black x Sumac. The leaves of ATx631 x RTx436 had the highest antioxidant activity relative to all other components of this variety. The antioxidant activity may be because of high levels of flavan-4-ols (Fig. 31; p. 104) and flavones will be discussed later.

A strong correlation was observed between DPPH and ABTS with total phenols for leaves (0.98 and 0.89 respectively), sheaths (0.94 and 0.95 respectively) and grains (0.93 and 0.99 respectively); $p<0.01$.

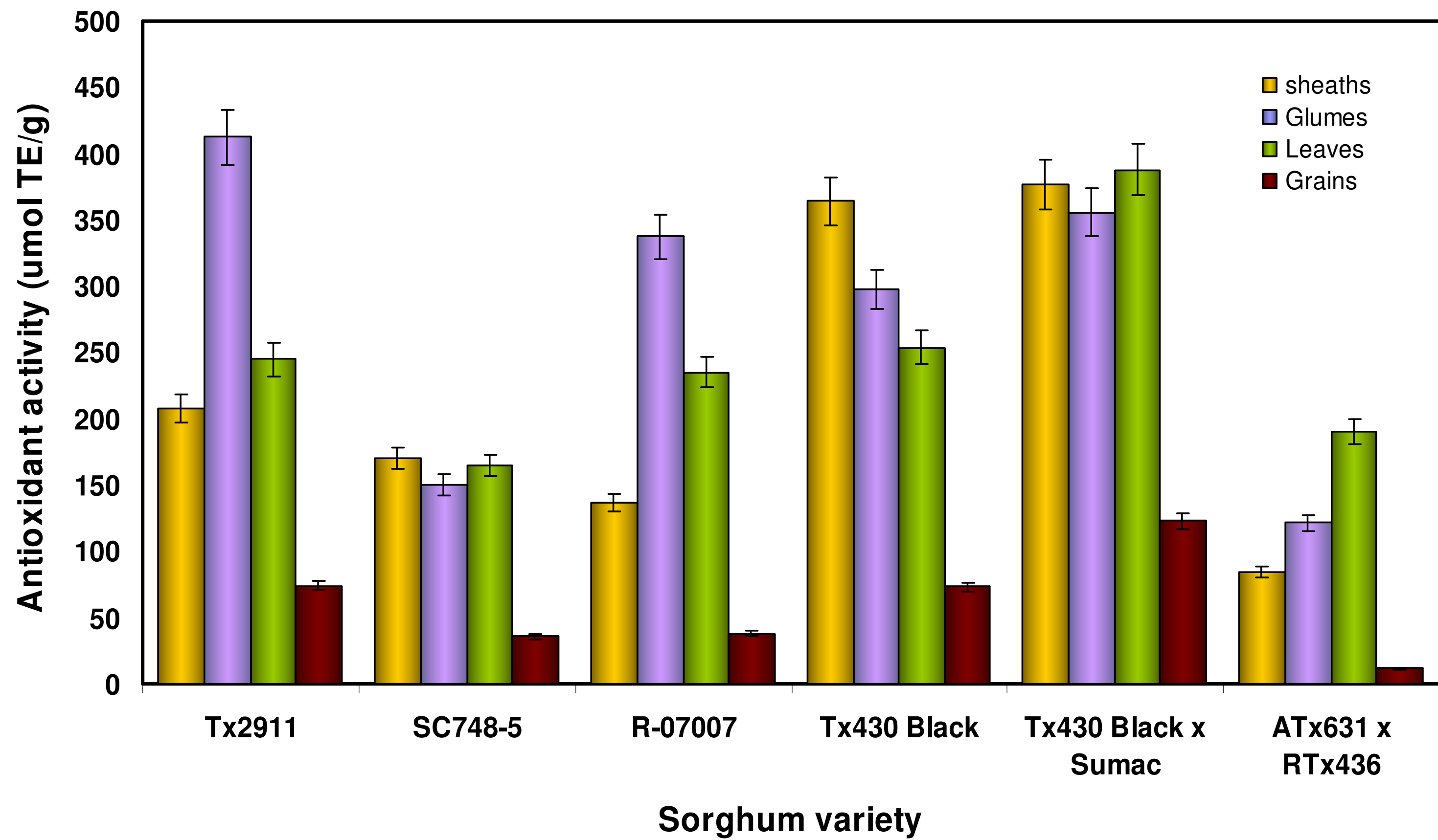


Fig. 33. ABTS antioxidant activity of sorghum components grown in College Station, TX 2008. Dry weight basis.

The low correlation between DPPH and total phenol observed for glumes may be due to the low DPPH values obtained for the glumes (Fig. 34) which is associated with interference from the anthocyanins leading to an underestimation of antioxidant activity. In systems with anthocyanins, color interference of the $DPPH^{\cdot}$ with anthocyanins has been reported to lead to an underestimation of antioxidant activity (Arnao 2000). The strong correlation observed between total phenol and antioxidant activity suggest total phenol content can be used to predict *in vitro* antioxidant potential in sorghum plant components. A similar correlation pattern was observed between ABTS and DPPH using sorghum grains (Awika et al 2003b; De-Beer et al 2003 and Dykes 2008).

Chlorophyll did not contribute to antioxidant activity. Simpson (2006) using thin layer chromatography showed that peanut leaves fractions with green pigments did not have significant antioxidant activity compared to other extracted components confirming chlorophyll is not a major antioxidant in leaves.

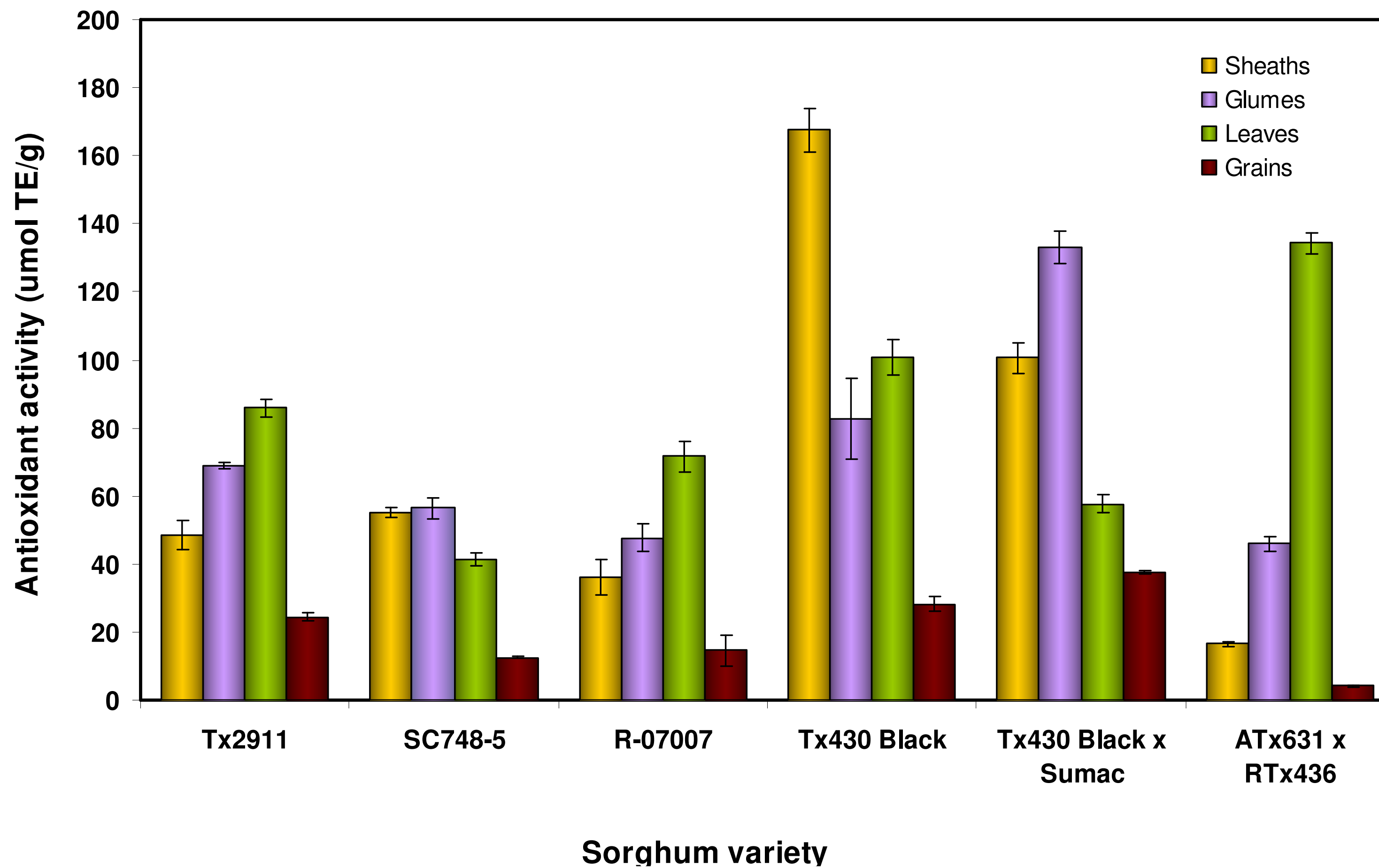


Fig. 34. DPPH Antioxidant activity of sorghum components grown in College Station, TX 2008. Dry weight basis.

3-Deoxyanthocyanins in Sorghum Components

Plant color had an effect on 3-deoxyanthocyanins. The red and purple-plant sorghum had higher 3-deoxyanthocyanins than the tan-plant sorghum (Table 6). Tan-plant sorghum did not have significant levels of 3-deoxyanthocyanins in the leaves, sheaths as well as the grains (Fig. 35). The sheaths of Tx430 Black with an intense color at harvest had high levels of 3-deoxyanthocyanins (9738 µg/g). The glumes of R-07007 (8767 µg/g) and Tx2911 (8738 ug/g) had intense red color at harvest and high levels of 3-deoxyanthocyanins (Fig. 35 & Table 6).

The amounts of 3-deoxyanthocyanins from leaves, sheaths and glumes of sorghum were very high compared to those in grains (Fig. 35 & Table 6). When sorghum plant components were intensely colored, high levels of 3-deoxyanthocyanins were observed, suggesting the colors in these components are mainly due to the presence of 3-deoxyanthocyanins. The results were in agreement with Sereme et al (1993), who reported that the sheaths and leaves of *Sorghum caudatum variety* Moneme kaya had 10. 8 % dry weight anthocyanins compared to the roots and grains in which anthocyanins levels did not exceed 1.6 %. Four 3-deoxyanthocyanins peaks (Luteolinidin, Apigeninidin, 5-Methoxyluteolinidin and 7-methoxyapigeninidin) were identified from all sorghum leaves with the exception of ATx631 x RTx436 which did not have luteolinidin and 5-methoxyluteolinidin.

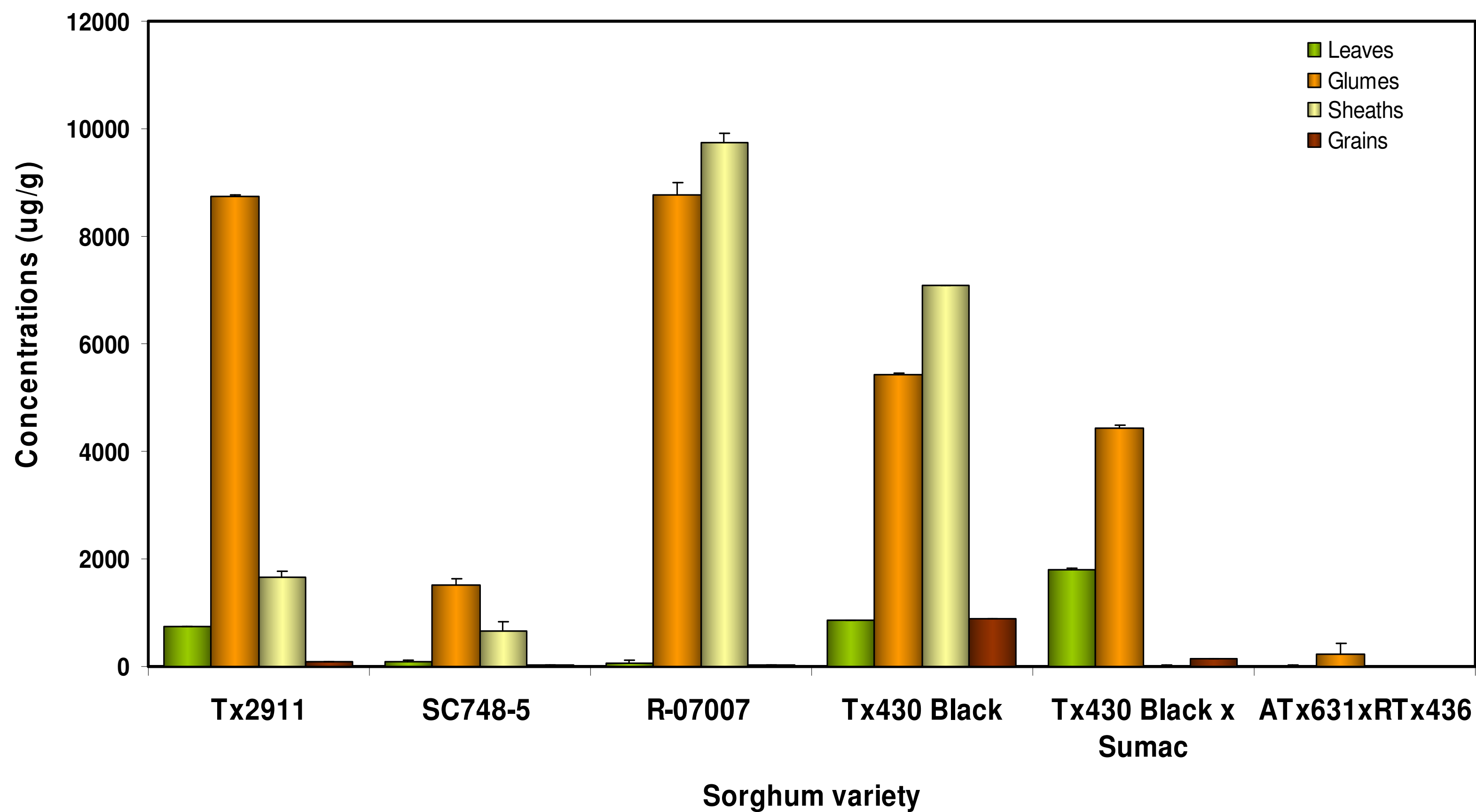


Fig. 35. Comparing 3-deoxyanthocyanin levels in different sorghum components grown in College Station, TX 2008. Dry weight basis.

Table 6

Summary of Flavonoids Identified in Sorghum Plant Components Grown in College Station, TX 2008

		Sorghum component				
Sorghum Varieties	Flavonoids [a]	Leaves	Sheaths	Glumes	Grains	Stalk
Tx2911	3-Deoxyanthocyanins	744.00±4.21	3629.10±28.08	8737.90±25.51	75.00±3.89	9.65±1.41
*Red	Flavones	244.37±12.19	344.58±9.60	1276.03±89.63	21.91±0.72	ND
	Flavanones	ND	6.84±0.57	1348.98±20.11	318.03±13.55	ND
SC748-5	3-Deoxyanthocyanins	94.70±4.60	1661.50±9.45	1502.00±8.45	17.7±0.56	NA
Purple	Flavones	265.53±6.03	731.99±6.27	147.75±2.03	54.81±0.58	NA
	Flavanones	173.92±1.04	265.74±12.78	159.85±5.19	911.25±35.83	NA
R-07007	3-Deoxyanthocyanins	60.70±3.35	660.00±6.56	8766.90±24.9	15.70±1.16	NA
Red	Flavones	815.45±2.33	681.39±13.92	754.25±42.33	21.68±0.68	NA
	Flavanones	1260.30±7.17	377.2±5.19	923.70±39.11	1375.06±38.84	NA
Tx430 Black	3-Deoxyanthocyanins	849.60±8.52	9738.40±54.6	5439.90±31.41	877.40±10.21	50.75±1.17
Purple	Flavones	379.42±10.45	319.74±41.62	857.34±45.79	69.80±0.37	ND
	Flavanones	ND	ND	ND	47.95±0.67	ND
Tx430 Black x Sumac	3-Deoxyanthocyanins	1810.60±20.11	7094.40±61.81	4431.70±34.82	156.50±4.10	NA
Red	Flavones	1448.50±8.31	708.99±7.46	408.38±15.67	19.41±2.43	NA
	Flavanones	ND	ND	ND	ND	NA
ATx631x RTx436	3-Deoxyanthocyanins	12.60±4.72	5.50±0.24	226.90±3.87	1.50±0.23	8.01±0.82
Tan	Flavones	255.46±10.93	236.00±7.94	775.89±6.51	79.43±2.59	ND
	Flavanones	ND	ND	ND	ND	ND
Sweet Sorghum (Collier)	3-Deoxyanthocyanins	6198.51±24.45	196.24±7.73	ND	ND	ND
	Flavones	ND	ND	NA	NA	NA
	Flavanones	ND	395.30±1.95	NA	NA	NA

[a] µg /g Dry matter basis. 3-Deoxyanthocyanins, flavones and flavanones were detected at 485, 340 nm, and 280 nm, respectively. ND =Not detected. NA= Not analyzed. * Secondary plant color.

The 3-deoxyanthocyanin profile varied among samples and among the various components analyzed (Figs. 36-40). The leaves of red-plant sorghums had higher levels of apigeninidin than luteolinidin. Tx2911 and Tx430 Black x

Sumac had higher levels of apigeninidin accounting for 72% of the total 3-deoxyanthocyanin levels (Fig. 36), while the purple-plant sorghum had higher levels of luteolinidin. Tx430 Black had higher levels of luteolinidin which accounted for 58% of its total 3-deoxyanthocyanins.

The red pigment found by Sereme et al (1993) in the sheaths and leaves of *Sorghum caudatum variety* Moneme kaya varieties identified as anthocyanins probably was 3-deoxyanthocyanins. Pigments extracted from the leaves of an intensely purple sorghum (sweet sorghum collier) obtained from Texas AgriLife Research had 6,189 ug/g 3-deoxyanthocyanin on dry weight basis (Table 6). Luteolinidin (3,291 ug/g) and 5-methoxyluteolinidin (1,934 ug/g) constituted 53 and 31 % of the total 3- deoxyanthocyanins detected.

The sheaths of Tx430 Black with its intense purple color had 9,738 ug/g 3-deoxyanthocyanins.The sheaths of red plant sorghum had higher levels of apigeninidin compounds, while those of purple plant sorghum had higher luteolinidin compounds. For example, Tx430 Black x Sumac had apigeninidin (2,836 µg/g) and 7-methoxyapigeninidin (870 µg/g) accounting for 40 and 36% respectively of total 3-deoxyanthocyanin identified. The sheaths of Tx2911 and R-07007 had only apigeninidin and 7-methoxyapigeninidin detected. Tx430 Black had high levels of luteolinidin (2,470 µg/g) and 5-methoxyluteolinidin (4,442 µg/g) accounting for 25 and 46 % respectively, of its total 3-deoxyanthocyanins (Fig. 37). The sheaths of the tan-plant sorghum (ATx631 x RTx436) did not have significant levels of 3-deoxyanthocyanins (Table 6).

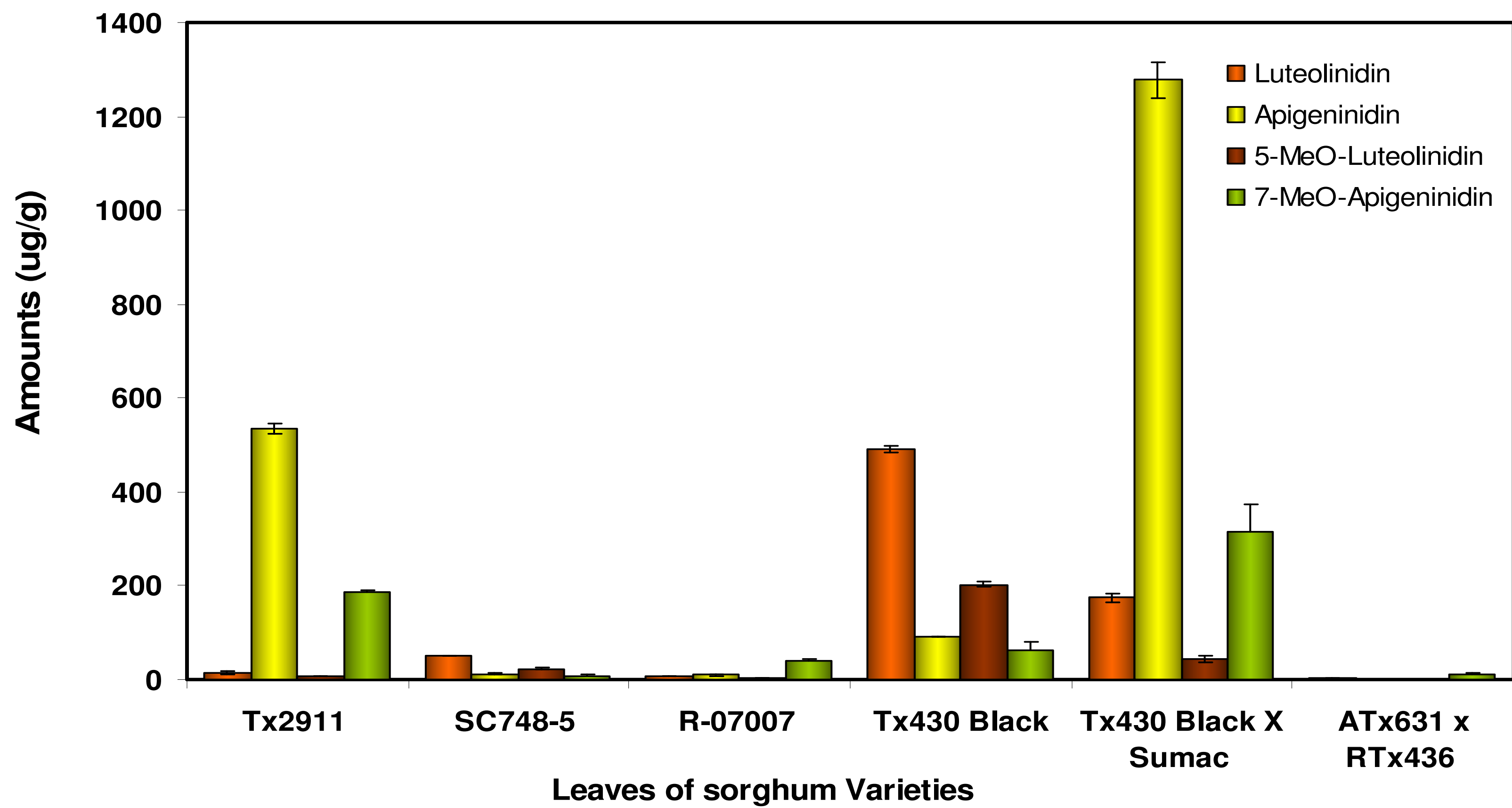


Fig. 36. 3-Deoxyanthocyanin profile of leaves of sorghums grown in College Station TX, 2008. Dry weight basis.

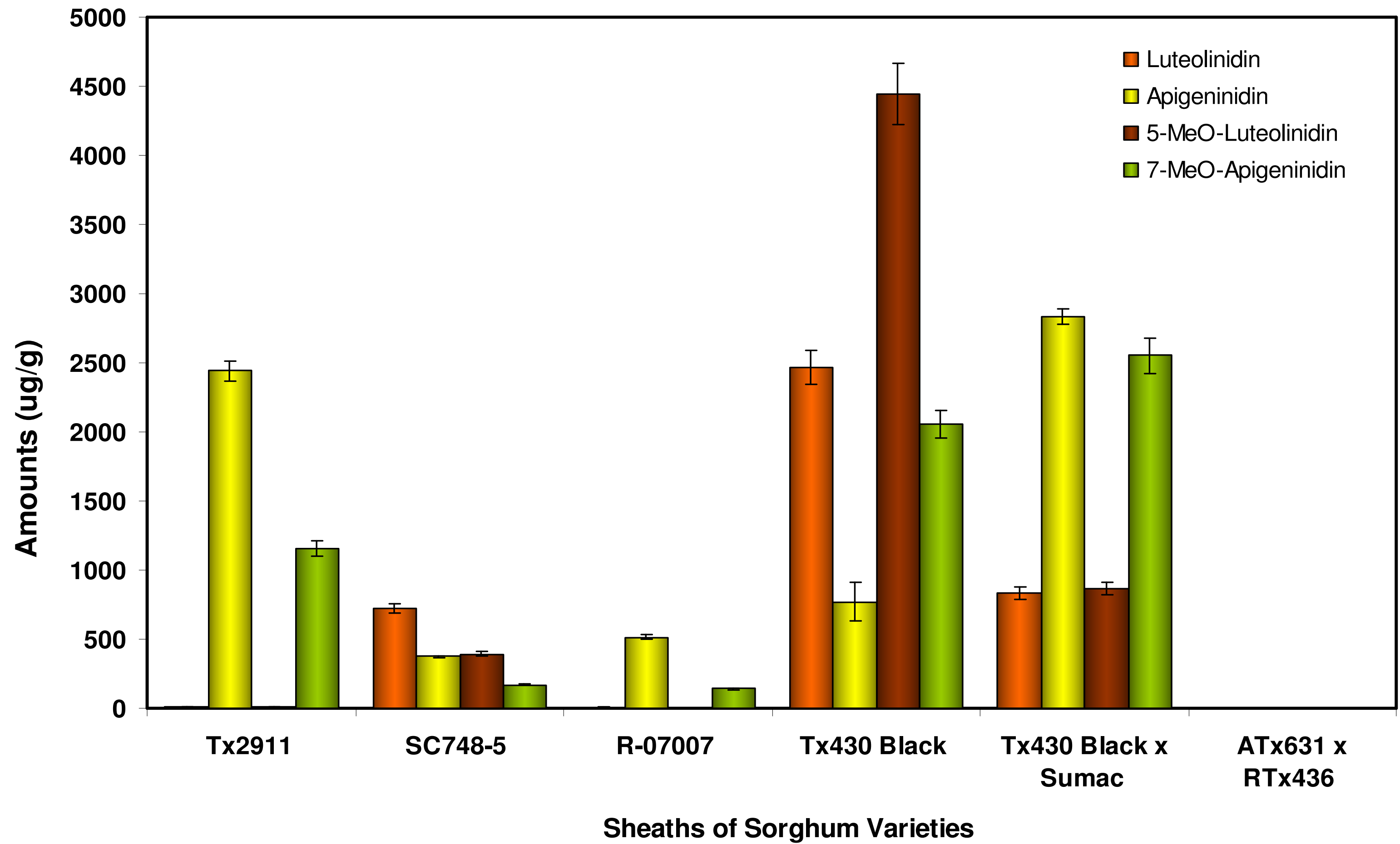


Fig. 37. 3-Deoxyanthocyanin profile of sheaths of sorghums grown in College Station TX, 2008. Dry weight basis.

Secondary plant color affected the levels of 3-deoxyanthocyanins in the glumes but did not affect the profile. The glumes of red and purple plant sorghums had higher levels of 3-deoxyanthocyanins than the tan plant sorghum. Apigeninidin and 7-methoxyapigeninidin were the predominant 3-deoxyanthocyanins in the glumes of sorghum varieties (Fig. 38). In R-07007 and Tx2911, apigeninidin alone constituted 78 and 67% of the total 3-deoxyanthocyanins.

The grains from all varieties had low levels of 3-deoxyanthocyanins compared to the other plant tissues with the exception of the stalks. The glumes and sheaths had higher levels of 3-deoxyanthocyanins than the leaves in all the sorghum varieties. The red and purple plant sorghum had higher levels of 3-deoxyanthocyanins than the tan plant sorghum. The red sorghum had higher levels of apigeninidin and 7-methoxyapigeninidin than luteolinidin and 5-methoxyluteolinidin while the purple plant had more luteolinidin compounds.
Tx430 Black had the highest 3-deoxyanthocyanins. Luteolinidin and 5-methoxyluteolinidin constituted 31 and 28% respectively of the total 3-deoxyanthocyanins (Fig. 39). Dykes (2008) reported a similar pattern for red sorghum grains which were higher in apigeninidin and 7-methoxyapigeninidin than in luteolinidin and 5-methoxyluteolinidin.

Secondary plant color did not affect levels of 3-deoxyanthocyanins in the stalks of the varieties evaluated. The stalks generally had low 3-deoxyanthocyanins relative to all other tissues, ranging from 10-51 µg/g (Fig. 40).

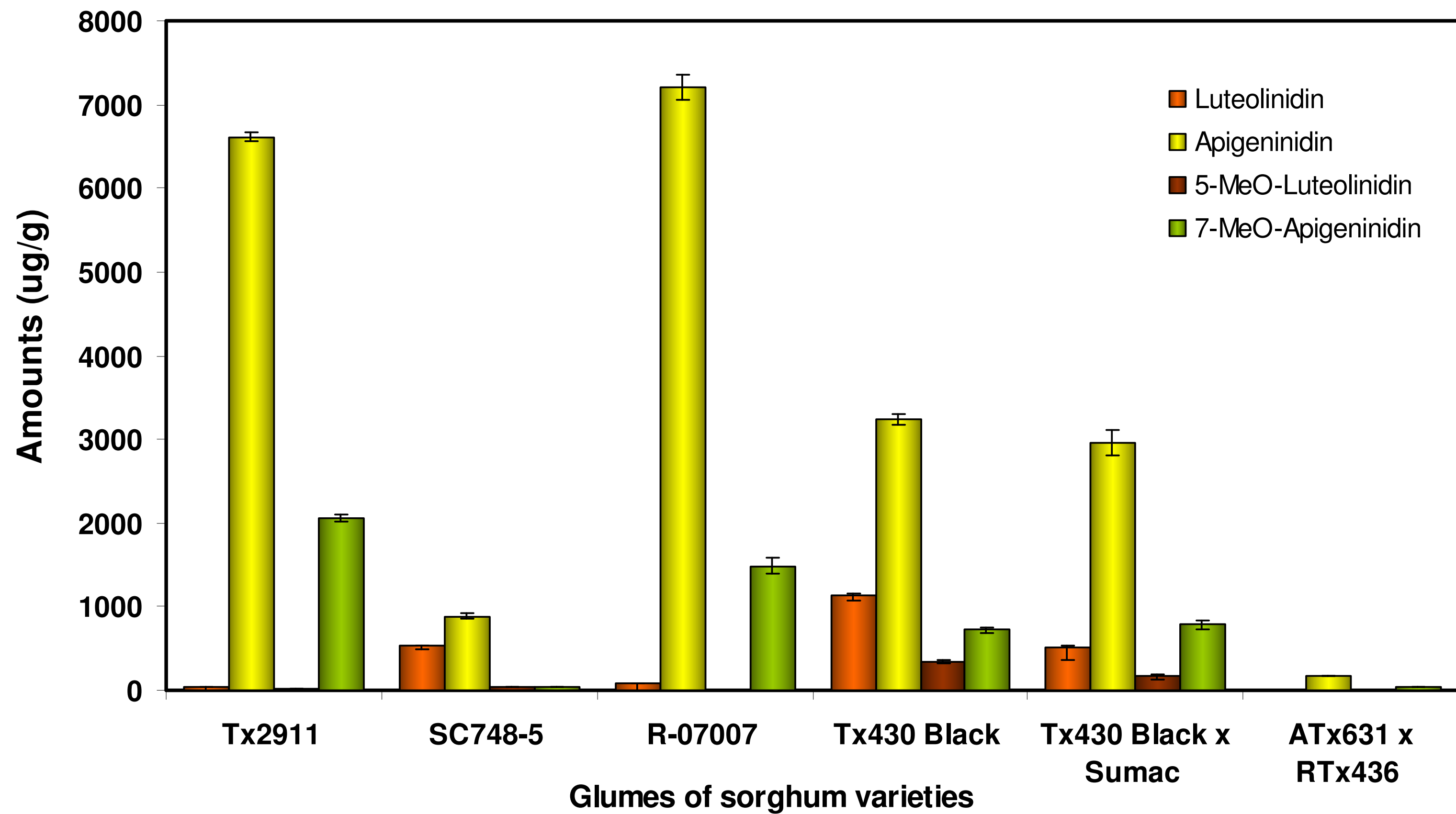


Fig. 38. 3-Deoxyanthocyanin profile of glumes of sorghums grown in College Station TX, 2008. Dry weight basis.

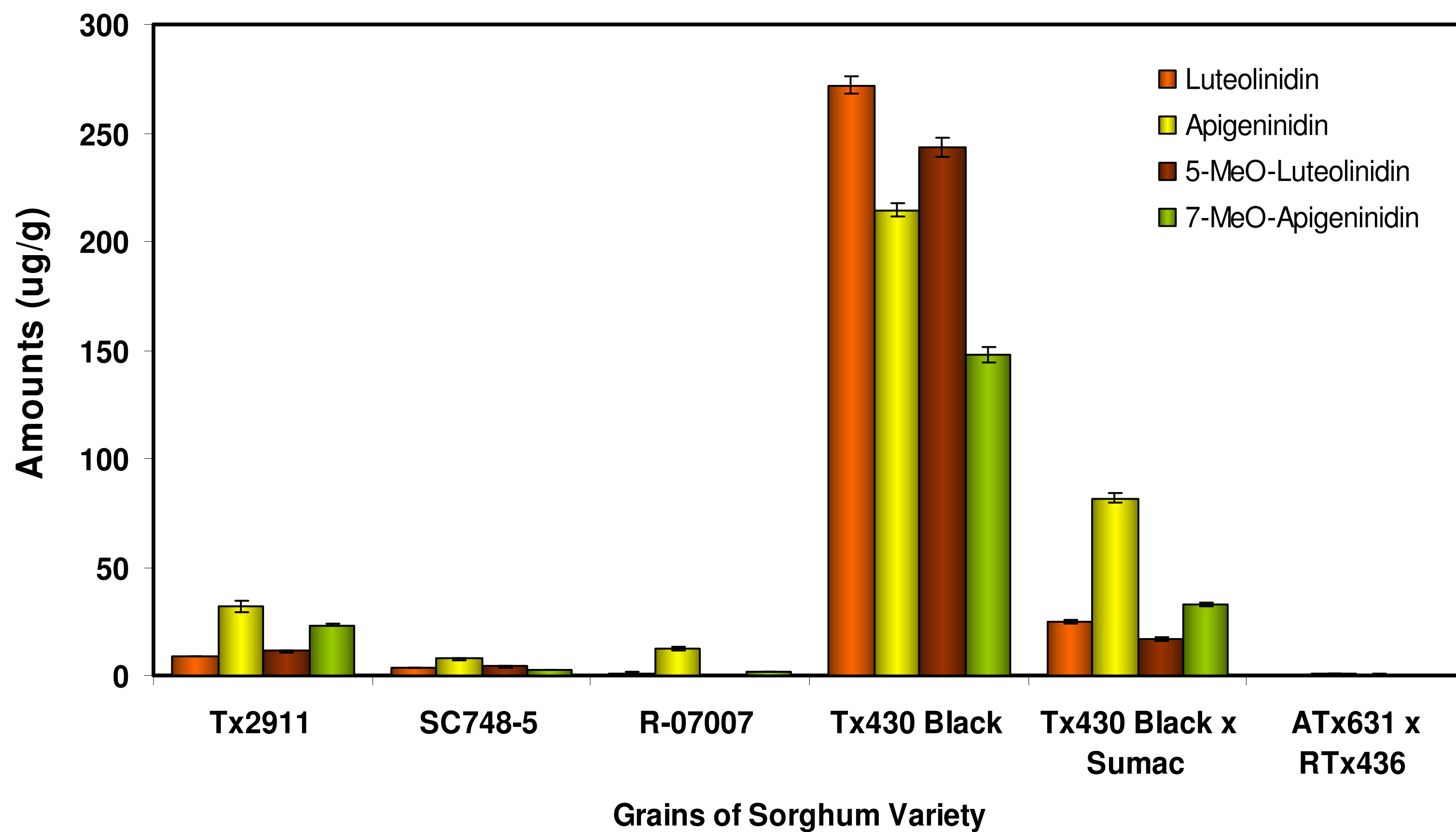


Fig. 39. 3-Deoxyanthocyanin profile of grains of sorghums grown in College Station TX, 2008. Dry weight basis.

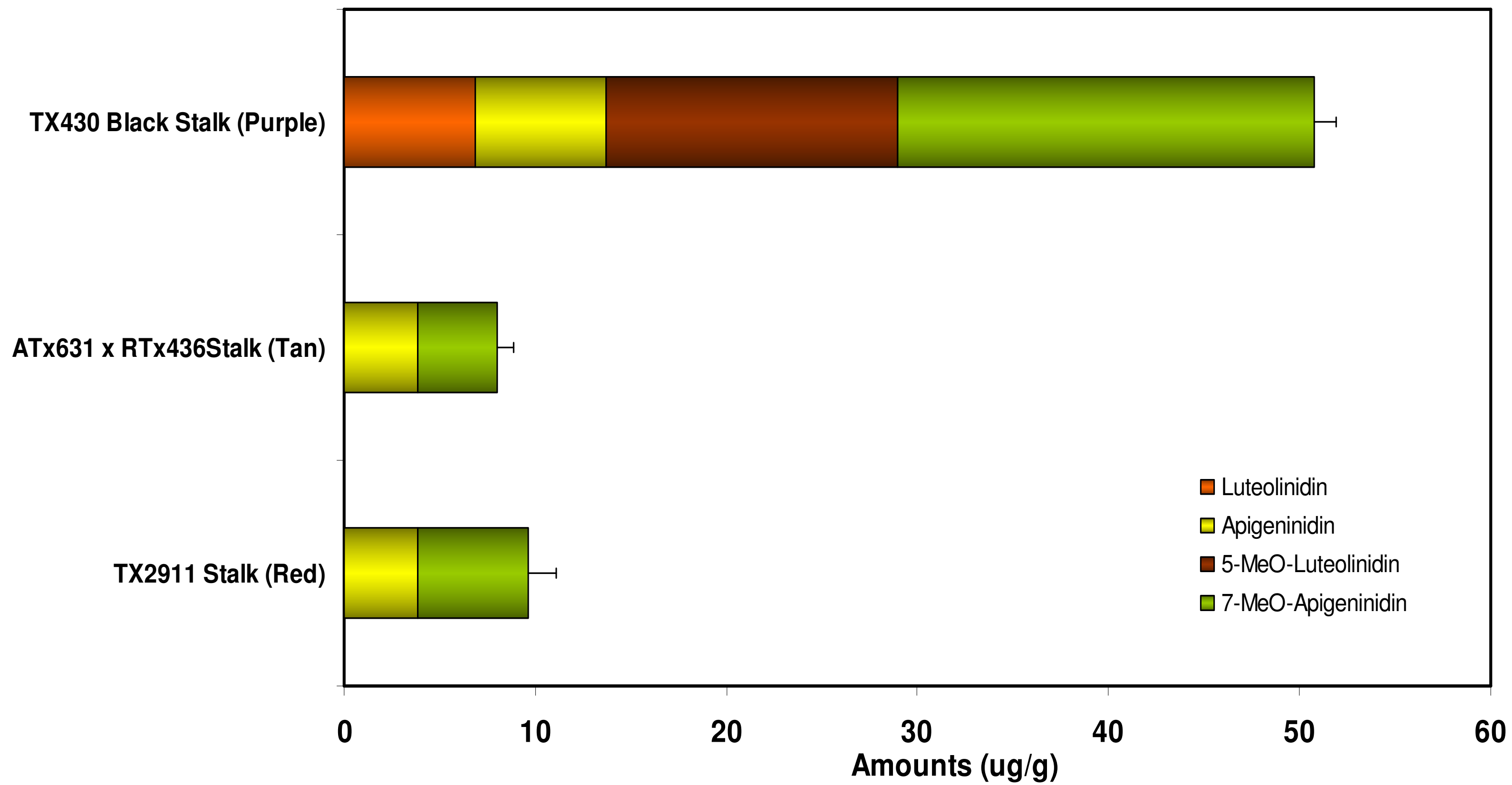


Fig 40. 3-Deoxyanthocyanin profile of stalks of different secondary plant color sorghums, grown in College Station TX, 2008. Dry weight basis.

The four main 3-deoxyanthocyanins were detected in the stalk of Tx430 Black, while in ATx631 x RTx436 and Tx2911, only apigeninidin and 7-methoxyapigeninidin were detected (Fig. 40). The leaves of Tx430 Black x Sumac had deep purple/red color in the field. The color was contributed mainly by the apigeninidin and 7-methoapigeninidin which was the predominant peak in the leaves of this sample. The grains of Tx430 Black had almost equal distribution of the 3-deoxyanthocyanins with luteolinidin compounds slightly higher. The sheath and leaves had higher levels of luteolinidin and 5-MeO-Luteolinidin, while the glumes had higher levels of apigeninidin and 7-MeO-Apigeninidin.

The sheaths, glumes and leaves of sorghums with purple-plant secondary color are excellent sources of the bright orange luteolinidin and 5-MeO-Luteolinidin, while the glumes, sheaths, and leaves of red-plant sorghum are suitable sources for the yellow apigeninidin and 7-MeO-Apigeninidin.

The sheaths, glumes and leaves of Tx430 Black x Sumac is predominant in apigeninidin and 7-MeO-Apigeninidin, although it still had high levels of luteolinidin and 5-MeO-Luteolinidin comparable to those in the purple plant sorghum. The Tx430 Black gene may be dominant, contributing to high luteolinidin and 5-MeO-Luteolinidin based on the Tx430 Black 3-deoxyanthocyanin profile.

A strong correlation was observed between anthocyanin levels measured by the colorimetric method of Fuleki and Francis (1968) expressed as luteolinidin

equivalent and by HPLC methods for leaves, sheaths, grains and glumes (Table 7). When all the components were taken together the correlation was 0.76. The strong correlation suggests that the anthocyanins measured colorimetrically were mainly 3-deoxyanthocyanins. Thus the colorimetric method can be used as a rapid, inexpensive method to screen for 3-deoxyanthocyanins in grain and non-grain components of sorghum.

Flavones in Sorghum Components

Flavone levels and profiles varied within components and varieties (Fig. 41). Flavones ranged from 244-1,449 µg/g in leaves, 148-1,276 µg/g in glumes, 236-732 µg/g in sheaths and 19-79 µg/g in grains (Table 6; p. 112).

Secondary plant color affected levels in the leaves and sheaths (Figs. 42 & 43). The leaves and sheaths of red-plant sorghums had higher levels of apigenin than luteolin while for purple and tan plant sorghums, luteolin was higher.

Table 7
Pearson's Correlation Coefficients of Anthocyanins Measured Colorimetrically and 3-Deoxyanthocyanins

	ACY measured colorimetrically			
3-Deoxy-ACY	Leaves	Sheaths	Glumes	Grains
measured by HPLC	0.85	0.96	0.87	0.98

Correlation is significant at $p<0.01$
ACY=Anthocyanins.

For example, apigenin was the major flavones in the leaves of Tx430 Black/Sumac (1,068 µg/g) and R-07007 (658 µg/g) accounting for 74 and 81% respectively of the total flavones detected in those varieties (Fig. 42). SC748-5 (197 µg/g), Tx430 Black (349 µg/g) and ATx631 x RTx436 (234 µg/g) had higher luteolin levels than apigenin, that accounted for 74%, 92% and 91% respectively of the total flavones in those varieties. Apigenin was the only compound identified in the leaves of Tx2911.

The sheaths of SC748-5 and ATx631 x RTx436 had higher levels of luteolin than apigenin, constituting 96 and 95% respectively of their total flavone levels (Fig. 43). R-07007 and Tx430 Black x Sumac had higher levels of apigenin than luteolin, constituting 82 and 58% respectively of the total flavones detected. Similar to the leaves, only apigenin was identified in the sheaths of Tx2911. Unlike the leaves of Tx430 Black in which both luteolin and apigenin were detected, the sheaths had luteolin as the only flavone detected.

Secondary plant color did not affect flavone profiles in the glumes, grains and stalks. The glumes of all varieties had apigenin as the predominant flavone with the exception of SC748-5 in which only luteolin was detected (Fig. 44). The glumes of R-07007 had apigenin as the only flavone while its grain had luteolin as the only detectable flavone. Similar to leaves and sheaths, apigenin was the only flavone detected in glumes and grains of Tx2911 (Figs. 44 & 45). The flavones in the leaves, sheaths and glumes of tan-plant sorghum may contribute to the total phenols and high antioxidant activities observed (Table 5; p. 99).

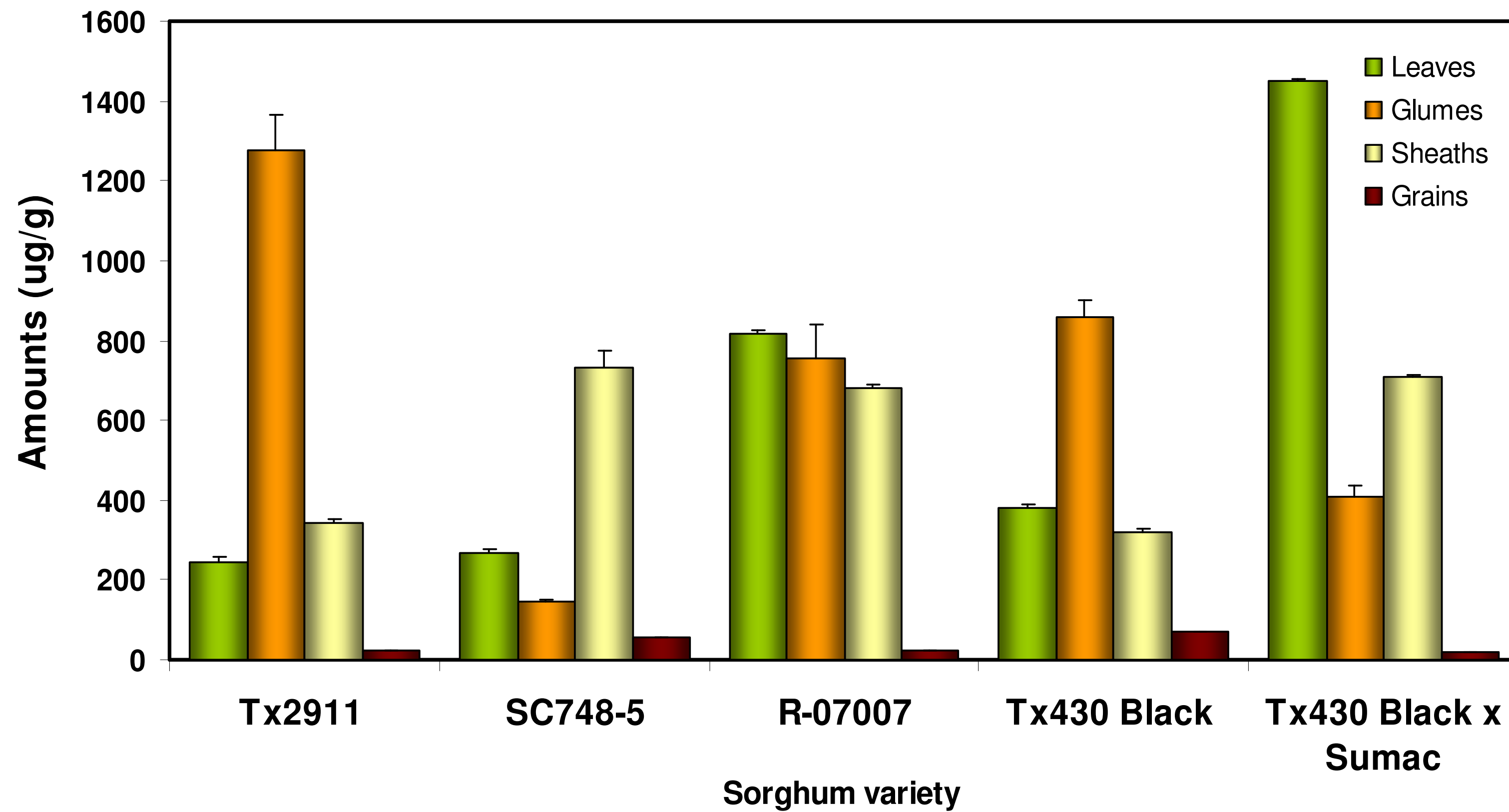


Fig. 41. Comparing Flavone levels in different sorghum components grown in College Station TX, 2008. Dry weight basis.

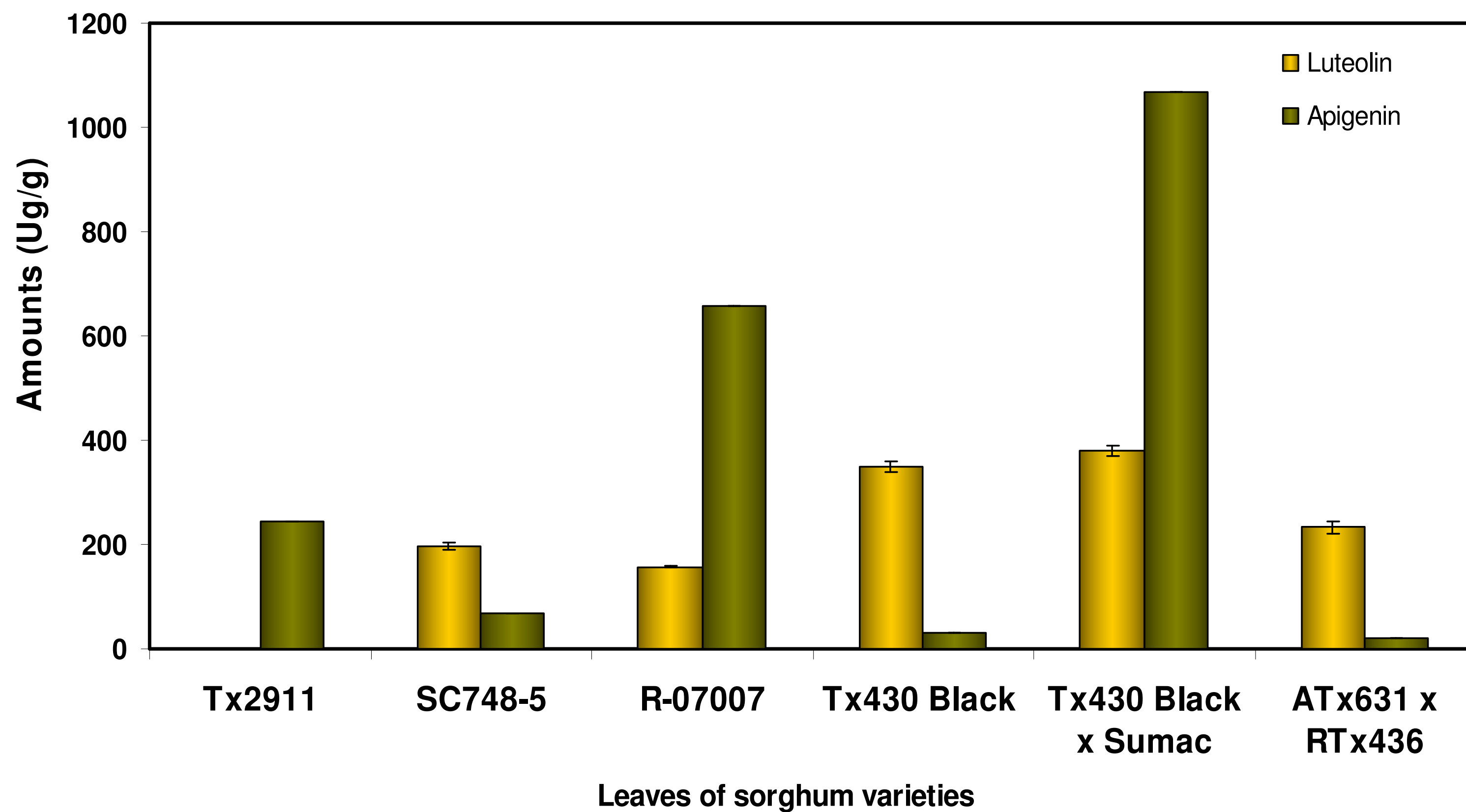


Fig. 42. Flavone profile of leaves of sorghums grown in College Station TX, 2008. Dry weight basis.

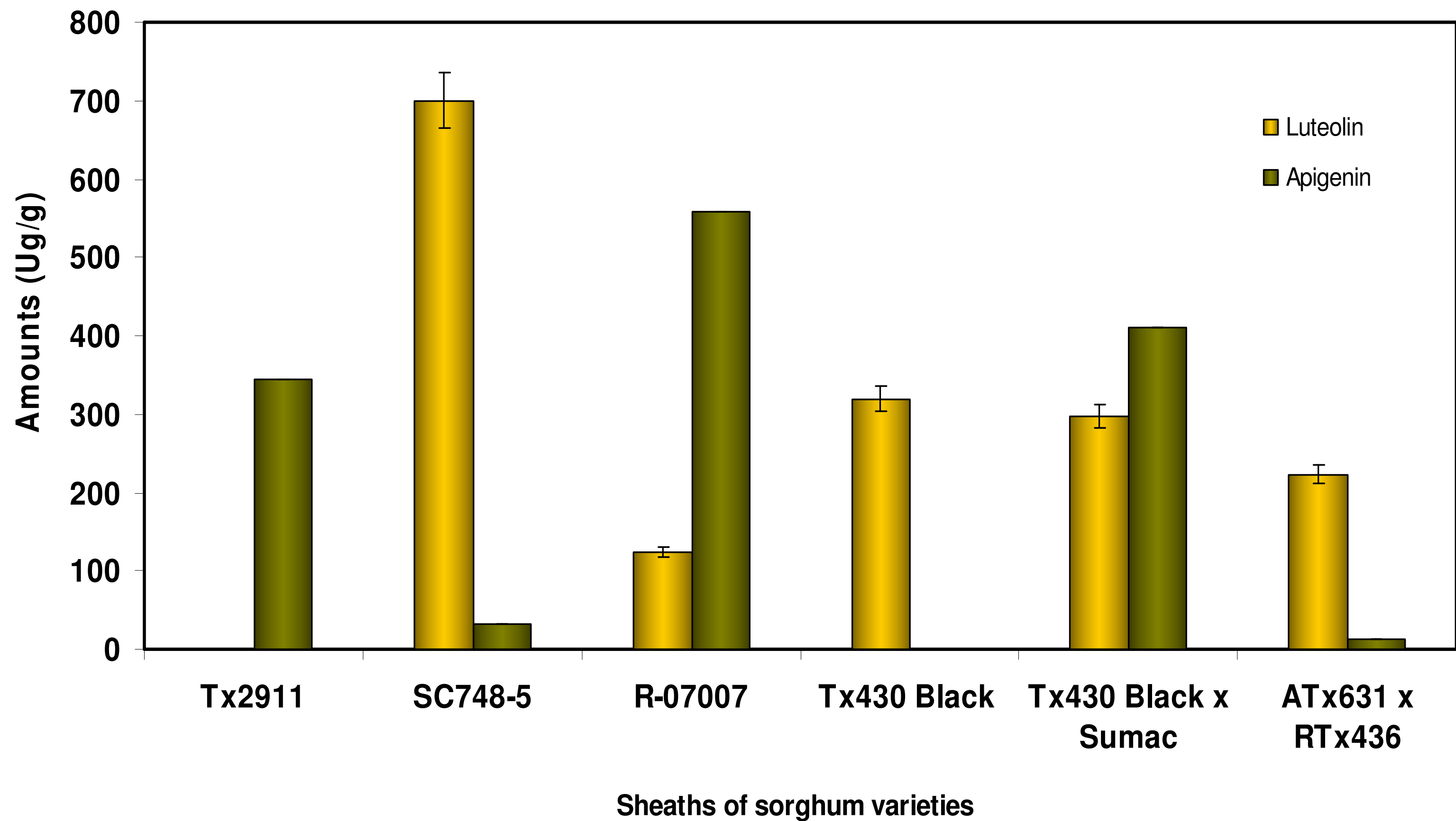


Fig. 43. Flavone profile of sheaths of sorghums grown in College Station TX, 2008. Dry weight basis.

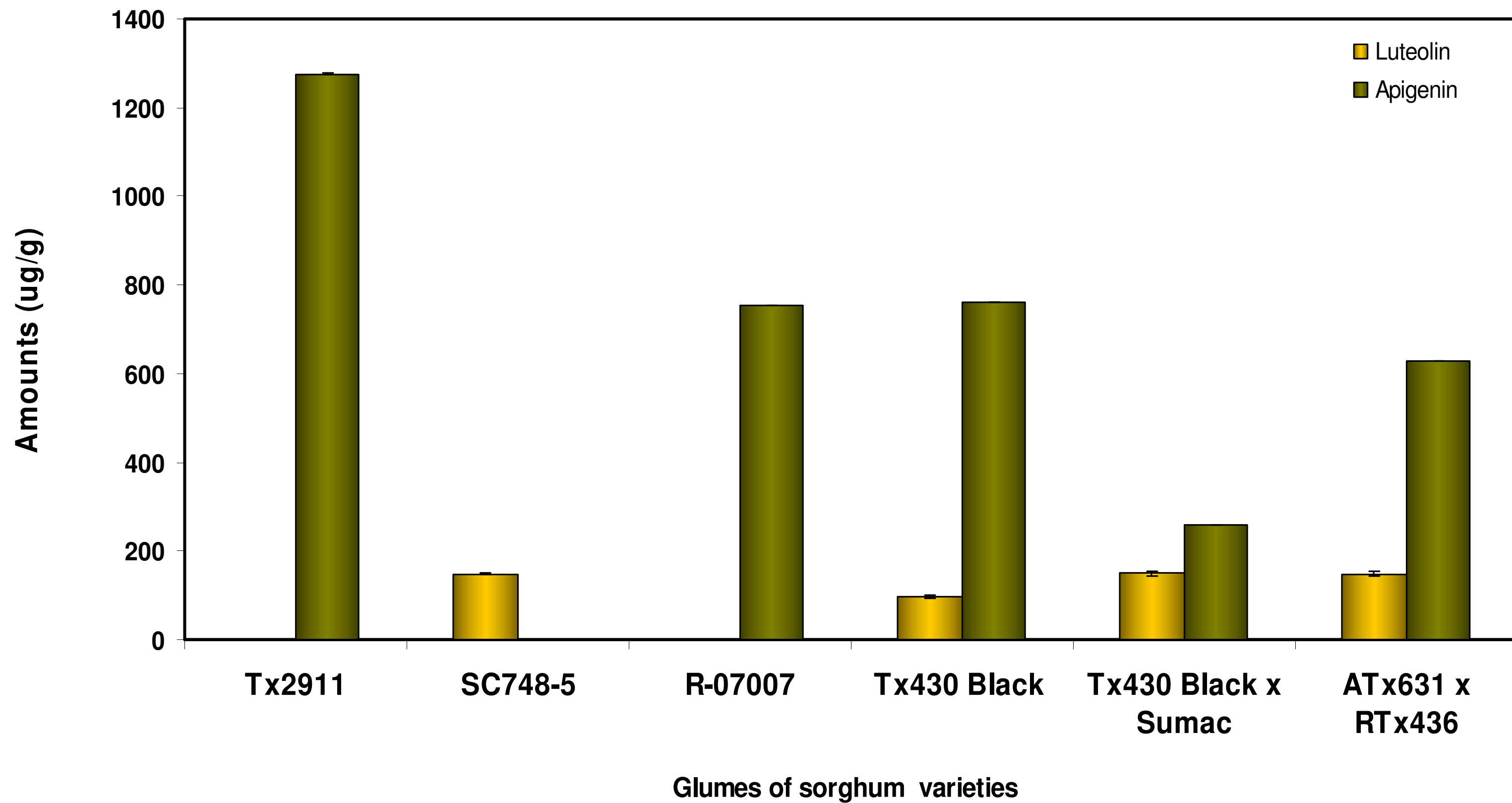


Fig. 44. Flavone profiles of glumes of sorghums grown in College Station TX, 2008. Dry weight basis.

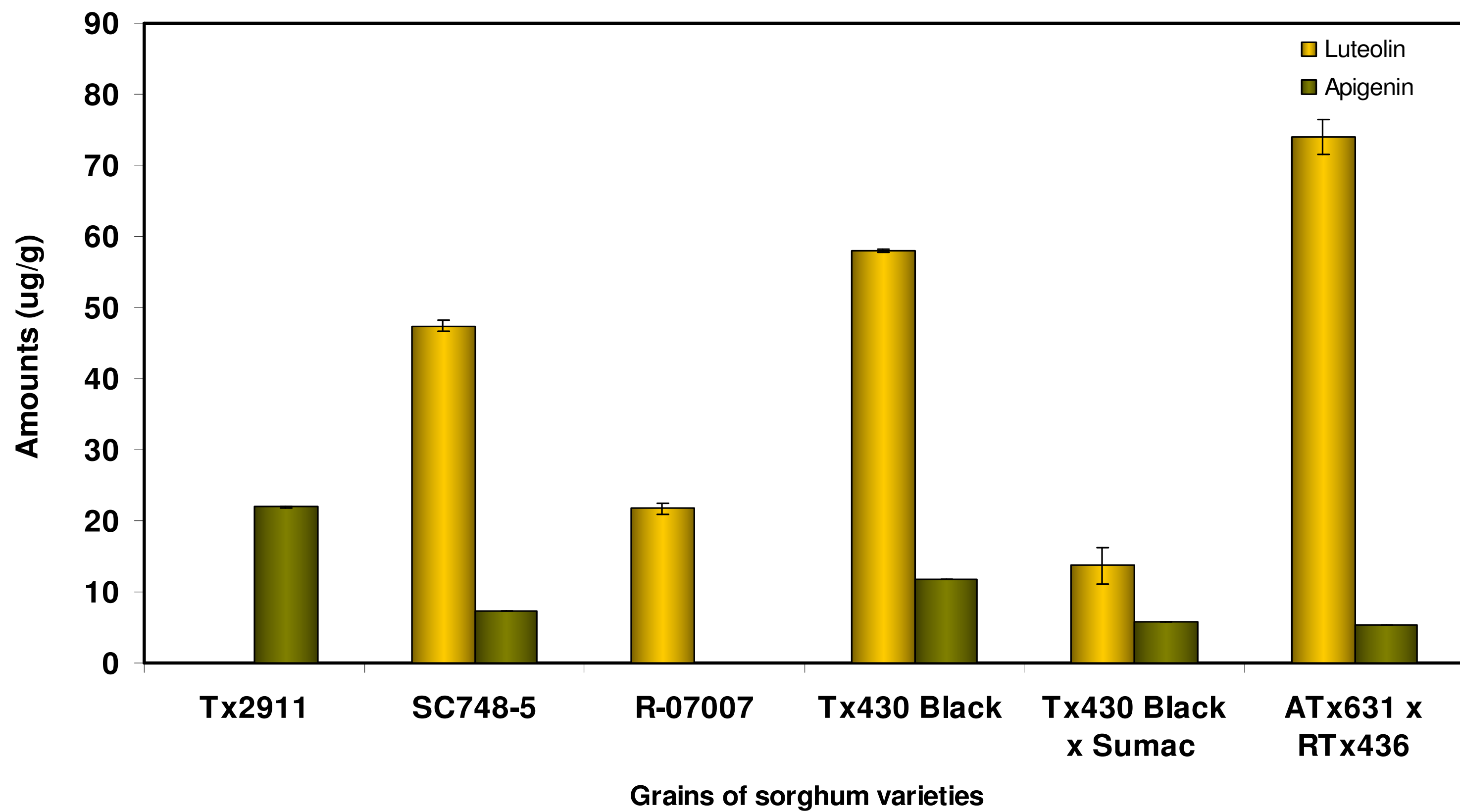


Fig. 45. Flavone profile of grains of sorghums grown in College Station TX, 2008. Dry weight basis.

The flavone for R-07007 and SC748-5 grains agreed with Dykes (2008) who found 34 and 30.8 µg/g in R-07007 grown in College station in 2006 and Lubbock in 2005 respectively; 47.3 and 46.2 µg/g in SC748-5 for the same locations and period respectively. Dykes (2008) found luteolin as the only flavone in Tx2911 grown in Lubbock in 2005 compared to both luteolin and apigenin found in Tx2911 grown in College Station in 2006.

These differences support the hypothesis that the environmental condition under which the sorghum matures influences its flavonoid composition. Low levels of flavones were detected in the stalks evaluated, ranging from 0-12 µg/g (Fig. 46). The stalk of Tx2911 (red plant) had no detectable levels of flavones, while only apigenin was detected in the stalks of Tx430 Black and ATx631 x RTx436.

Luteolin and apigenin are major dietary flavones, commonly found in aromatic herbs (parsley, rosemary & thyme). Both flavones have been identified in sorghum and sorghum compares favorably with other common sources of these compounds (Fig. 47). The leaves, sheaths and glumes of red, purple and tan plant sorghums are viable sources of flavones with potential health applications. Flavones have anticancer, anti-inflammatory, antiallergic, and analgesic properties (Block et al 1998; Hirano et al 2004; Horinaka et al 2005; Matsui et al 2005; Cherng et al 2007; Ziyan et al 2007). They are used as vascular relaxation agents and for the treatment of corneal neovascularization (Block et al 1998; Xu et al 2007).

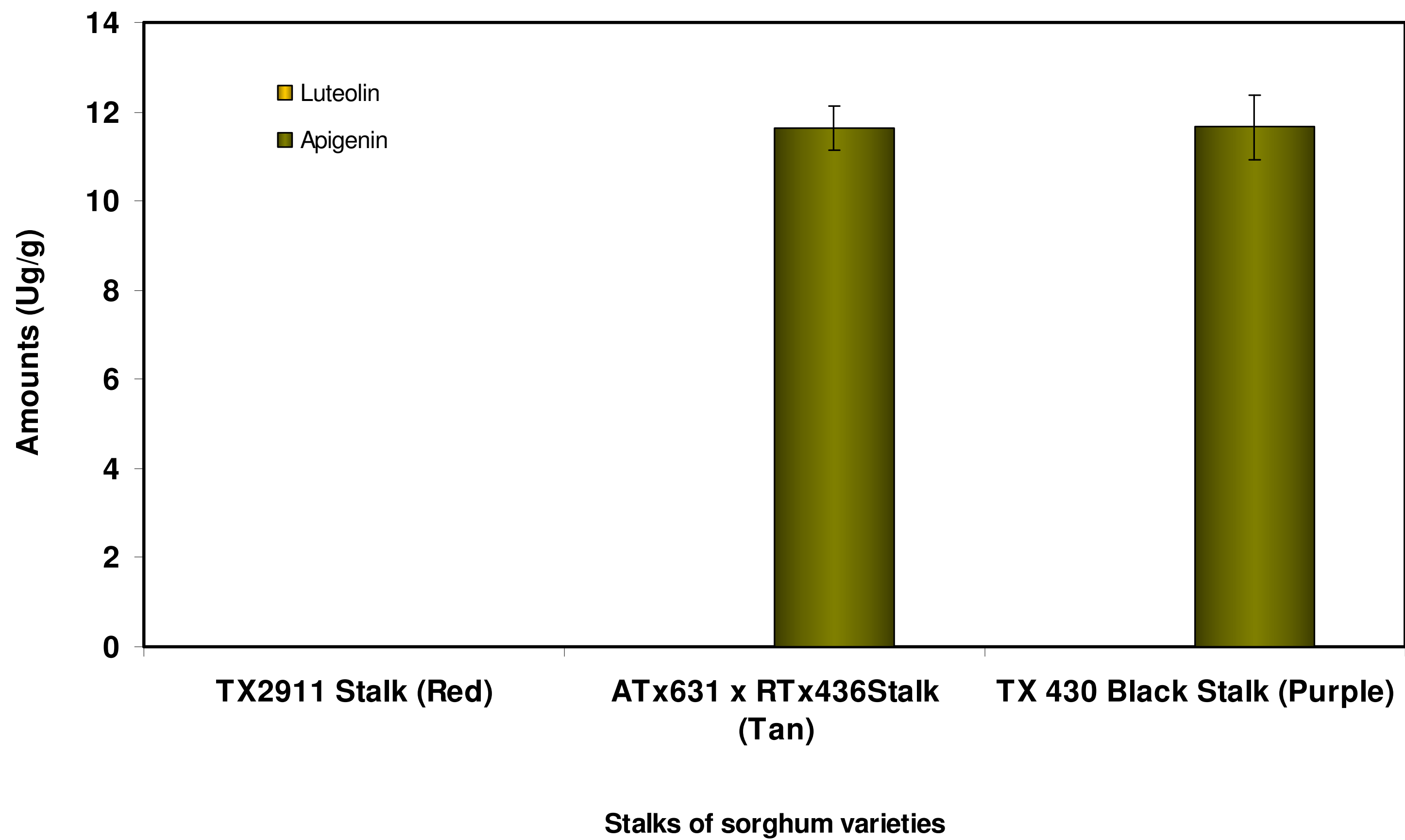


Fig. 46. Flavone profile of stalks of different secondary plant color sorghum, grown in college station TX, 2008. Dry weight basis.

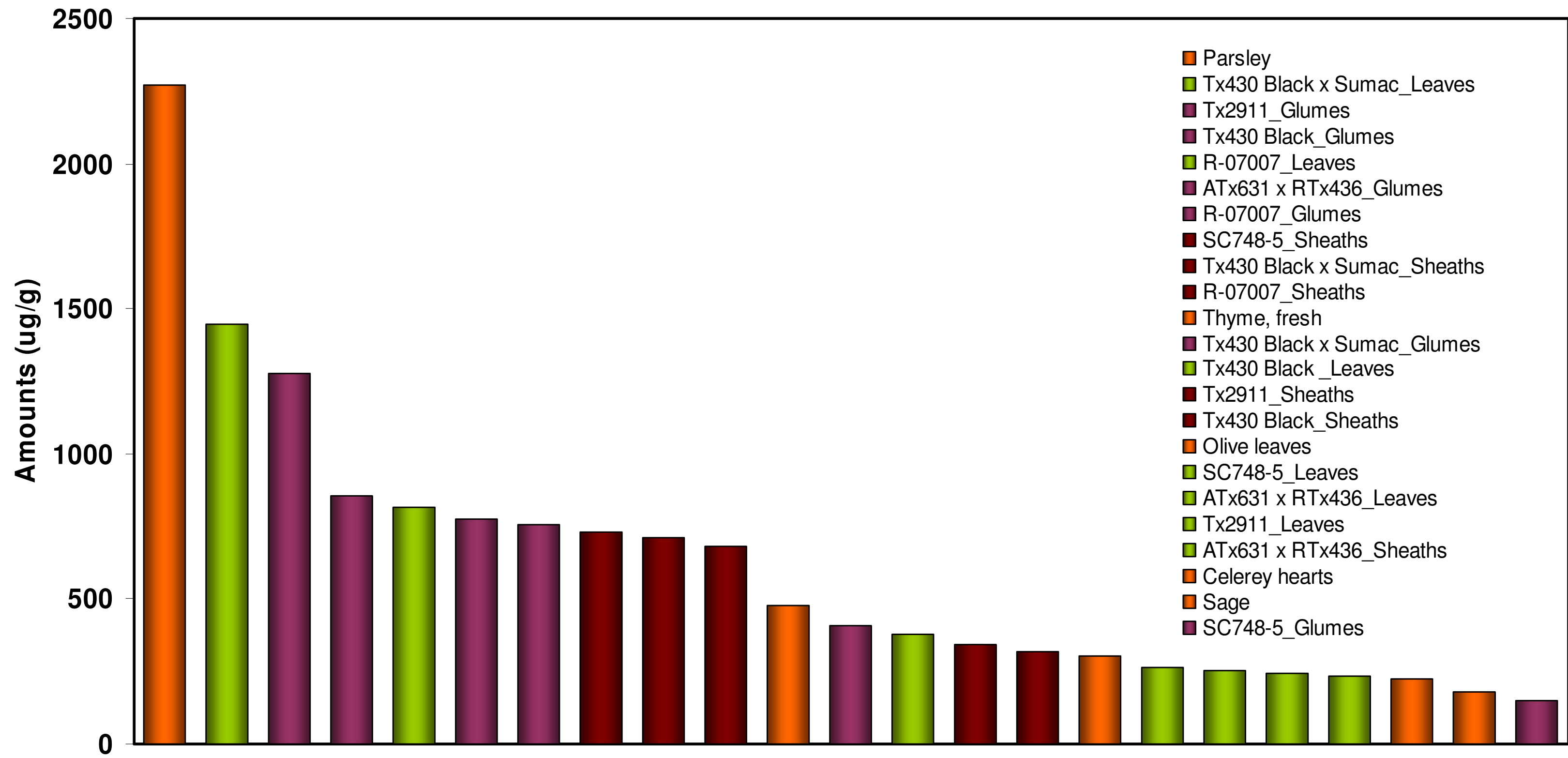


Fig. 47. Flavones (ug/g) in sorghum compared to common sources. (Data from USDA Database for the Flavonoid content of selected Foods, Release, 2.1).

The glumes of Tx2911 gave the most viable apigenin while the sheaths of SC748-5 gave the most luteolin from the sorghum varieties analyzed (Figs. 48 & 49). In addition to luteolin and apigenin, flavone-glycosides, which had not been previously identified in sorghum, were detected in the non-grain sorghum tissues. Non-grain sorghum tissues, with the exception of the stalks provide excellent sources of large quantities of flavones and flavone-glycosides.

The grains of sorghum are low in flavones compared to the non-grain tissues. The grain of sorghum provides an indication of the presence or absence of flavones in the non-grain tissues but cannot be used to predict the flavone profiles. The profiles found in the leaves and sheaths suggest that one might use such information to predict the profile of the others, but not the levels.

Characterization of Flavone-Glycosides

The total flavones reported were based on retention times of commercial standards and UV-Vis spectral characteristics. The known peaks for luteolin and apigenin (1 & 2 respectively) were minor peaks compared to the unidentified peaks; a, b, c, d, e and f (Fig. 50) measured at 340 nm.

Flavone-glycosides were detected in the leaves, sheaths and glumes of sorghum. Tentative identifications of the flavone-glycosides (Table 8) were based on spectral and mass spectrometric characteristics, showing distinctive fragmentation patterns of pseudomolecular ions $[M-H]^-$.

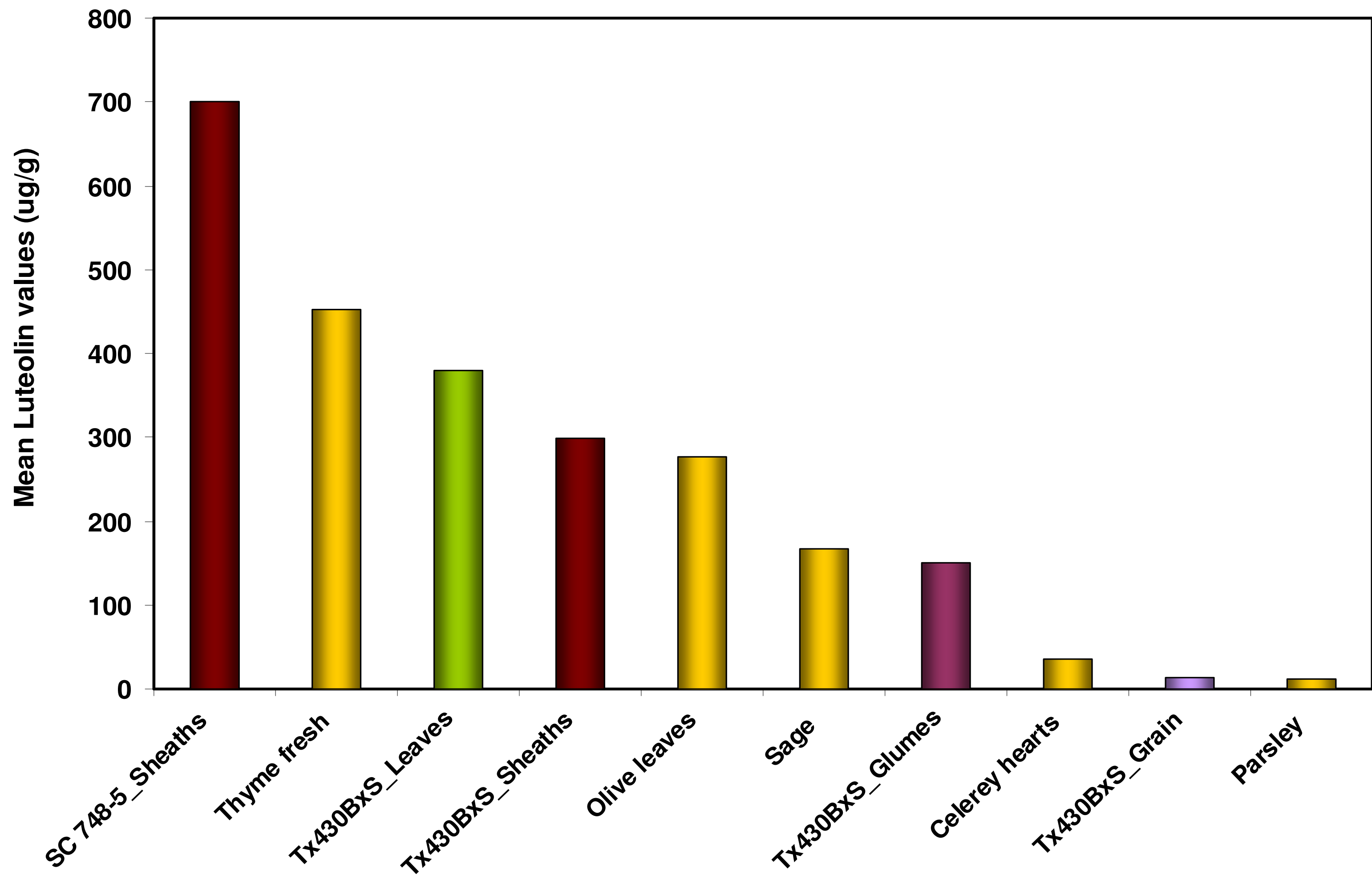


Fig. 48. Luteolin in sorghum compared to common sources. (Data from USDA Database for the Flavonoid content of selected Foods, Release, 2.1). Tx430BxS= Tx430 Black x Sumac.

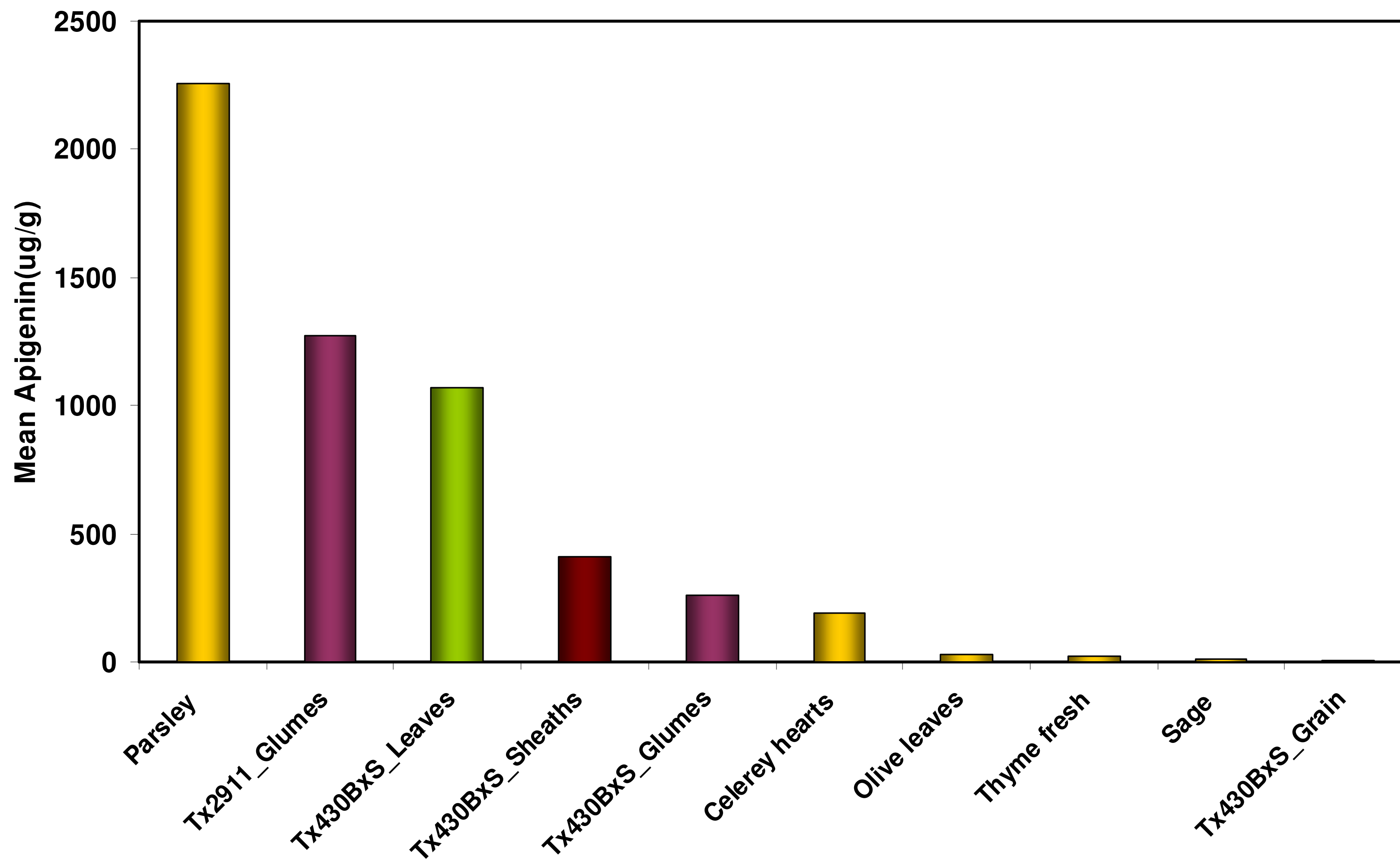


Fig. 49. Apigenin in sorghum compared to common sources. (Data from USDA Database for the Flavonoid content of selected Foods, Release, 2.1).Tx430BxS= Tx430 Black x Sumac.

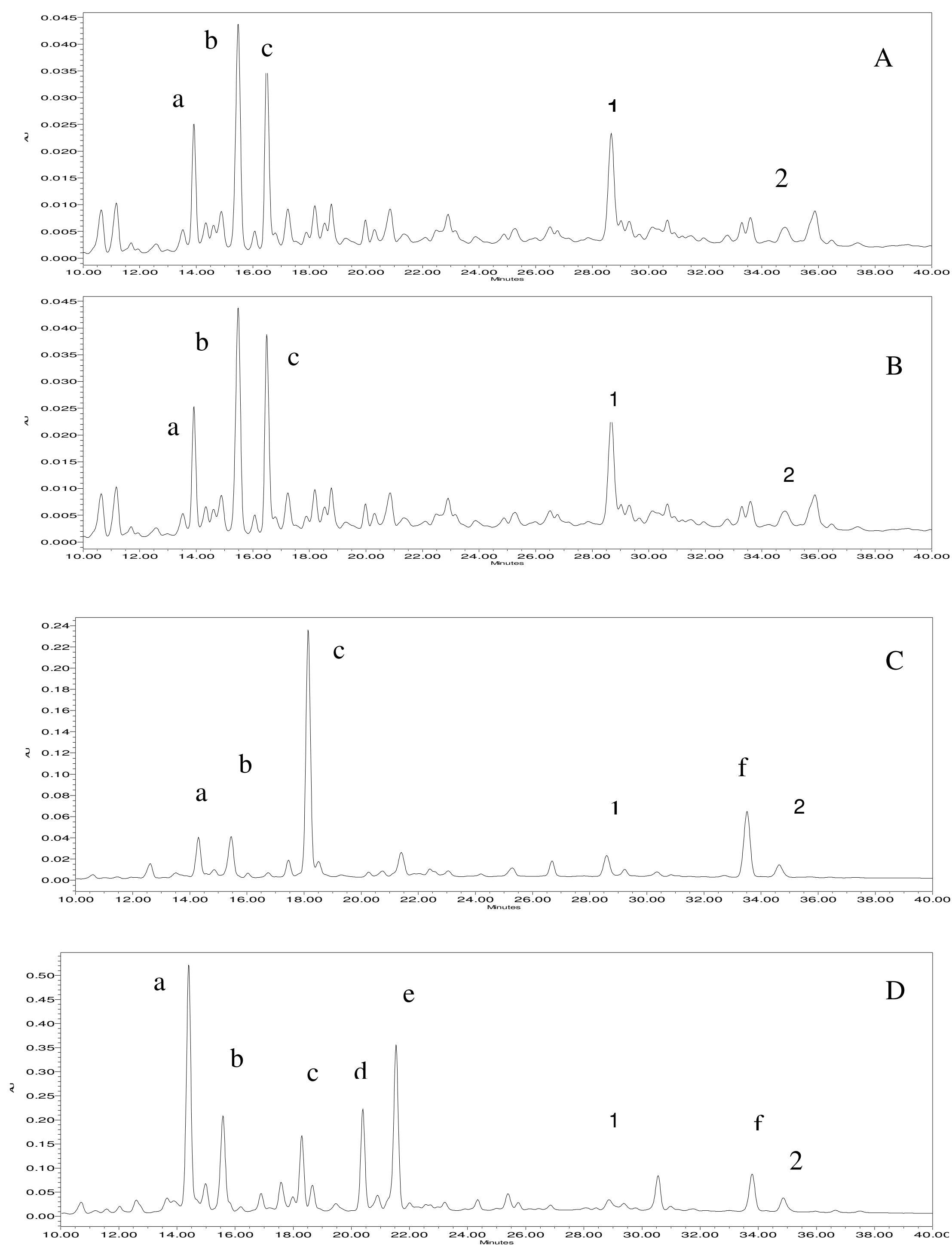


Fig. 50. HPLC Chromatograms of sheaths and leaves of sorghum grown in College Station TX, 2008. A=Tx430 Black sheaths; B=Tx430 Black x Sumac sheaths; C= Tx430 Black x Sumac leaves; D= Tx 2911 leaves at 340nm. 1=Luteolin; 2= Apigenin. A,b,c,d,e,f.

The fragmentation yielded product ion signals at $[M\text{-}H\text{-}60]^-$, $[M\text{-}H\text{-}90]^-$, and $[M\text{-}H\text{-}120]^-$ in the negative ion mode MS^n analyses (Gattuso et al 2007; Caristi et al 2006; Pereira et al 2005; Ferreres et al 2003; Voirin et al 2000).

Luteolin and apigenin-*C*-glycosides were detected in the leaves, sheaths and glumes of sorghum (Caristi et al 2006; Pereira et al 2005).

The tentative identifications were based on typical characteristic loss of ions (absorption at 350-360 nm) of $[M–H–18]^–$, $[M–H–60]^–$, $[M–H–90]^–$, $[M–H–120]^–$, $[M–H–120–90]^–$, $[M–H–120–120]^–$ (Caristi et al 2006). Fragment ions corresponding to luteolin (*m/z*=285.2, $[M\text{-}H]^-$) and apigenin (*m/z*=269.1, $[M\text{-}H]^-$) aglycones were also present. More detail identification needs to be done to confirm these compounds using appropriate standards and NMR analyses. Acid hydrolysis test could be done to confirm the presence of *C* as oppose to *O*-glycosides.

The principal feature of O-glycosides fragmentation product ion of mass spectra $[M\text{-}H]^-$ are the formation of Y^- and $(Y\text{-}H)^{\bullet -}$ ions . An example of O-glycoside fragmentation is illustrated (Fig. 51) for apigenin-*O*-glycosides (March et al 2006). Y^- is formed as a result of the loss of 162 Da and re-arrangement, while $(Y\text{-}H)^{\bullet -}$ radical anion is formed by scission with the loss of 163 Da (March et al 2004; March et al 2006). The product ion showed three major fragmentations of the glycan that yield *m/z* 341, *m/z* 311 and also m/*z* 413 from loss of a water molecule.

Table 8

Characterization of Non-3-Deoxyanthocyanin Flavonoids Present in Sorghum Non-Grain Materials

Peak No.	RT (min)	Compound*	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)
1	17.4	Apigenin	337	269.2	269.2, 197.4, 133.1
2	17.7	Apigenin-*C*- glycoside	258, 352	563.1	**503.1, 473.1, 443.1, 383.1, 353.1, 341.1**
3	19.2	Apigenin-*C*- glycoside	272, 352	563.0	545.1, 473.1, 443.1, 383.1, 353.1, 294.2, 250.9
4	19.3	Unidentified flavone glycoside	347	367.0	367.1, 307.2, 270.1, 195.7, 191.2, 179, 135.2
5	22.3	Luteolin-glucoside	327, 330, 347	447.1	429.4, 327.1, **285.2**, 190.9
6	23.9	Flavone-glucoside	340,347	497.2	497, 451.3, 399, 335.1, 290.2, 177.5
7	24.5	Unidentified flavone glycoside	347	622.4	555.9, 544.9, 521.2, 460.1, 371.6, 245.3, 188.7
8	26.1	Apigenin-*O*-glucoside	267, 337	431.2	413.5, 334.8, 311, **269.2. 285**
9	30.1	Unidentified flavone glycoside	347	489.0	489.1, 457.1, 445.1, 337.1, 323.2, 280, 233, 161
10	30.2	Unidentified procyanindin glycoside	347	723.3	723.2, 677.5
11	35.5	Apigenin-gylcoside	347	531.0	498.9, 431.1, 412, 311.1, **269.2**

* Tentative identification based on similarities on spectral characteristics and ESI-MS^n fragmentation patterns.

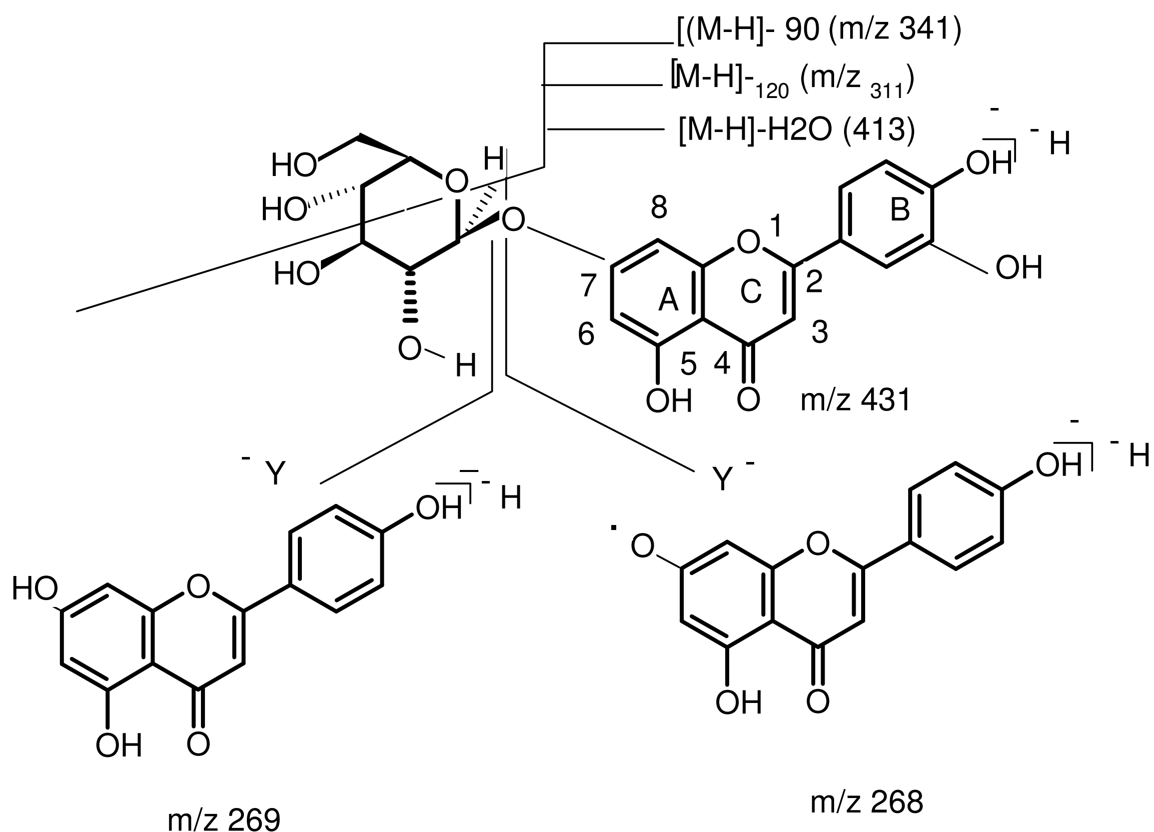


Fig. 51. Tentative identification of [M-H]$^-$ of apigenin-*O*-glycosides *m/z* 431.

Flavanones in Sorghum Components

Flavanones ranged from non-detectable to 1,375.1 µg/g (Table 6; p. 112). Secondary plant color did affect flavanones in the leaves, sheaths, glumes and grains. All components of R-07007 and SC748-5 had detectable levels of flavanones. In Tx2911 flavanones were detected in the glumes and grains only and in the grains of Tx430 Black (Fig. 52). Flavanone profile varied among the sorghum varieties and also among components within the same variety (Figs.

53-56). The leaves and the glumes had higher levels of naringenin than eriodictyol while the sheaths had higher levels of eriodictyol.

Flavanones were detected only in leaves of SC748-5 and R-07007, with naringenin accounting for 70 and 62% respectively of the total flavanone levels in these two varieties (Fig. 53). The sheaths of R-07007 had 75% of its flavanones from eriodictyol (Fig. 54). In SC748-5 eriodictyol and naringenin were evenly distributed constituting 49 and 51% respectively of its total flavanone levels. 7 ug/g of eriodictyol was detected as the only flavanone in the sheaths of Tx2911.

The Glumes of Tx2911 had the highest level of flavanones (1349 /g) with only naringenin detected. R-07007 had 932.7ug/g of total flavanones with 45 and 55 % of eriodictyol and naringenin respectively. The glumes of SC748-5 and TX2911 had only naringenin as detectable flavanones (Fig. 55). The glumes of Tx430 Black, Tx430 Black x Sumac and ATx631 x RTx436 had no detectable flavanones.

Sorghum grains with lemon yellow pericarp had the highest levels of flavanones. The grains of R-07007 had the highest levels of flavanones with eriodictyol constituting 84 % of the total flavanones and SC748-5 had 51 % eriodictyol constituting of its total flavanones (Fig. 56).

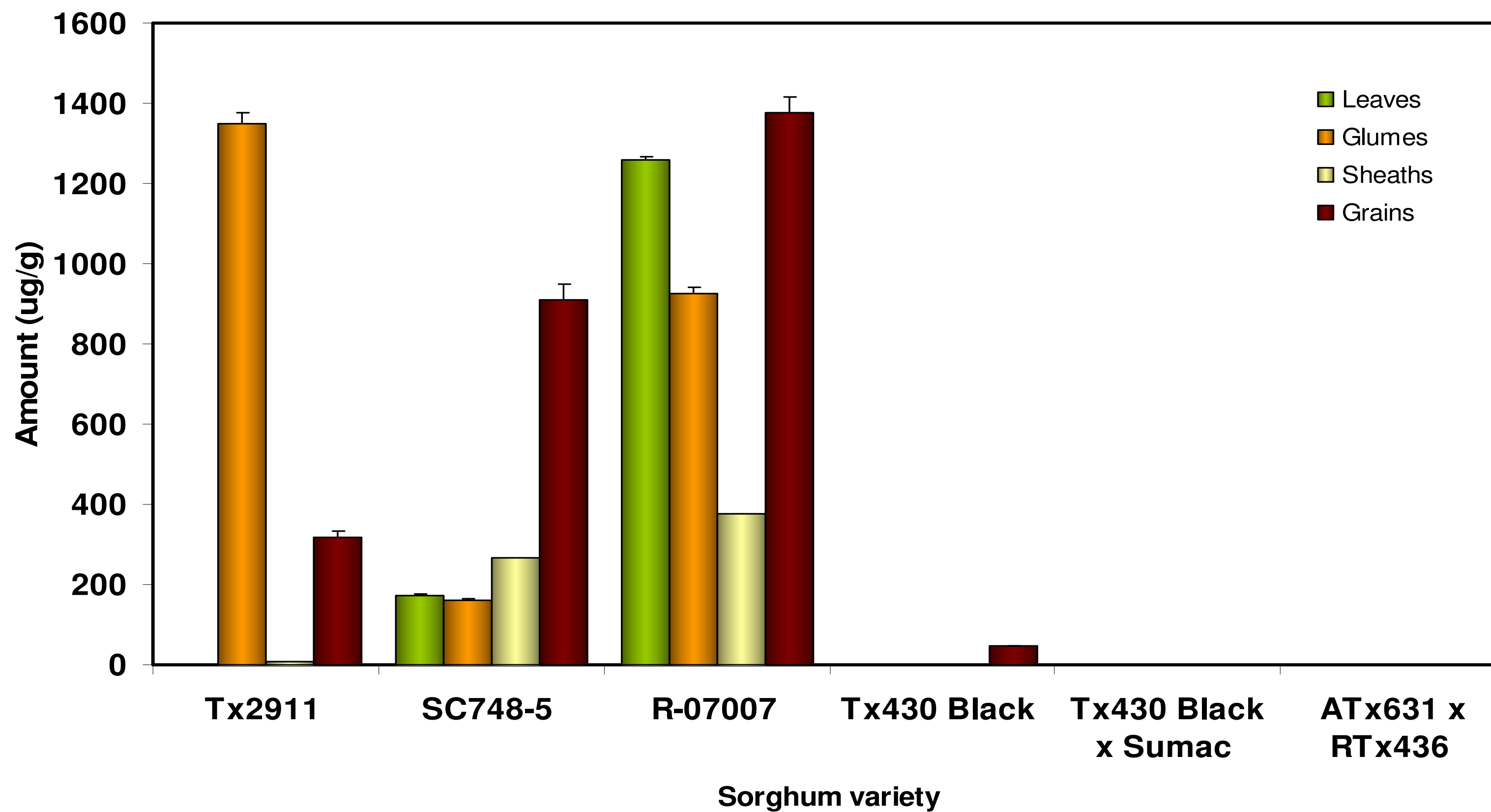


Fig. 52. Comparing Flavanones in different sorghum components grown in College Station TX, 2008. Dry weight basis.

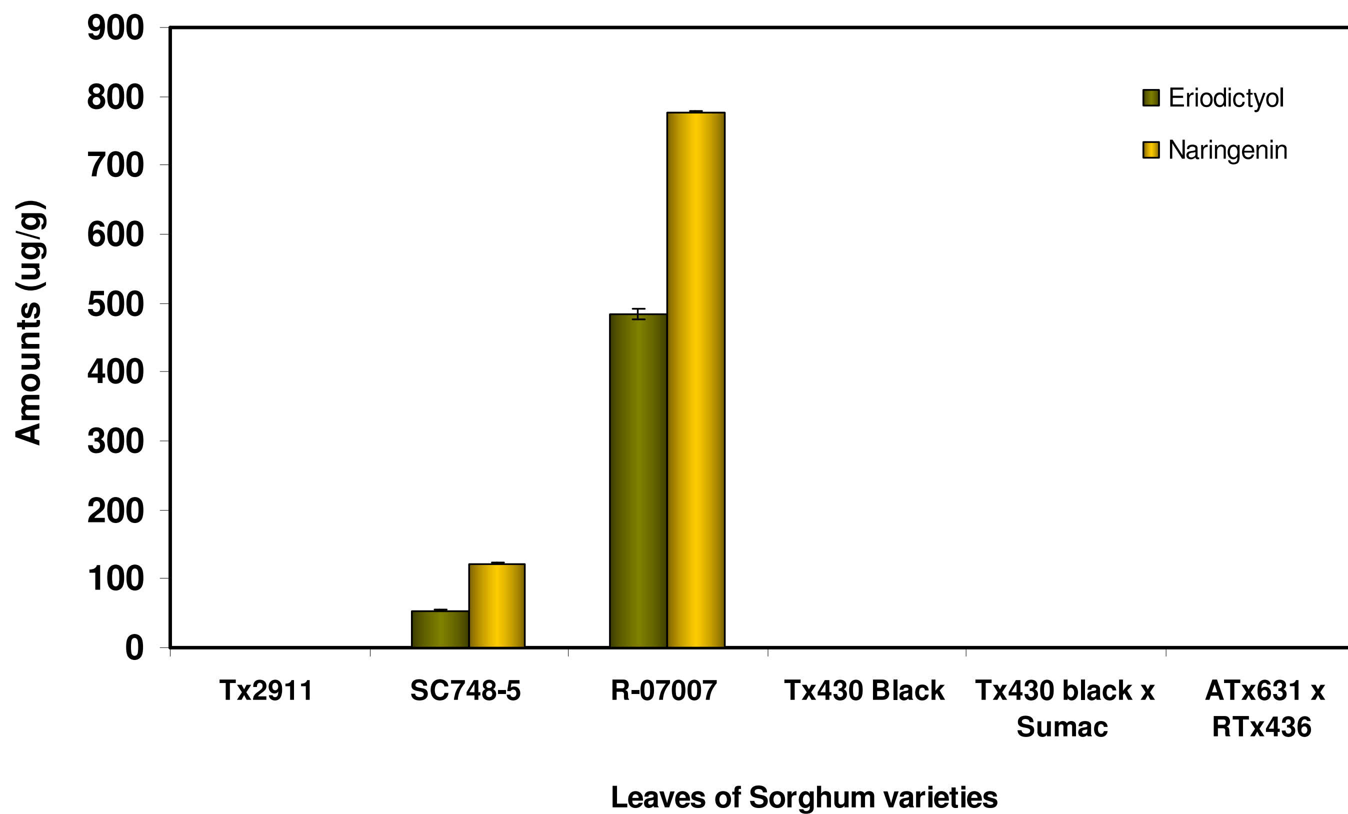


Fig. 53. Flavanones in leaves of sorghums grown in College Station TX, 2008. Dry weight basis.

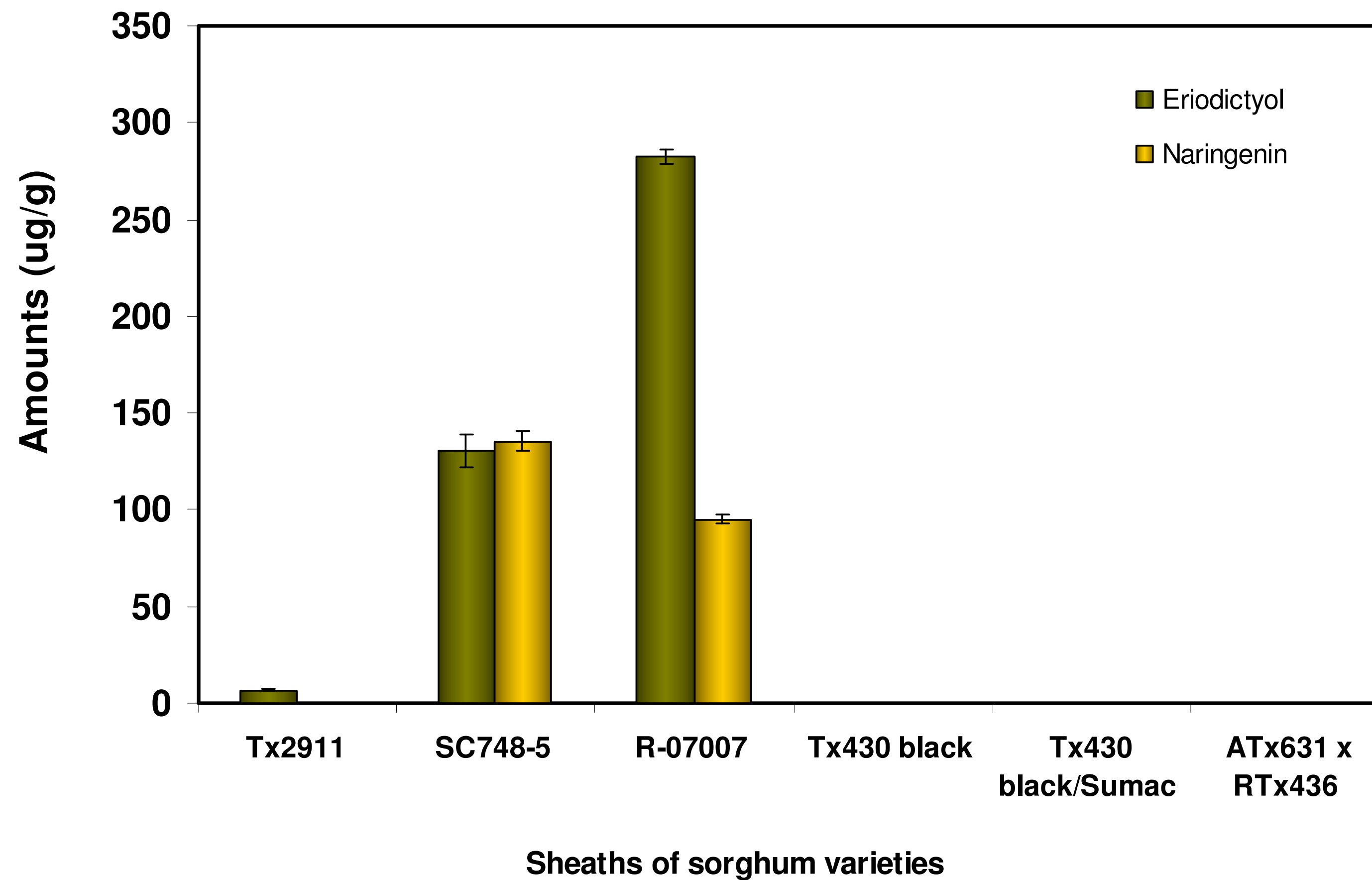


Fig. 54. Flavanones in sheaths of sorghums grown in College Station TX, 2008. Dry weight basis.

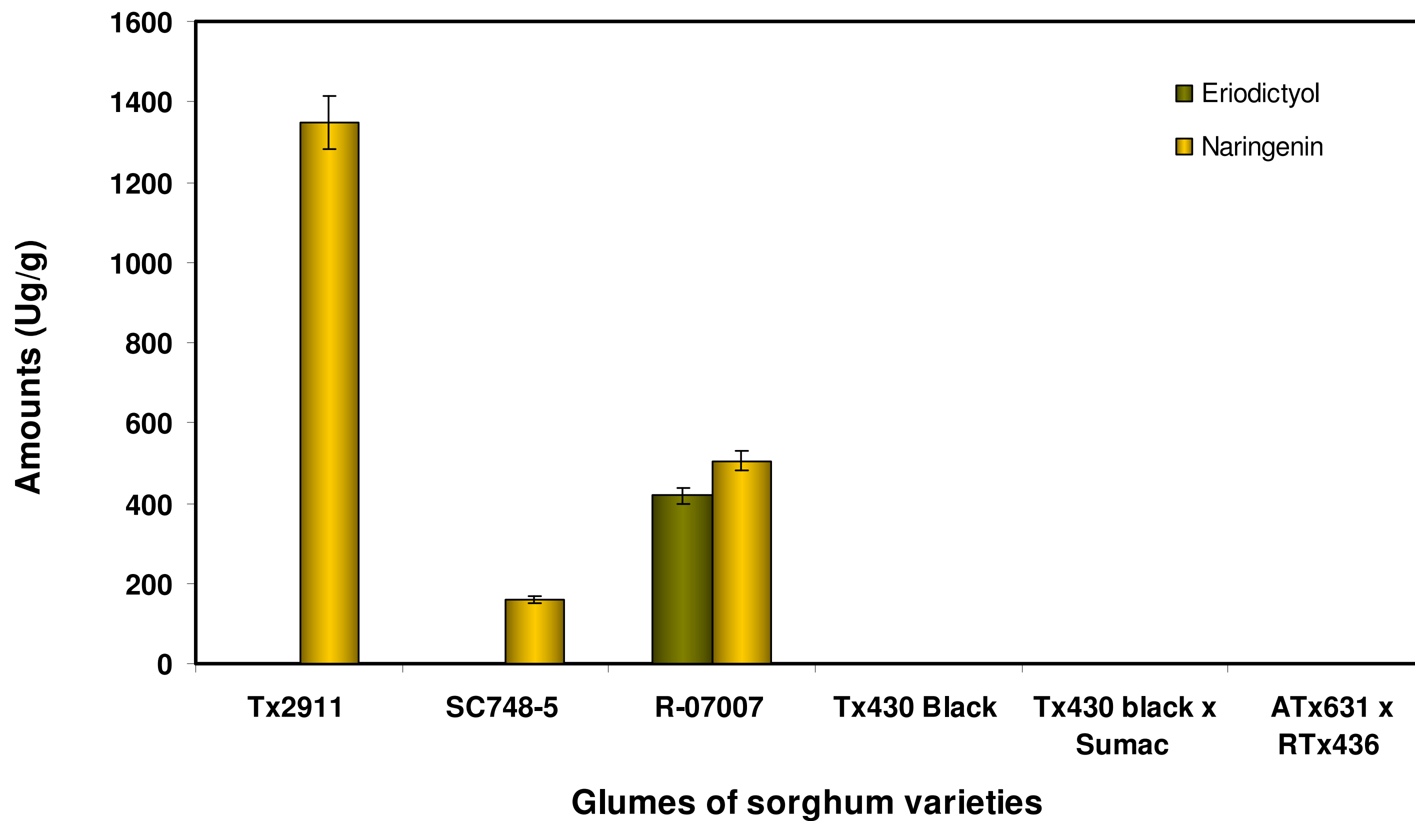


Fig. 55. Flavanones in glumes of sorghums grown in College Station TX, 2008. Dry weight basis.

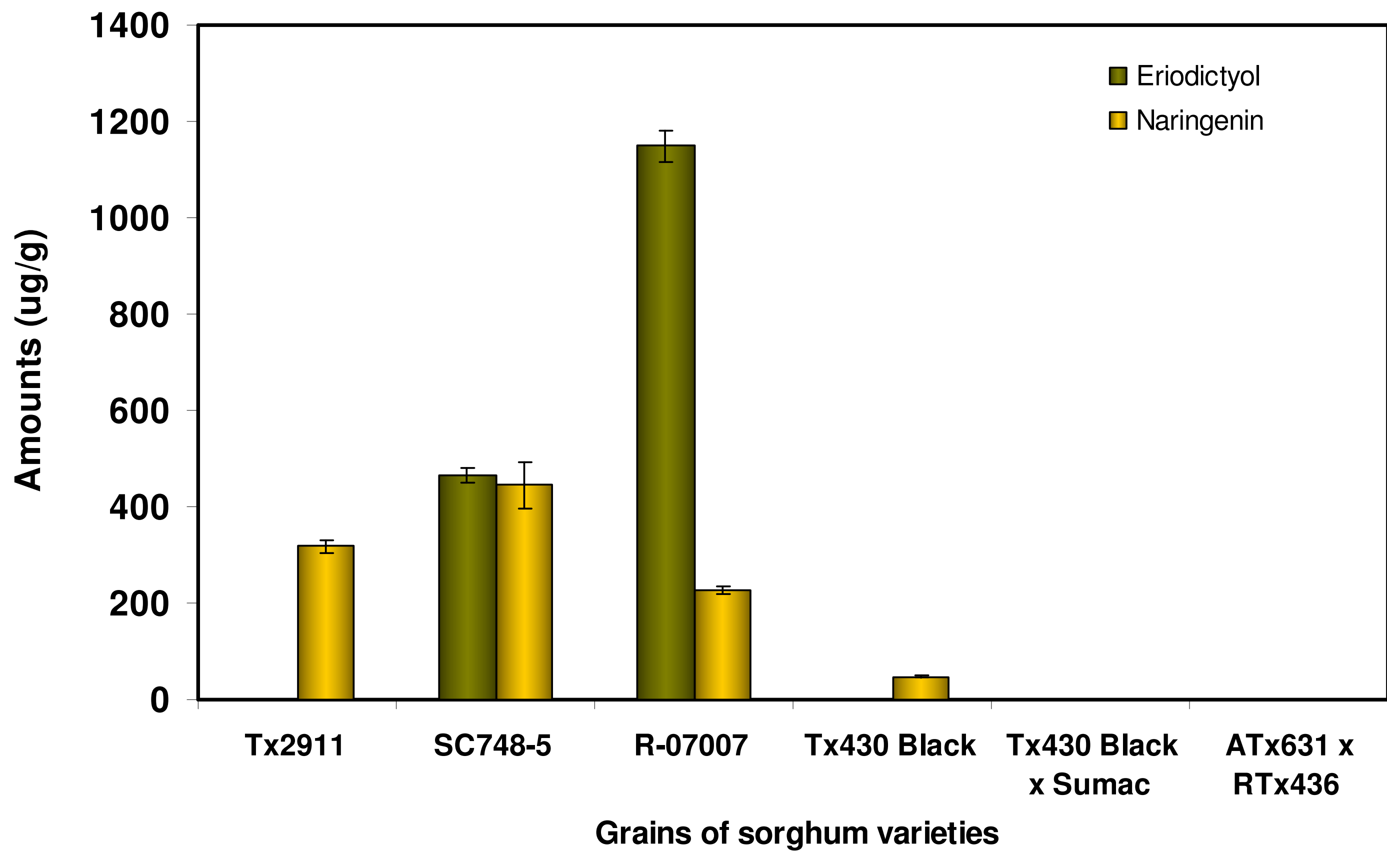


Fig. 56. Flavanones in grains of sorghums grown in College Station TX, 2008. Dry weight basis.

Grains of Tx2911 and Tx430 Black had only naringenin as detectable flavanone. The results for Tx2911 were in agreement with the results of Dykes (2008) who detected naringenin as the only flavanone in this variety at levels of 240 and 209 µg/g from different locations and different crop years.

The glumes and sheaths of R-07007 as well as the glumes of Tx2911 are viable sources of flavanones. These sources have flavanones as high as the lemon-yellow pericarp sorghums. With the exception of flavanones, sorghum leaves, sheaths and glumes are the most viable source of flavonoids compared to grains.

The grains and non-grain tissues of selected sorghum varieties are excellent sources of flavanones (Figs. 57 & 58). R-07007 grains are a viable source of eriodictyol, while the glumes of Tx2911 are a viable source for naringenin. The lemon-yellow pericarp sorghums are potent sources of flavanones as reported by Dykes (2008).

Each component had its unique profile and it was not evident that the profile of one component can be used to predict the flavanone profile of another. Tx430 Black x Sumac and ATx631 x RTx436 did not have detectable levels of flavanones in any component. There were no detectable flavanones in the stalks of Tx430 Black (purple plant), Tx2911 (red plant) nor ATx631 x RTx436.

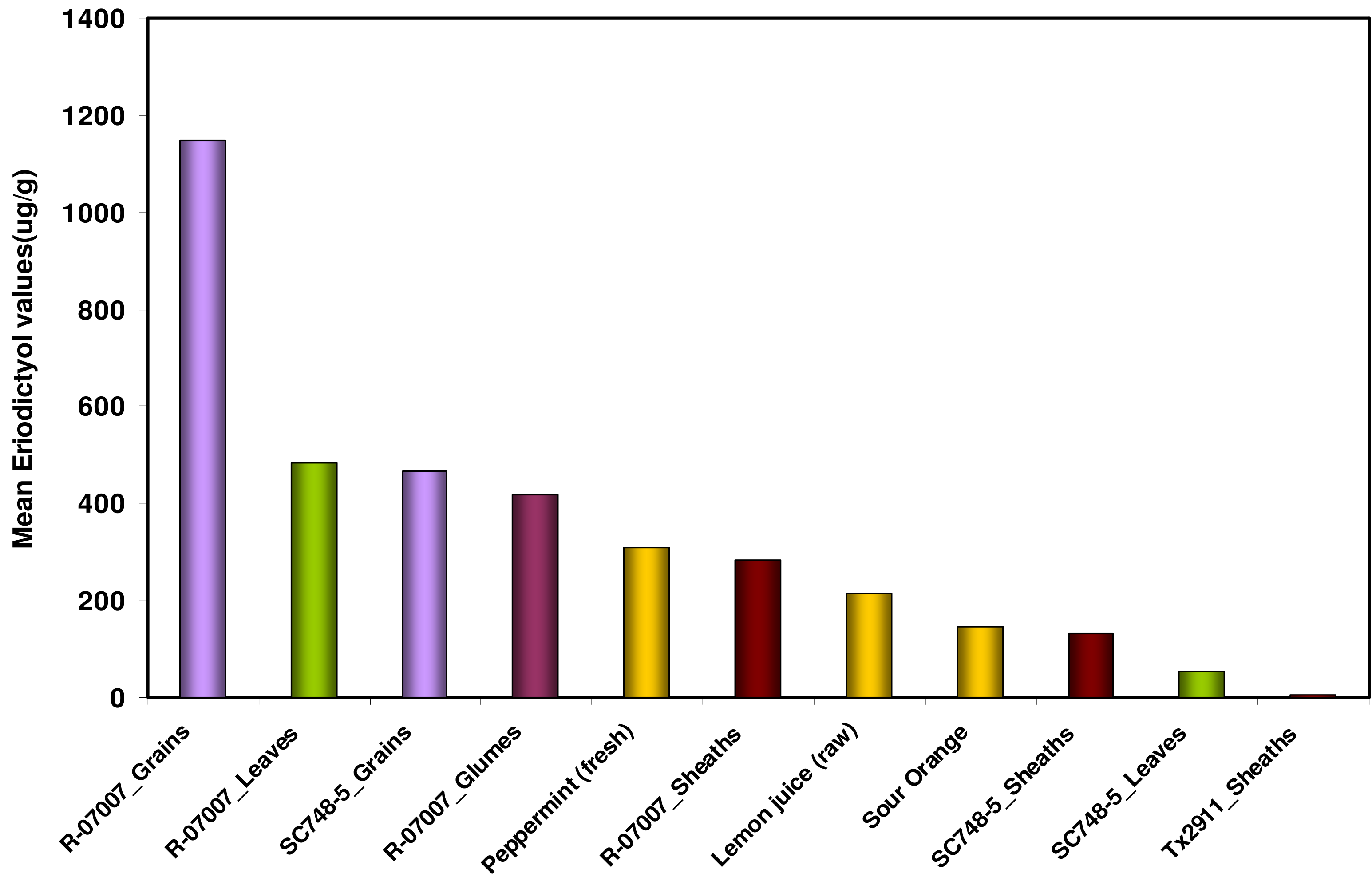


Fig. 57. Eriodictyol in sorghum components compared to common sources. (Data from USDA Database for the Flavonoid content of selected Foods, Release, 2.1).

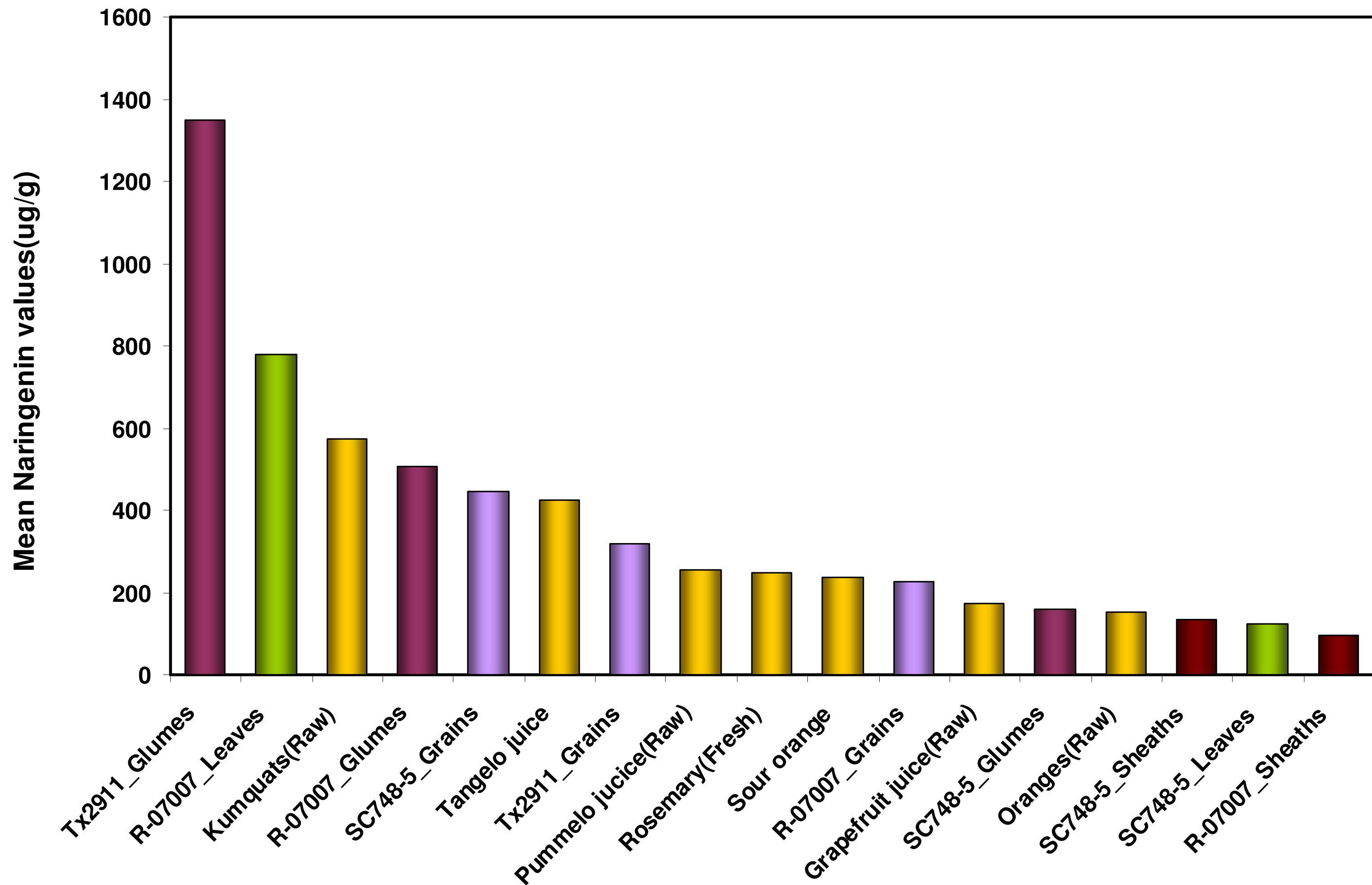


Fig. 58. Naringenin in sorghum components compared to common sources. (Data from USDA Database for the Flavonoid content of selected Foods, Release, 2.1).

Non-grain sorghum tissues are potential sources for all classes of sorghum flavonoids. These tissues provide sources of sorghum bioactive compounds up to between 78-170 folds more than grains in some varieties. This offers a possibility for concentrated amounts of sorghum bioactive compounds with high antioxidant capacity compared to their respective grains for large scale applications.

The levels of flavonoids in non-grain sorghum tissues compared favorably with common dietary sources (Figs. 48-49, 57 &, 58). There is a potential of sorghum biomass by-product of ethanol production as a source of raw material for these compounds. This will add value to the market potential of sorghum and its importance as source of dietary flavonoids with applications in food, nutraceutical, cosmetic and pharmaceutical industries.

CHAPTER V

OPTIMIZING EXTRACTION OF PHENOLIC COMPOUNDS FROM SORGHUM

Introduction

The commonly used solvent for extraction of phenolic compounds from sorghum is 1% HCl/methanol. The residues obtained after extraction with 1% methanol/HCl remain dark indicating incomplete extraction. Other solvents have been used for the extraction of phenolic compounds from fruits, vegetables and cereals with varying degrees of success. For example, aqueous acetone has been considered a good solvent for the extraction of procyanidins, anthocyanins and other phenols in fruits and vegetables (Kallithraka et al 1995; Garcia-Viguera et al 1998) although Lu and Foo (2001) observed significant anthocyanin interaction with aqueous acetone.

Awika et al (2004b) reported modification of the HPLC-spectral characteristic of 3-deoxyanthocyanins associated with formation of pyrano-3-deoxyanthocyanins and significantly lower levels of detectable 3-deoxyanthocyanins in sorghum when aqueous acetone was used.

Enzyme assisted extraction of phenolic compounds from fruits is utilized (Landbo and Meyer 2001; Buchert et al 2005; Kim et al 2005). Enzymes are used for biomass digestion in ethanol production to increase yield. The use of enzymes could provide an eco-friendly process for extraction of phenolic compounds from sorghum and from sorghum biomass co-products.

The objective of this work was to evaluate food-friendly extraction methods for use in nutraceuticals and food systems.

Materials and Methods

Tx430 Black and Black PI Tall grown in College Station, TX in 2001 and 2006 respectively were used. The black sorghum grains were decorticated to yield 15 % bran using a PRL dehuller (Nutama Machine Co., Saskatoon, Canada).

Reagents

Organic acids (citric acid, tartaric acid, acetic acid) and alpha (α)-amylase were obtained from Sigma (St. Louis, MO). Spezyme® CP and Optimash™ BG enzymes were provided by Genencor (Danisco Inc, NY), while Validase® AFP 1000L, Validase® ANC L, Validase® TRL and Validase® BNP L, were provided by Valley Research (Indiana, USA). All solvents were HPLC grade.

Extraction Using Different Organic Acids

Black sorghum brans were ground through a cyclotec mill (UDY Cor, Fort Collins, CO (0.5 mm mesh) prior to extraction. Acetic acid (AA), citric acid (CA) and tartaric acid (TA) at 0.5%, 1% and 5% in 70% aqueous ethanol and 1% HCl/methanol were used for extraction of phenolic compounds from black

sorghum bran. Citric acid at 0.5 % in 70 (30% water) and 100 % methanol and ethanol were also used.

For all analyses, 0.1 g of bran was weighed into centrifuge tubes, the solvents were added, and shaken for 2 hours at low speed on an Eberbach shaker (Eberbach Cor, MI). The extracts were centrifuged at 2790 x g for 15 minutes in a Sorvall SS-34 centrifuge (DuPont Instruments, Wilmington, DE) and then decanted. The supernatants were analyzed for total phenols and antioxidant capacity using the Folin Ciocalteu and ABTS methods respectively.

Extraction for HPLC Using Different Organic Acids

Ground samples (0.1 g) were extracted using 10 mL of 0.5%, 1% and 5% organic acids [acetic acid (AA), Citric acid (CA) and Tartaric acid (TA)], in 70% aqueous ethanol/30% water. 0.5 % CA in 70% aqueous ethanol and 100 % methanol and ethanol were also used. 1% HCl/methanol was used as a reference. Extraction and analyses were carried out as described in Chapter III (p. 47 & 52).

Enzyme Extraction

Ground samples (1.0 g dry weight) were extracted in a 20% slurry containing appropriate buffer solutions at optimum pH levels per manufacturers' specifications. Incubation was at the optimum temperature of the enzyme (Table 9). A second set of samples was extracted using different pH buffers (2, 3, 4.5

and 7.4) as controls. Extractions were done for 72 hours in a water bath while shaking. The enzyme activities were stopped by freezing the samples at -8°C.

Table 9
Enzymes and Their Optimum Conditions of Activity*

Enzymes	Optimum pH	Optimum Temperature
Spezyme® CP	4.5	55°C
Optimash™ BG	4.5	65°C
Validase® AFP 1000L	3.5	55°C
Validase® ANC L	4.5	55°C
Validase® TRL	4.5	55°C
Validase® BNP L	7.0	55°C

CP&TRL =Cellulases; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase ;BG= Beta Glucanses/Xylanase * Based on manufacturer's specifications

The samples were dried in a speed vac at ambient temperature. Samples were shaken in methanol for 1 hour, centrifuged at 2790 x g for 15 min; the supernatants were collected for colorimetric and HPLC analyses.

Colorimetric and HPLC Analyses

All colorimetric and HPLC analysis were conducted as described in Chapter III (p. 47 & 52)

Microscopy

Within 24 hours of extraction and/or enzyme treatment, the residues of Tx430 Black bran were placed on a slide with 50% glycerol/water prior to examination with a Zeiss Universal Microscope equipped with a 100-W mercury arc lamp and Zeiss Neofluor objectives as described by McDonough (1986). Nile Blue (0.01% w/v) in distilled water was used to stain neutral lipids in the epicarp layer and aleurone cells. The stain was applied to the surface of the slide, and viewed immediately.

Results and Discussion

Total Phenol Levels as Indication of Solvent Efficiency

Total phenol levels ranged from 14.0-17.5 mg GAE/g (Fig. 59). 1% HCl/methanol gave 15.0 mg GAE/g total phenols. Extracts from all combinations of CA, TA and AA in 70% ethanol gave total phenol levels comparable to using 1% HC/methanol. Tartaric acid extracted more phenols (16.3-17.3 mg GAE/g) than other acids.

The differences in total phenols extracted were not statistically significant for the solvents. Thus it is not cost effective to use high concentrations of organic acids for extraction of phenolic compounds from black sorghum bran. Although TA at 1 and 5 % extracted the most phenols (17.3 mg GAE/g) it is expensive and uneconomical compared to the other organic acids. 0.5% CA in 70% aqueous ethanol extracted compared favorably with 1% HCl/methanol.

Antioxidant capacity as indication of solvent efficiency

Antioxidant capacity of extracts using the individual solvents ranged from 50.6-156.2 µmol TE/g (Fig. 60). Extraction with 1% HCl/methanol gave the highest level of antioxidant activity. The antioxidant activities of extracts from all combinations of citric acid in 70 % aqueous ethanol compared favorably with antioxidant activity of extracts obtained with1% HCl/methanol.

As the levels of organic acid increased, the antioxidant activity decreased. At 5% in 70% aqueous ethanol all organic acids gave lower antioxidant capacity compared to 0.5 and 1 % acid in 70% aqueous ethanol.

CA is the most efficient of all the three organic acids tested. At 0.5% the antioxidant activity and the total phenols compared favorably with 1% HCl/methanol extracts. Citric acid is inexpensive and cost effective if used in large scale extractions.

Comparing Extraction Efficacy Based on 3-Deoxyanthocyanins

Organic acids in 70% ethanol gave about 50% less 3-deoxyanthocyanins than 1% HCl/methanol (3474.2 ug/g). TA and CA at 0.5, 1 and 5% in 70% aqueous ethanol extracted more 3-deoxyanthocyanins than acetic acid. No significant differences were observed in the total levels of 3-deoxyanthocyanins extracted by similar levels of CA and AA but TA extracted larger amounts (Fig. 61). Overall at all levels, AA extracted less than CA and TA. No significant difference exists between the amounts of 3-deoxyanthocyanins extracted by TA and CA.

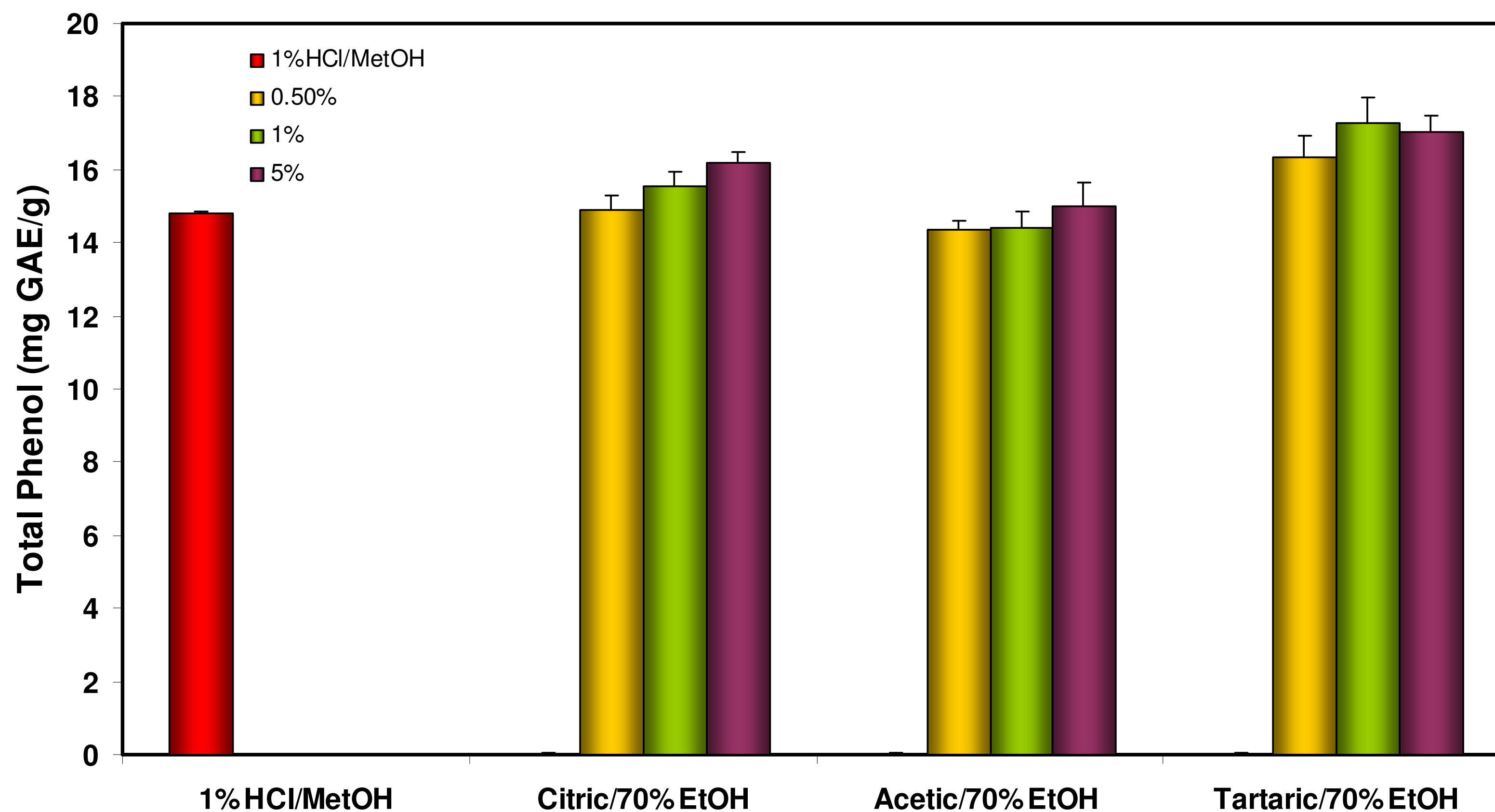


Fig. 59. Total Phenols from Tx430 Black sorghum bran using different extraction solvents 70% EtOH= 70% ethanol: 30%H_2O (70%aqueous ethanol).

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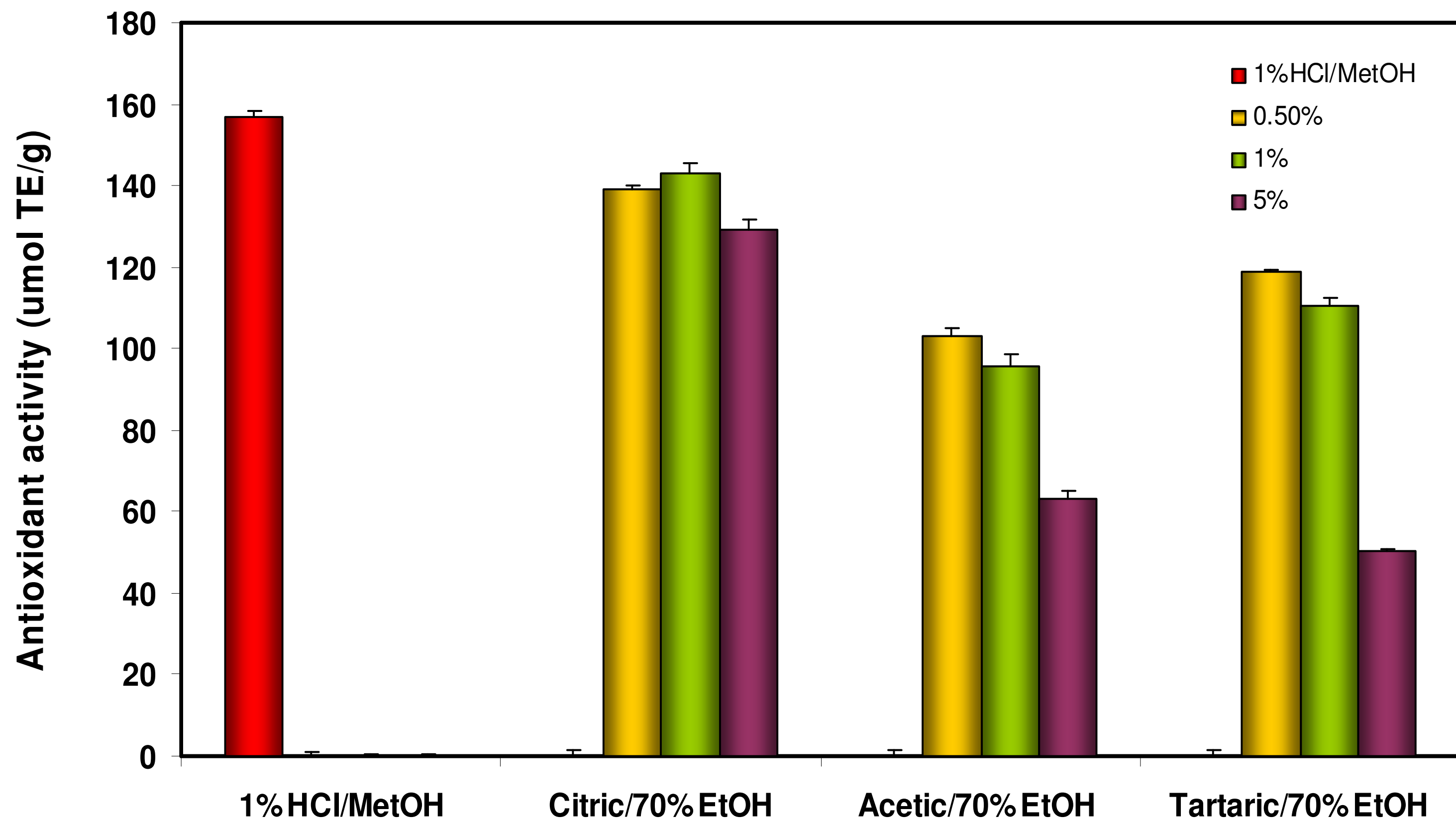

Fig. 60. Antioxidant activity (ABTS) of Tx430 Black sorghum bran using different solvents. 70% EtOH= 70 ethanol %:30% H_2O (70%aqueous ethanol).

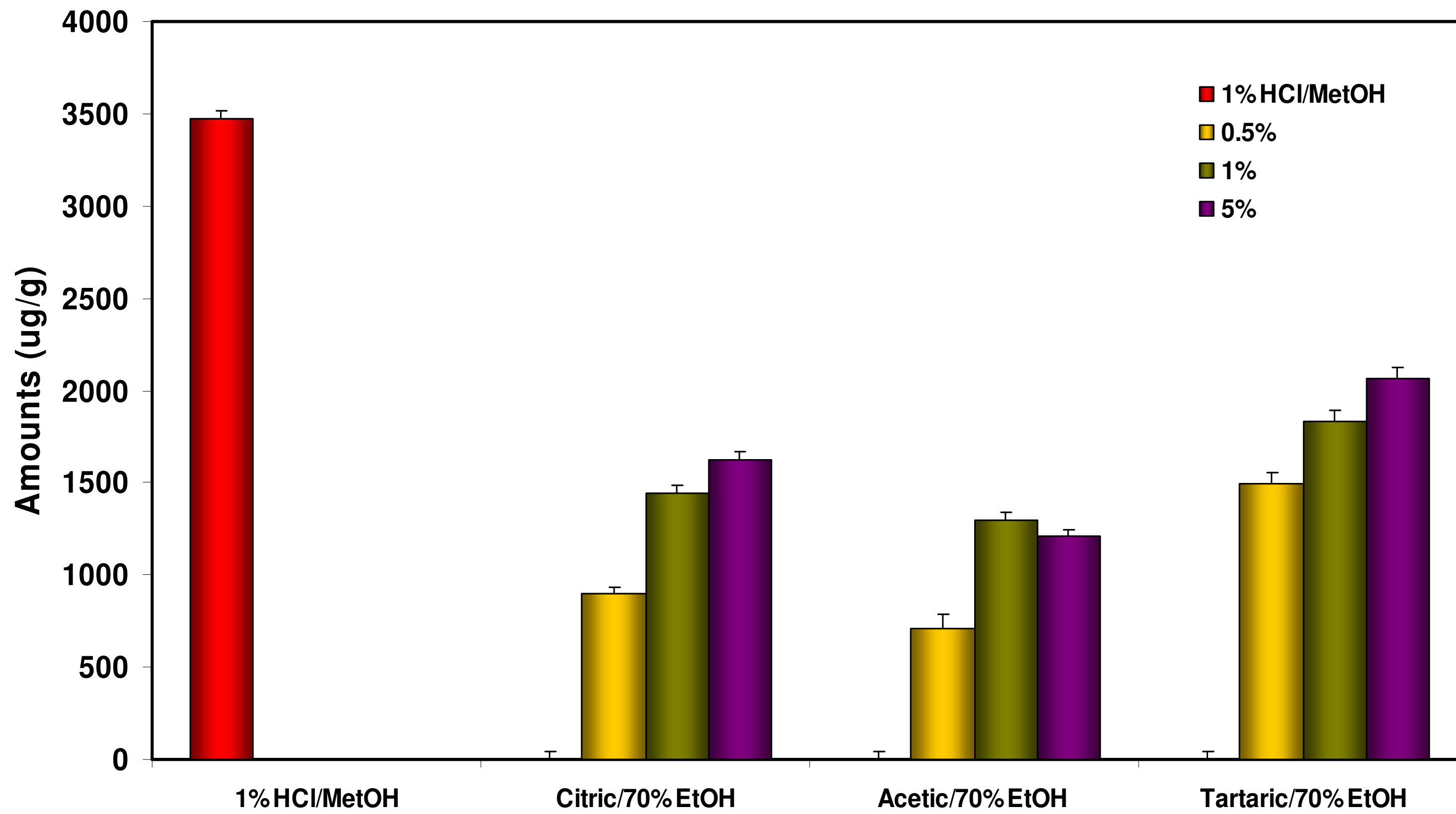

Fig. 61. 3-Deoxyanthocyanins in Tx430 Black sorghum bran using different levels of acid and solvent combinations .

3-Deoxyanthocyanin profiles and proportions from extracts of 1% HCl/methanol were not different from 3-deoxyanthocyanin profiles in extracts obtained with all the other acid/solvent combinations (Fig. 62). This suggests that any of these acids/solvent combinations can be used as a substitute for 1% HCl/MetOH providing a more food friendly extraction solvent for phenolic compounds from sorghum.

Flavone Levels

Flavone levels in the extracts ranged from 147-219 ug/g (Fig. 63). Flavone levels in extracts using various levels of acids (0.5, 1 and 5%) had no significant differences across all the acids used, except when 1% HCl/ methanol were used. Increasing acid levels did not increase levels of extracted flavones. Although TA extracted slightly more flavones than citric acid at 0.5%, CA.

The flavones in extracts from all levels of acid/ solvent combinations were similar to levels obtained with 1% HCl/ methanol (Fig. 64). 0.5 % CA in 70% aqueous ethanol was proposed as a food friendly solvent for the extraction of 3-deoxyanthocyanins from sorghum, however the use of food grade 1%HCl/ethanol might provide a more valuable substitute for 1%HCl/methanol.
At 0.5%, CA in 70% aqueous EtOH compares favorably with 1%HCl in methanol in the extraction of phenolic compounds from black sorghum bran. The quantities of flavonoids extracted with the use of 0.5% CA was about half those obtained with 1% HCl/methanol.

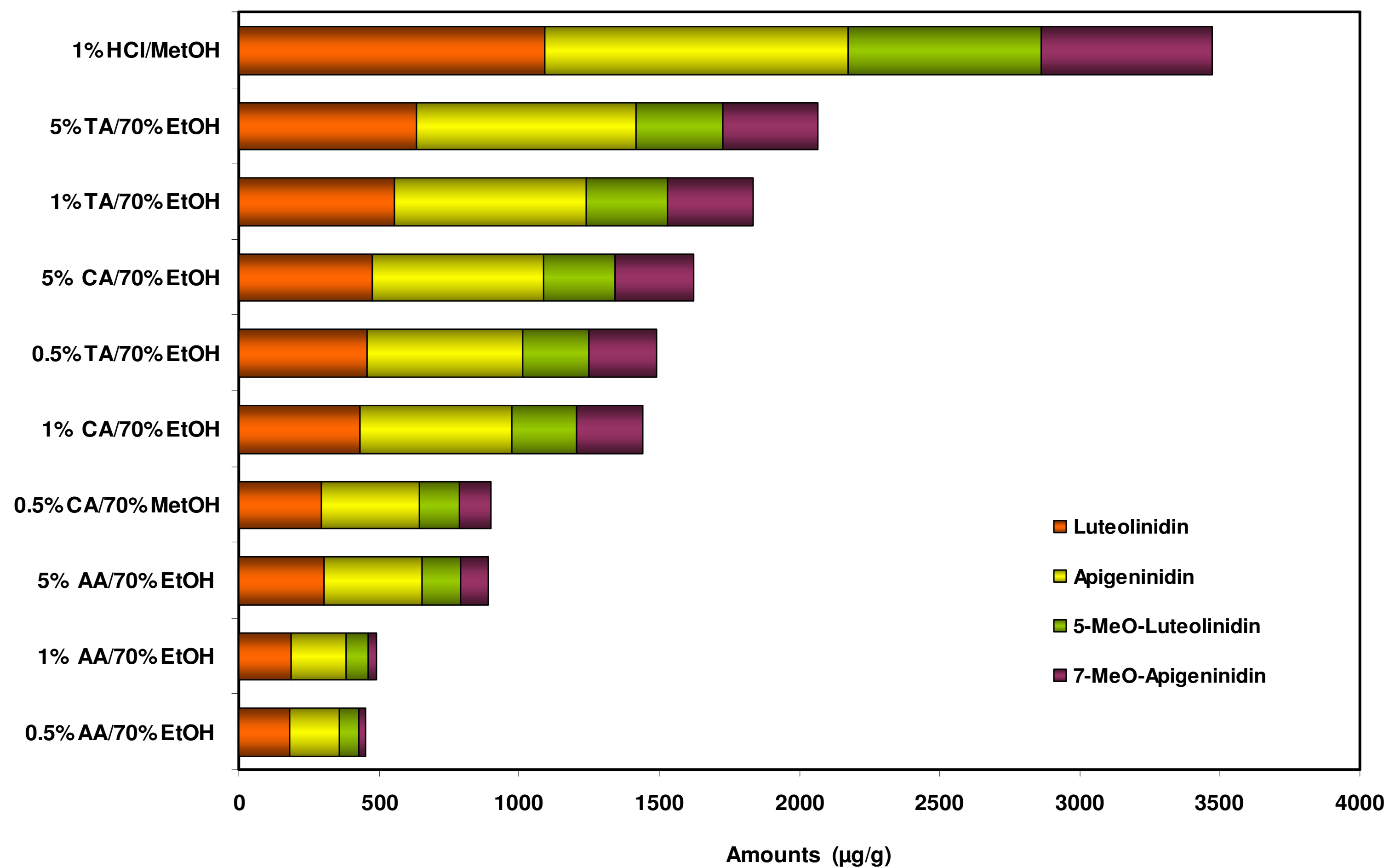

Fig. 62. 3-Deoxyanthocyanin profile of Tx430 Black sorghum bran using different levels of acid and solvent combinations. CA=Citric acid, TA=Tartaric acid, AA=Acetic acid .

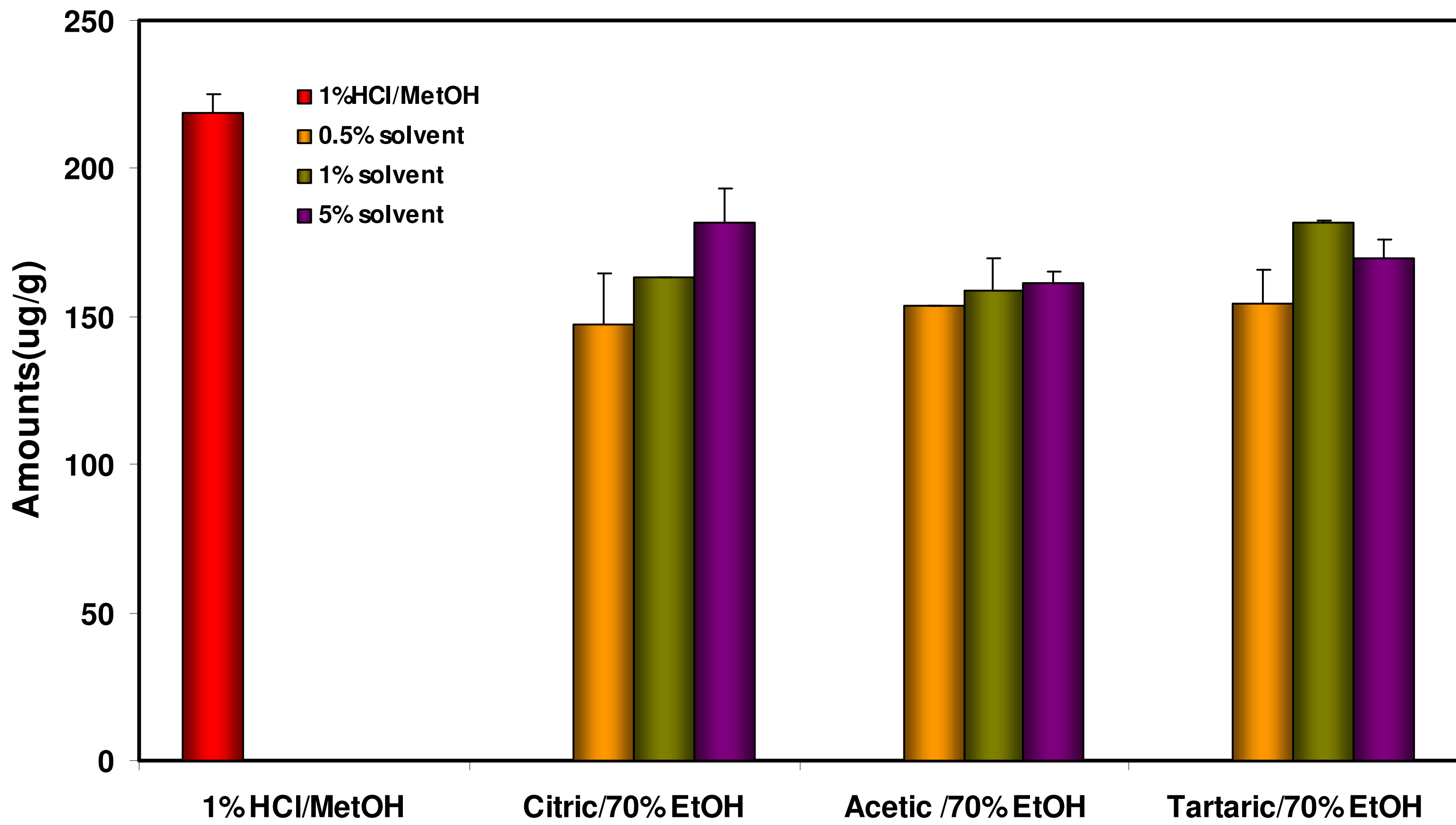

Fig. 63. Flavone levels in Tx430 Black sorghum bran using different levels of acid in 70% ethanol compared to 1 % HCl / methanol.

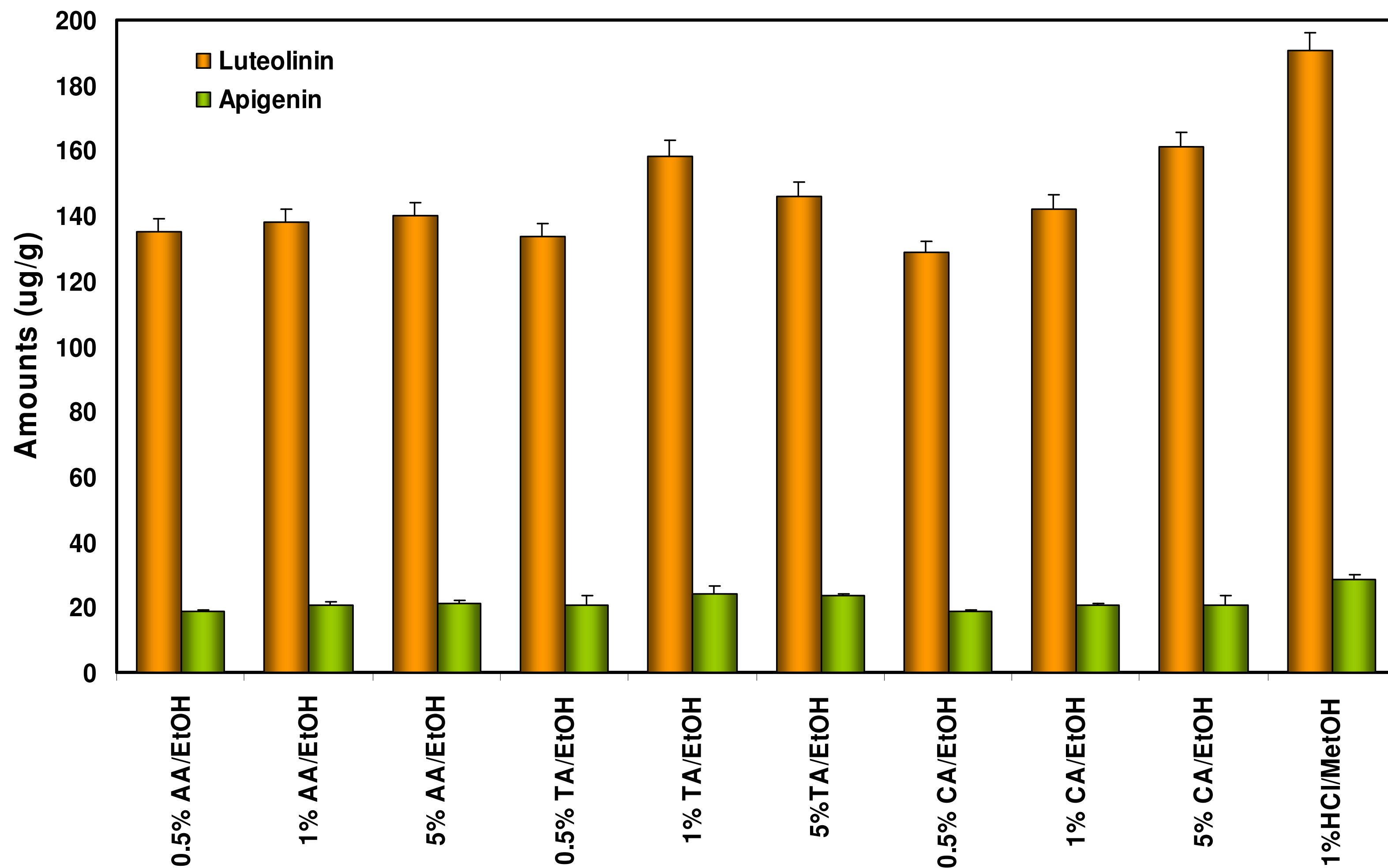

Fig. 64. Flavone profile in Tx430 Black sorghum bran using different extraction solvents. AA= Acetic acid, TA=Tartaric acid, CA=Citric acids.

However, methanol is not acceptable in food preparation. Hydrochloric in ethanol might be a better solvent for food systems.

The profiles of 3-deoxyanthocyanins and flavones (Figs. 62 & 64) obtained from both solvents also suggest that CA can be used in place of HCl for extraction of phenolic compounds from sorghum to obtain the same compounds extracted by 1% HCl/methanol although the quantities are reduced (Figs. 65 & 66).

The use of citric acid in ethanol, like 1% HCl in methanol did not completely extract the phenolic compounds in black sorghum bran. The residues obtained from both extraction solvents still remained black suggesting that more work still needs to be done to determine the most effective solvent for the extraction of phenolic compounds from sorghum grains and brans.

Validation of Extraction Solvent

After a number of runs it was evident that extraction using food acids in 70% aqueous ethanol was more efficient than using 100% ethanol (Fig. 67). 0.5% citric acid in 70% aqueous ethanol (CA/70% in EtOH) was selected as the best combination for use in subsequent experiments because it was more cost effective than the other combinations. Higher concentrations of citric acid were inefficient in extracting phenolic compounds. The phenolic compounds were generally difficult to extract as the residues still remained dark after all extractions. The brans of Black PI Tall and Tx430 Black were used to verify the

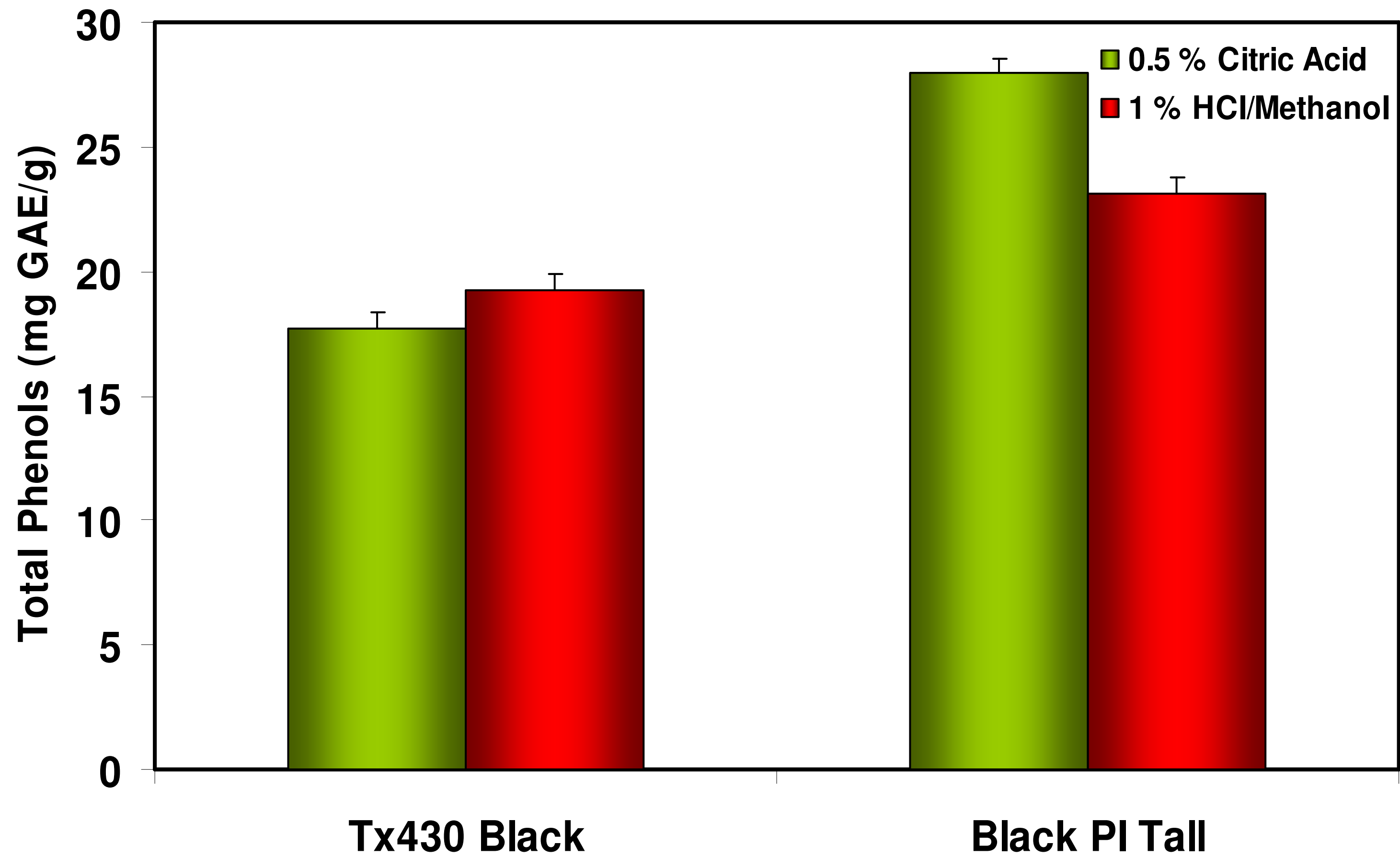

Fig. 65. Extractability of total phenols from black sorghum brans using of 0.5% CA / 70% aqueous ethanol and 1% HCl/ methanol.

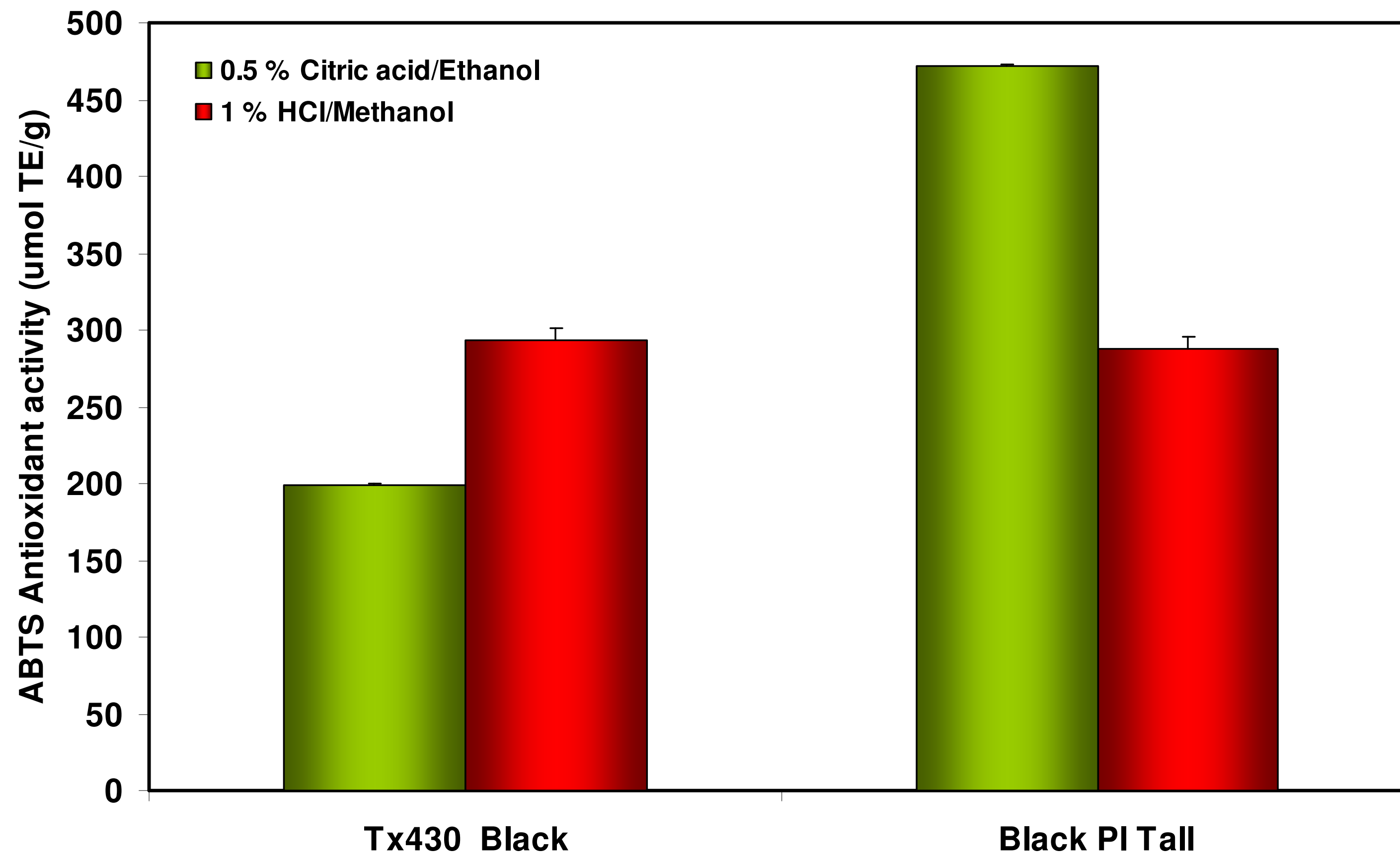

Fig. 66. Antioxidant activity of black sorghum brans extracted with 0.5% Citric acid/ 70% aqueous ethanol and 1% HCl/ methanol.

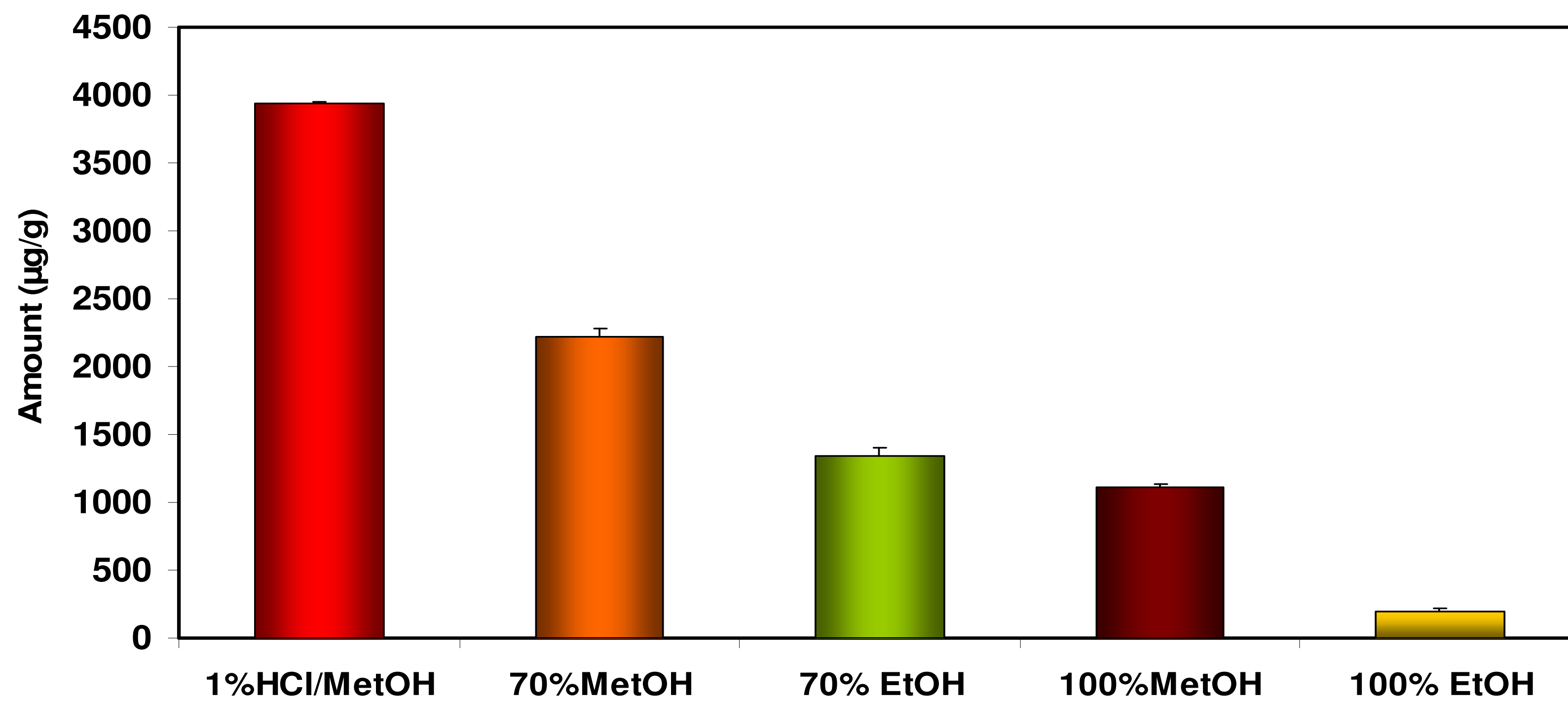

Fig. 67. 3-Deoxyanthocyanins in Tx430 Black sorghum bran using 0.5% CA in methanol and ethanol.

potential of 0.5 % CA in 70% aqueous ethanol for extraction of phenols from sorghum. Total phenols (Fig. 65) and antioxidant activity (Fig. 66) obtained from the brans were similar to those obtained with extracts using 1% HCl/methanol. These confirmed previously discussed results. This extraction solvent was used in determining the stability of 3-deoxyanthocyanins from black sorghum bran under different processing conditions (Cárdenas-Hinojosa 2007).

The 3-deoxyanthocyanin profile was similar to 1% HCl/ methanol extract profile and the chromatogram from Tx430 Black and Black PI Tall extracted with 0.5% Ca/70% aqueous ethanol contained luteolinidin (1), apigeninidin (2), 5-methoxyluteolinidin (3) and 7-methoxyapigeninidin (4) (Fig. 68).

Effects of Storage on Extractability of Phenolic Compounds from Black Sorghum Bran

Tx430 Black sorghum bran, decorticated and stored for 3 years at 8°C and fresh bran from the same sorghum grain stored in a freezer were extracted with 1% HCl/methanol (Fig. 69). Total phenols were lower in the stored bran compared to fresh bran when both acidified methanol and methanol were used. Reduction in total phenols was 47 and 50% for 1% HCl/methanol and methanol respectively. Antioxidant activity of stored Tx430 Black bran was 60% lower compared to the fresh bran using 1% HCl/methanol (Fig. 70).

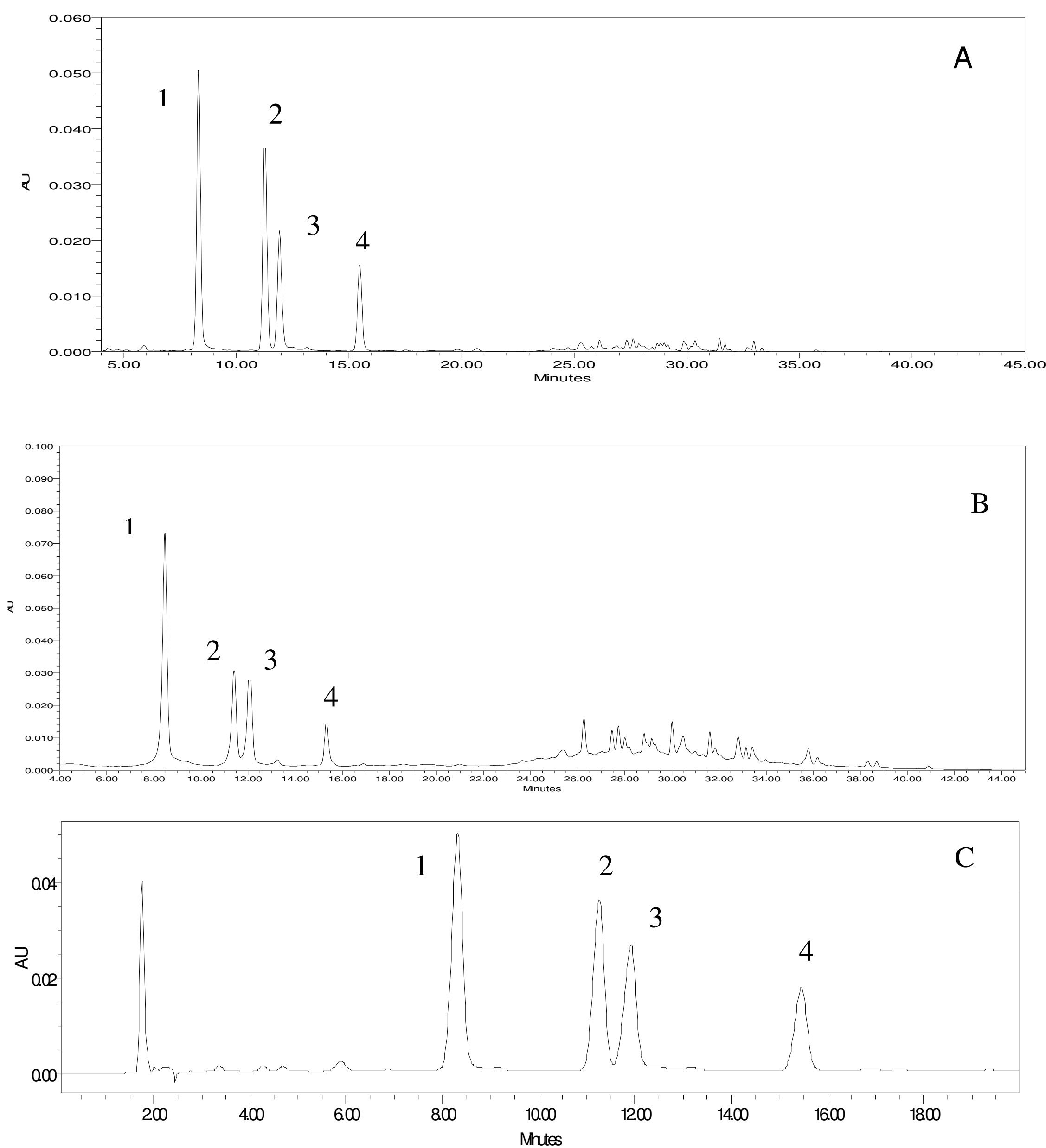

Fig. 68. HPLC chromatograms of A= Tx430 Black bran; B= Black PI Tall bran using 0.5% Ca/70% aqueous EtOH; C=Tx430 black bran using 1% HCl/MetOH at 485 nm. CA=citric acid; 1=Luteolinidin; 2 =Apigeninidin; 3= 5-MethoxyLuteolinidin; 4=7-MethoxyApigeninidin.

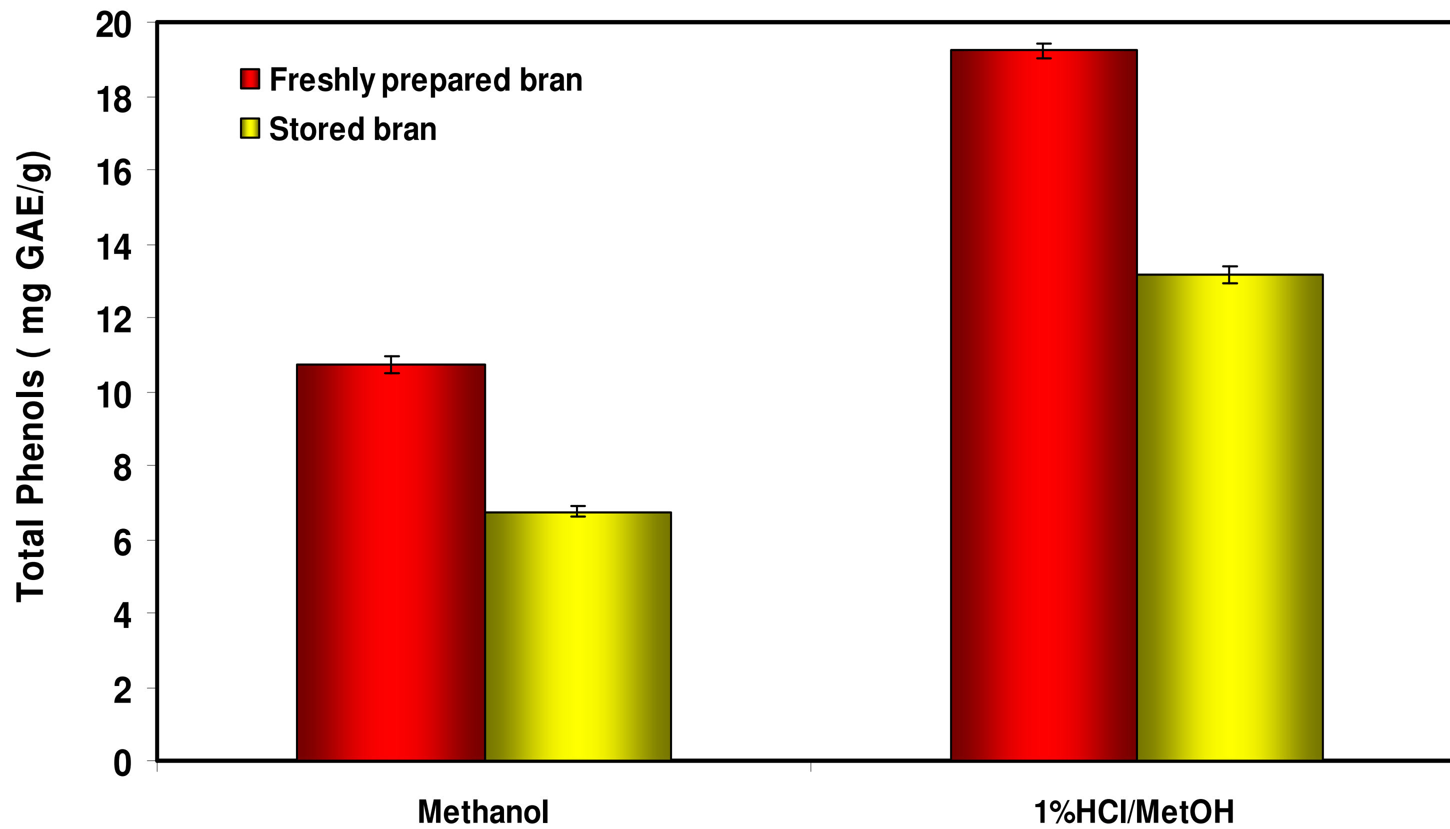

Fig. 69. Total phenols of Tx430 Black bran stored for 3 years compared to freshly decorticated bran.

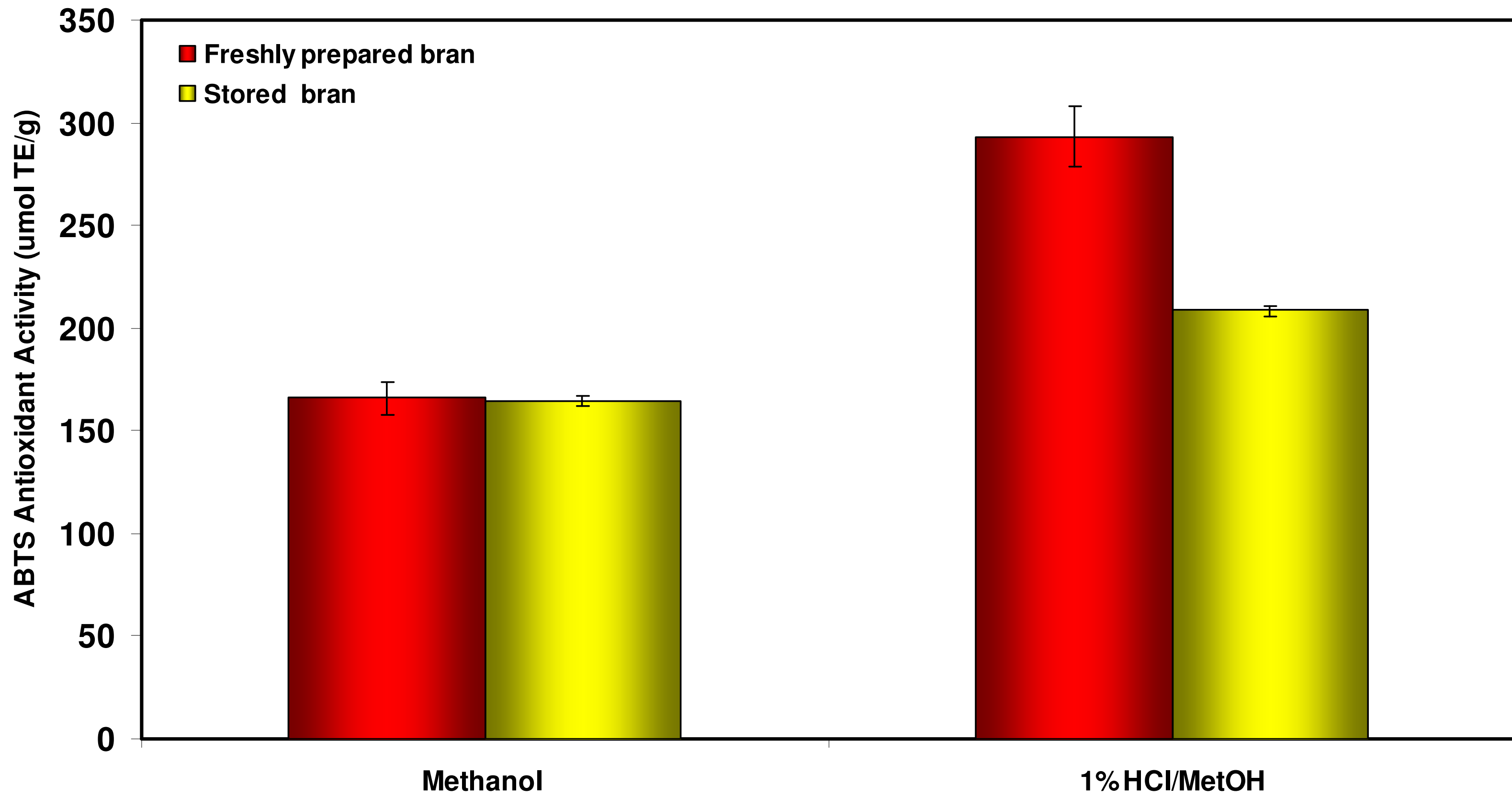

Fig. 70. Antioxidant Activity of Tx430 Black bran stored for 3 years compared to freshly decorticated bran.

More effective methods of disrupting the cell wall components need to be established to enable proper quantification of phenolic compounds in sorghum grain and bran. Storage of bran results in break down, deterioration or binding of phenols. Decortications might release or activate phenol catabolizing enzymes present in sorghum such as polyphenol oxidases (PPO) and peroxidases (POX). Mayer and Harel (1979) reported two types of PPO activities in sorghum: the monophenolase activity located in the endosperm and the o-diphenolase activity localized in the pericarp. PPO and POX affect post harvest quality of foods by causing degradation (browning) and the development of off-flavors in raw and un-blanched cereals (Marsh and Galliard, 1986; Hatcher and Kruger, 1993). These enzymes are capable of modifying phenolic compounds in sorghum and other cereals during processing (Dicko et al 2006). The appearance of colored products in wheat flour is attributed to the oxidation of endogenous phenols by PPO (Marsh and Galliard, 1986; Hatcher and Kruger, 1993). Sorghum bran should be processed as needed and cannot be stored for a long time for optimum performance.

Enzyme Assisted Extraction of Phenolic Compounds from Sorghum

The unit of activity of each enzyme was calculated from the respective enzyme activity and the specific gravities specified by the manufacturer (Table 10).

Table 10
Enzyme Activity Levels Calculated Based on Manufacturer's Specification

Volume (µl)	ANC-L	AFP	BG	BNP-L	CP	TRL
1000	140	8	79	17	26	54
800	112	6	63	14	15	43
600	84	5	47	10	20	32
400	56	3	32	7	10	22
200	28	2	16	3	5	11

CP&TRL =Cellulases ; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase ; BG= Beta Glucanses/Xylanase

Total Phenols Extracted with Enzymes

Upon treatment with six different enzyme systems, all extracts had an intense color with AFP treated samples showing the highest intensity. Enzyme treated samples had higher levels of total phenols than samples extracted at different pH buffers without enzymes (Fig. 71). The total phenol levels increased as the enzyme unit increased.

Bran incubated with proteases resulted in higher total phenol values. For example, AFP produced 350-725% increased in total phenol levels compared to the control, BPN gave 175-650% increased and CP and TRL gave 275-475 and 150-300% increased respectively compared to the controls. This suggests that the phenolic compounds in sorghum are mainly associated with protein and cellulose.

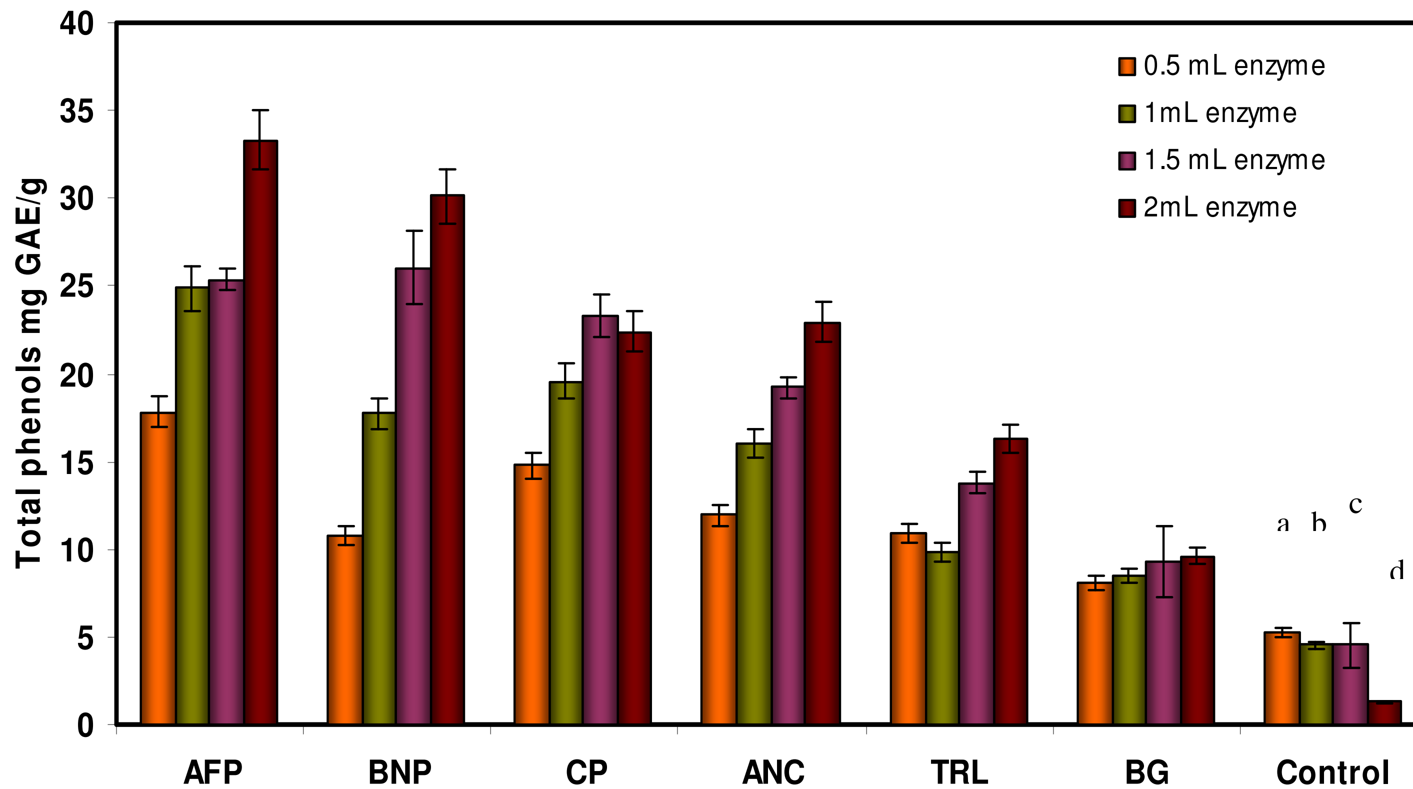

Fig. 71. Effect of varying enzyme units on total phenols of Tx430 Black sorghum bran. CP&TRL =Cellulases; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase; BG= Beta Glucanase. Control were without enzymes but different pH levels (a= 3, b=4.5 ,c=7.4 and d=distilled water) . Table 8 =Enzyme activity units.

Sorghum phenols are concentrated in the pericarp (Awika et al 2003a). 10% decorticated Sorghum bran has 30% starch, 18% hemicelluloses, 11% cellulose, 11% protein, 10% crude fat and 3% ash (Corredor et al 2007). The starch in bran is from the endosperm during the decortication process. An enzyme complex (ANC) with cellulase and hemicellulase gave 200-475 % more phenols while beta glucanase gave total phenols of 150% more compared to the controls. Sorghum contains very little beta-glucans. Cellulases, hemicellulases and proteases will increase phenols extractability from sorghum, by breaking the association between phenols and the cell wall components.

Distilled water gave the lowest total phenols. This confirms that the extraction of phenolic compounds from sorghum is different from fruits and vegetables where water is used to extract phenolic compounds. Because of the structure of sorghum cell walls, an aggressive method is required to extract phenols. That is why 1% HCl in methanol has been used for the extraction of phenols from sorghum.

1% HCl in methanol gave 16 mg GAE/g of phenols. Extraction with cellulase and proteases gave about 45-106 % more phenols. When phenols were extracted in sequence, first by extracting with 100% methanol, followed by the enzyme systems acting on the residues, about 300 % increased in total phenols was obtained compared to the control (Fig. 71). Enzymes had an effect on the extractability of phenols from sorghum bran.

Overall it is difficult to determine if the values by the Folin-Ciocalteu assay for sorghum phenols extracted were truly total phenols because HPLC analysis of the extracts did not show any of the peaks expected in black sorghum bran extracts.

Antioxidant Activities from Enzyme Extraction

The ABTS antioxidant activity of Tx430 Black bran extracted with enzymes gave 72-770 % higher values than samples extracted with different pH solutions as controls. ANC a cellulose/hemicellulase complex gave 390-647% increased and TRL a cellulase gave 304-471% increased compared to the controls. BNP gave 373-770% more antioxidant activity compared to the control. BG and AFP had the least effects compared to the other enzymes with only 97-141% and 72-120% increase respectively compared to the controls. Values increased as the enzyme activity units increased (Fig. 72).

Sequential extraction using 100% methanol followed by enzymes gave antioxidant activity resulted in 227-263 % increased in antioxidant activity resulted in 227-263 % increased in antioxidant activity compared to the controls. The increased levels of antioxidant activity for enzyme treated samples suggest that phenolic compounds contributing to antioxidant activity were present in the extracts and certainly more were released by the enzyme treatment. The fate of the phenolic compounds after leaching is still unknown. Further work needs to be done to understand the fate of these compounds.

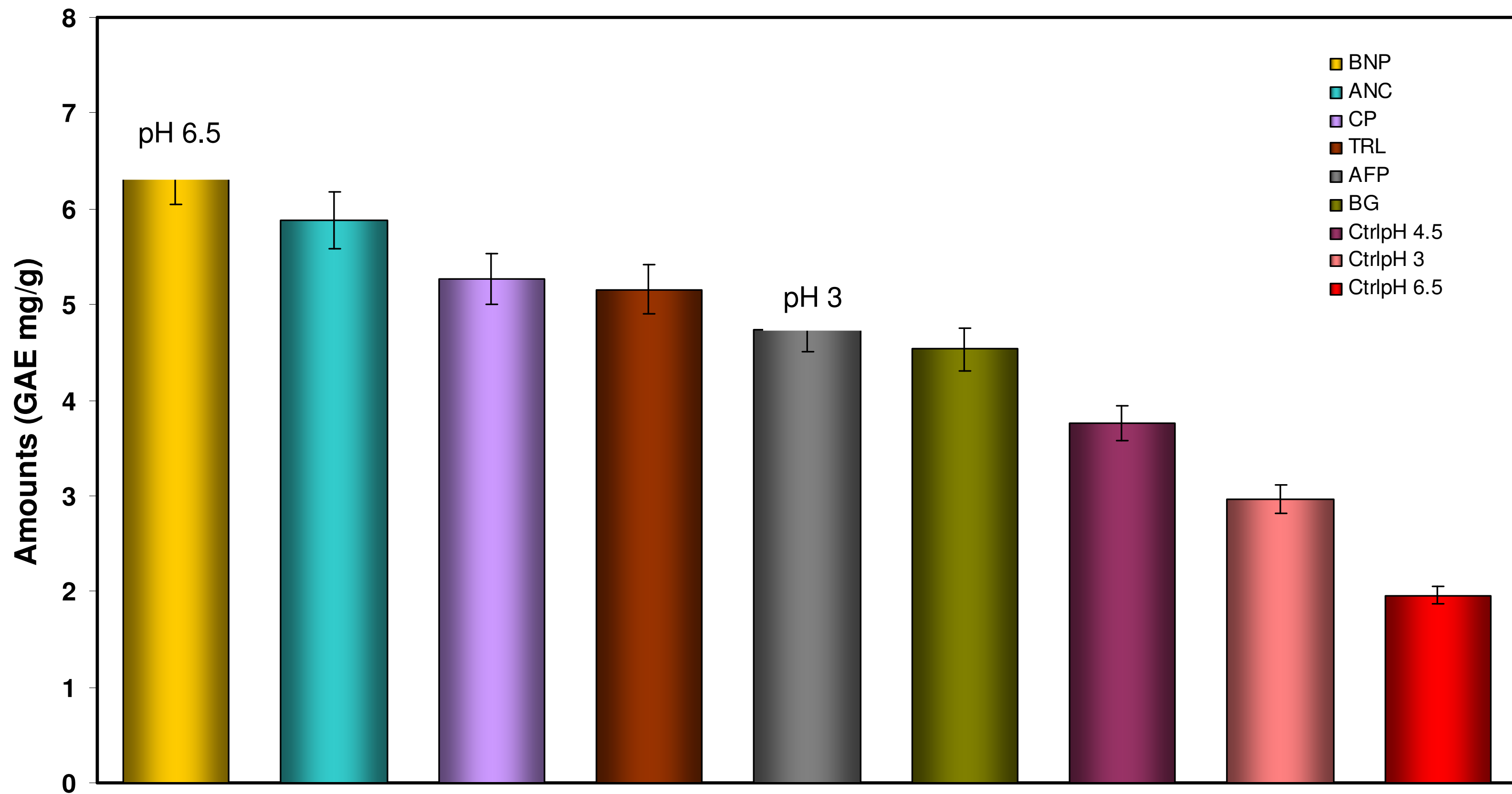

Fig. 72. Total Phenols in Tx430 Black sorghum bran extracted with enzymes. CP&TRL =Cellulases; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase ; BG= Beta Glucanase. Table 8 =Enzyme activity units.

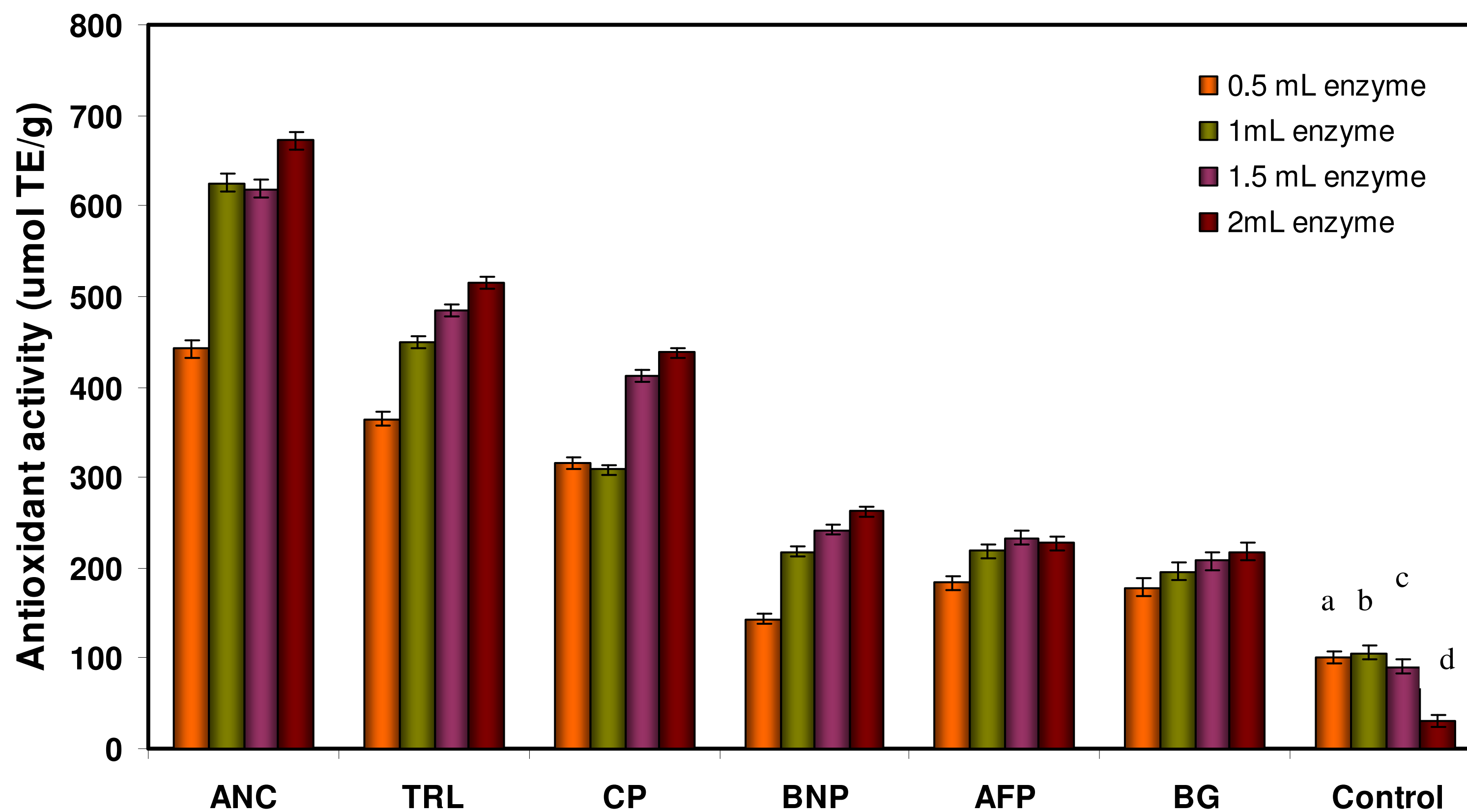

Fig. 73. ABTS of Black sorghum bran from different enzyme assisted extractions. CP&TRL =Cellulases ; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase ; BG= Beta Glucanse. Control were without ezymes but different pH levels (a=3, b=4.5 ,c=7.4 and d=distilled water).

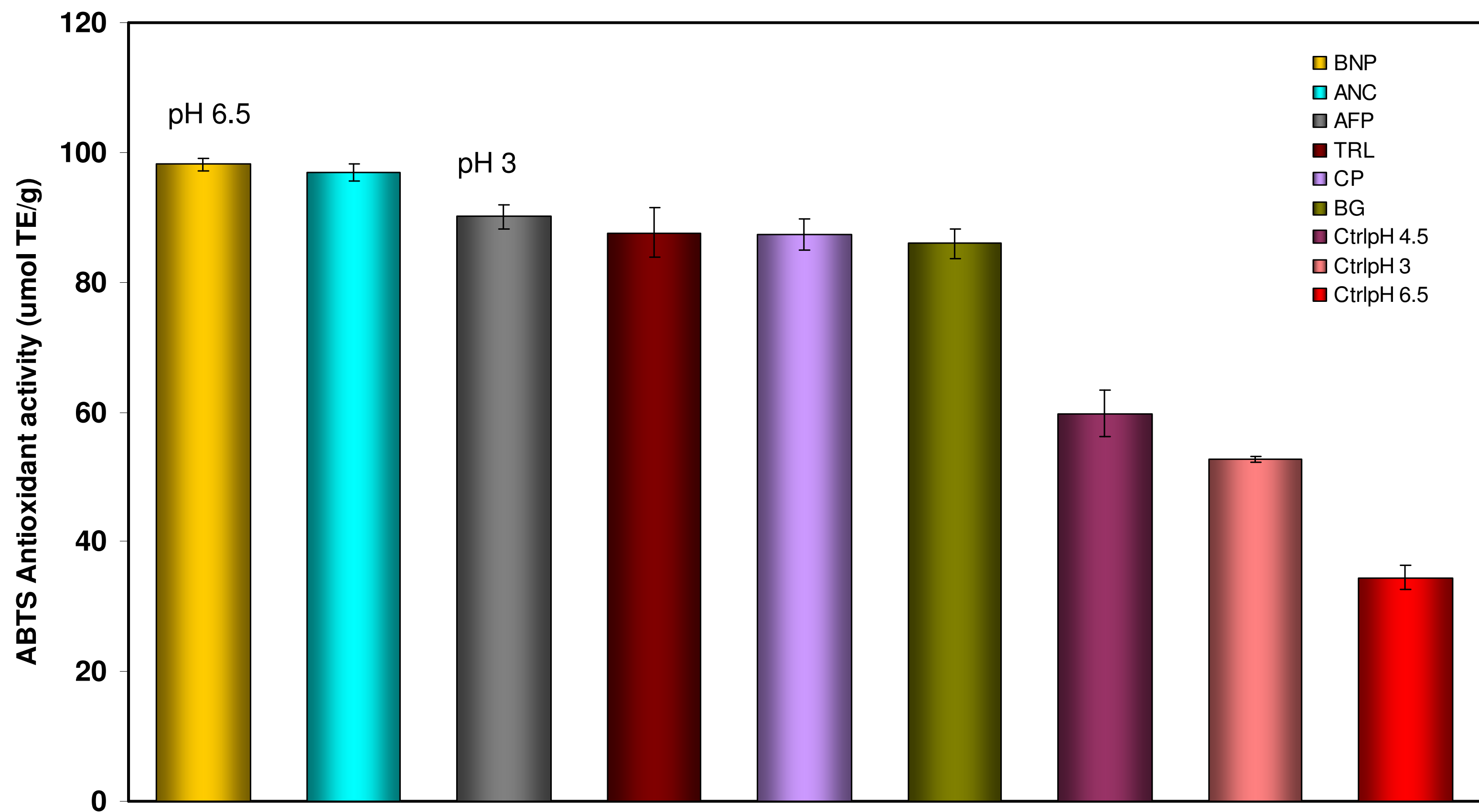

Fig. 74. Antioxidant activity potential of from enzyme-assisted extraction. CP&TRL =Cellulases ; AFP & BNP L =Proteases; ANC L Cellulase/Hemicellulase ; BG= Beta Glucanase.

3-Deoxyanthocyanins from Enzyme Extraction

HPLC analysis of the extracts obtained with enzymes showed no traces of 3-deoxyanthocyanin, although they had an intense orange color. Perhaps the 3-deoxyanthocyanins released by the enzyme treatment formed complexes that could not be detected or did not match the spectral characteristics of the four 3-deoxyanthocyanins that are present in black sorghum bran. The 3-deoxyanthocyanins may also be trapped in the gel matrix that was formed in the process of enzyme extraction.

Microscopic Analysis of Enzyme Extracted Residues of Black Sorghum Bran

Microscopic examination of residues to investigate the effect of the enzyme on the cell wall structural components and to determine the fate of phenolic compounds after leaching revealed that the cell wall components were disrupted and phenolic compounds likely leached out. These phenolic compounds however could not be quantified.

Microscopic examination of residues from extracts using pH 4.5 (control) showed the epicarp, mesocarp and starch granules were not disrupted (Fig. 75). The pigments were intact in all components. This suggests that the control solvent used did not extract any phenolic compounds.

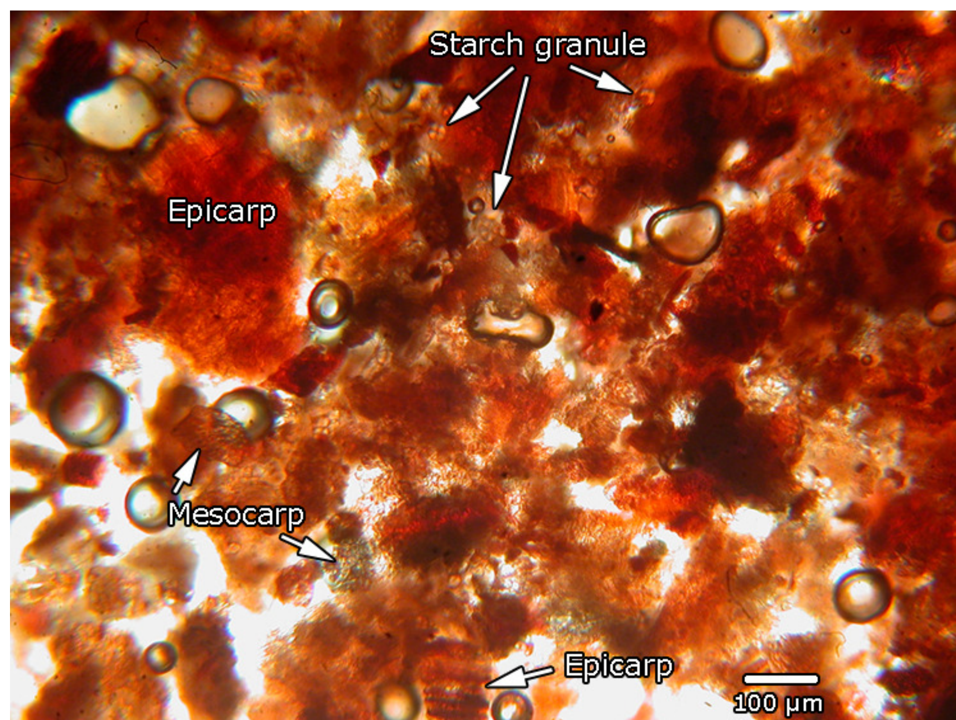

Fig. 75. Tx430 Black bran extracted for two hours at pH 4.5. 100X. (control). Epicarp, mesocarp and starch granules. Pigment still intact in all components.

The use of different enzymes resulted in the breakdown of different cell components. For example, extraction with cellulases (TRL) hydrolyzed the seed coat (Figs. 76 & 77) and although the cell wall and cell content were still intact, irregular pigment leaching could be seen.

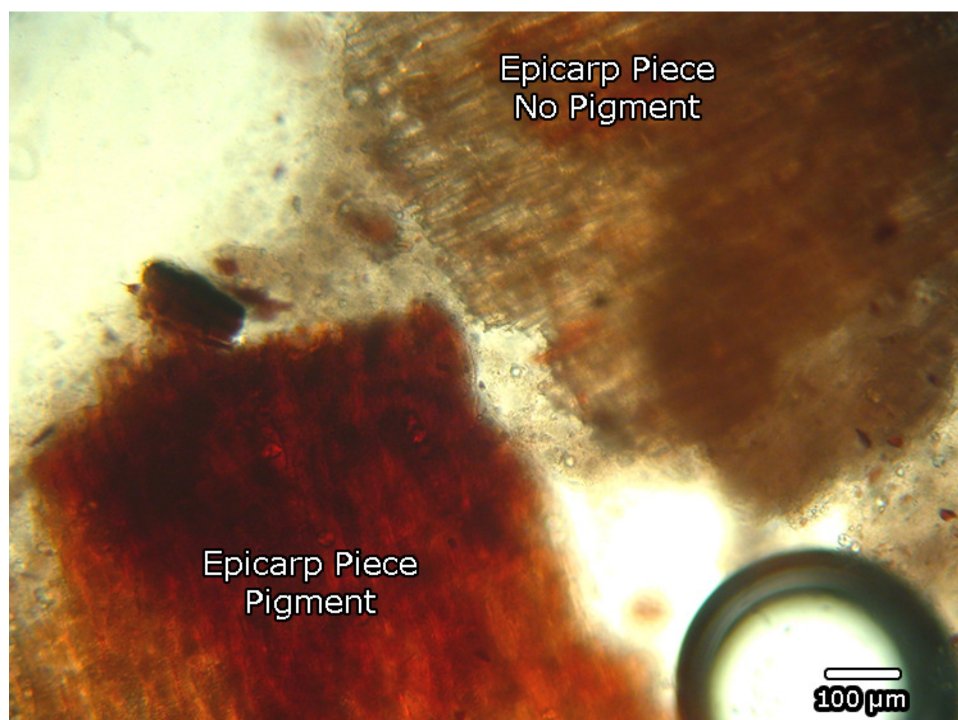

Fig. 76. Bran after using 2 mL (108 enzyme units) TRL (Cellulase). 100X. Epicarp is still intact. Irregular leaching of pigments can be seen.

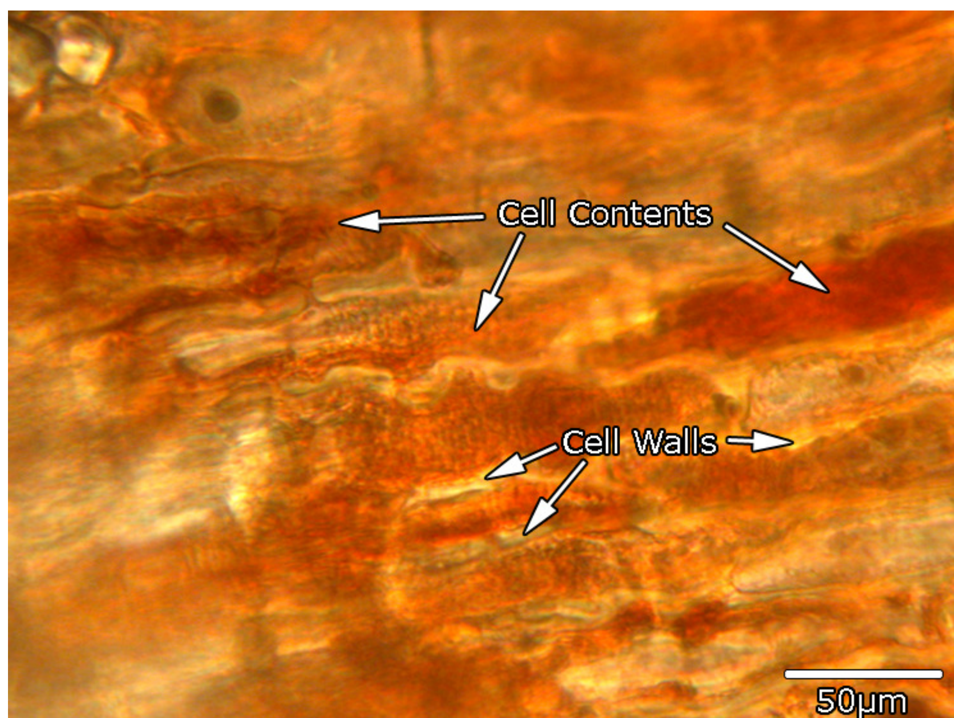

Fig. 77. Epicarp with contents using 2mL (108 enzyme units) TRL(Cellulase). 250X. Seed coat taken off, cell wall and cell contents still intact. Pigment not leached.

Different enzymes degraded bran differently (Figs. 78 & 79). There was loss of pigments in the mesocarp while the epicarp cells still retained the pigments. This suggests that a combination of enzymes might result in a synergistic effect that could be controlled for optimum benefits in the food industry if the chemistry of extraction of phenolic compounds from sorghum using enzymes is better understood.

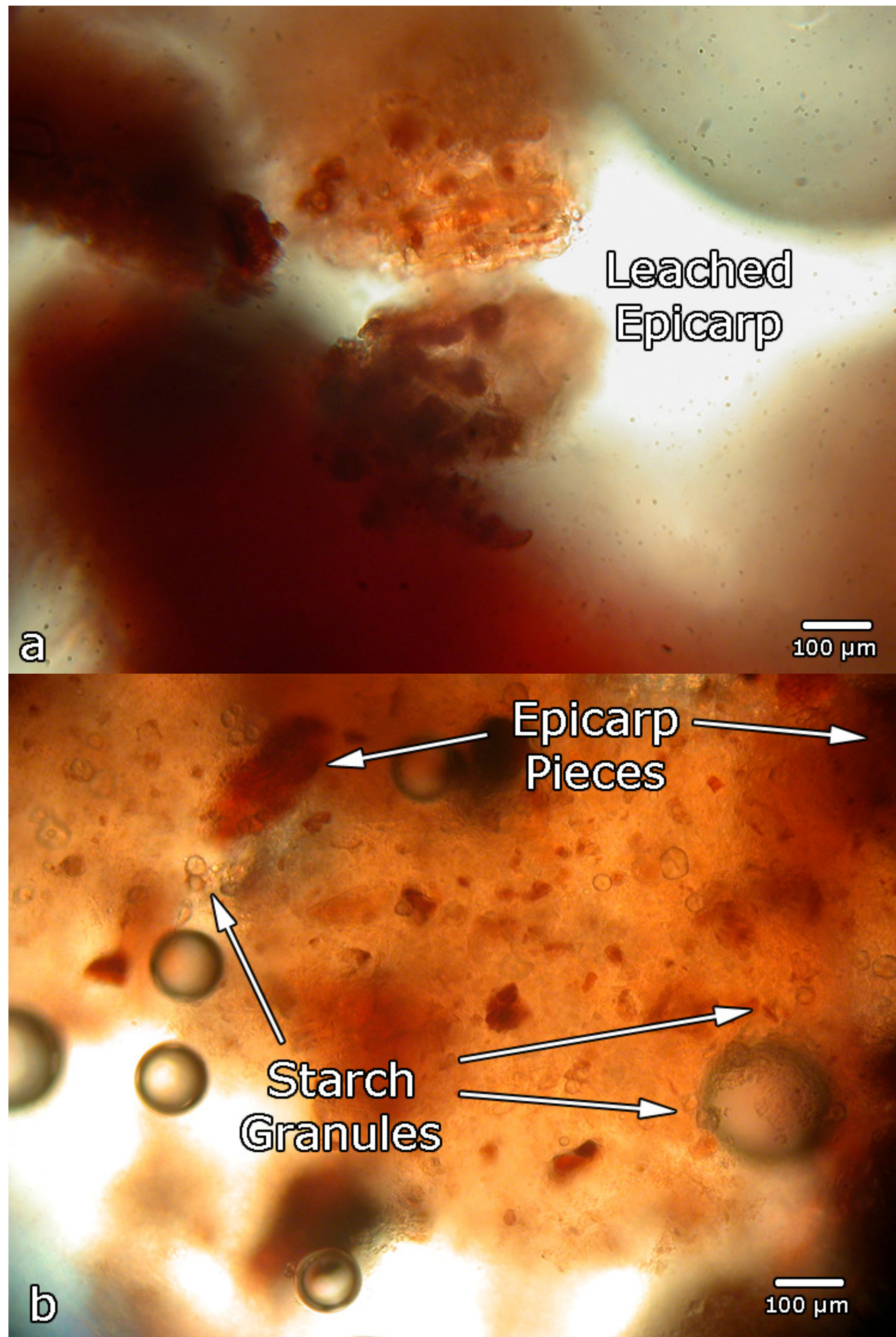

Fig. 78. CP (Cellulase) 2mL (52 enzyme units) [a] and AFP (Protease) 1.5 mL (118 enzyme units) [b], treated bran.100X. Different enzymes degrade bran layer differently.

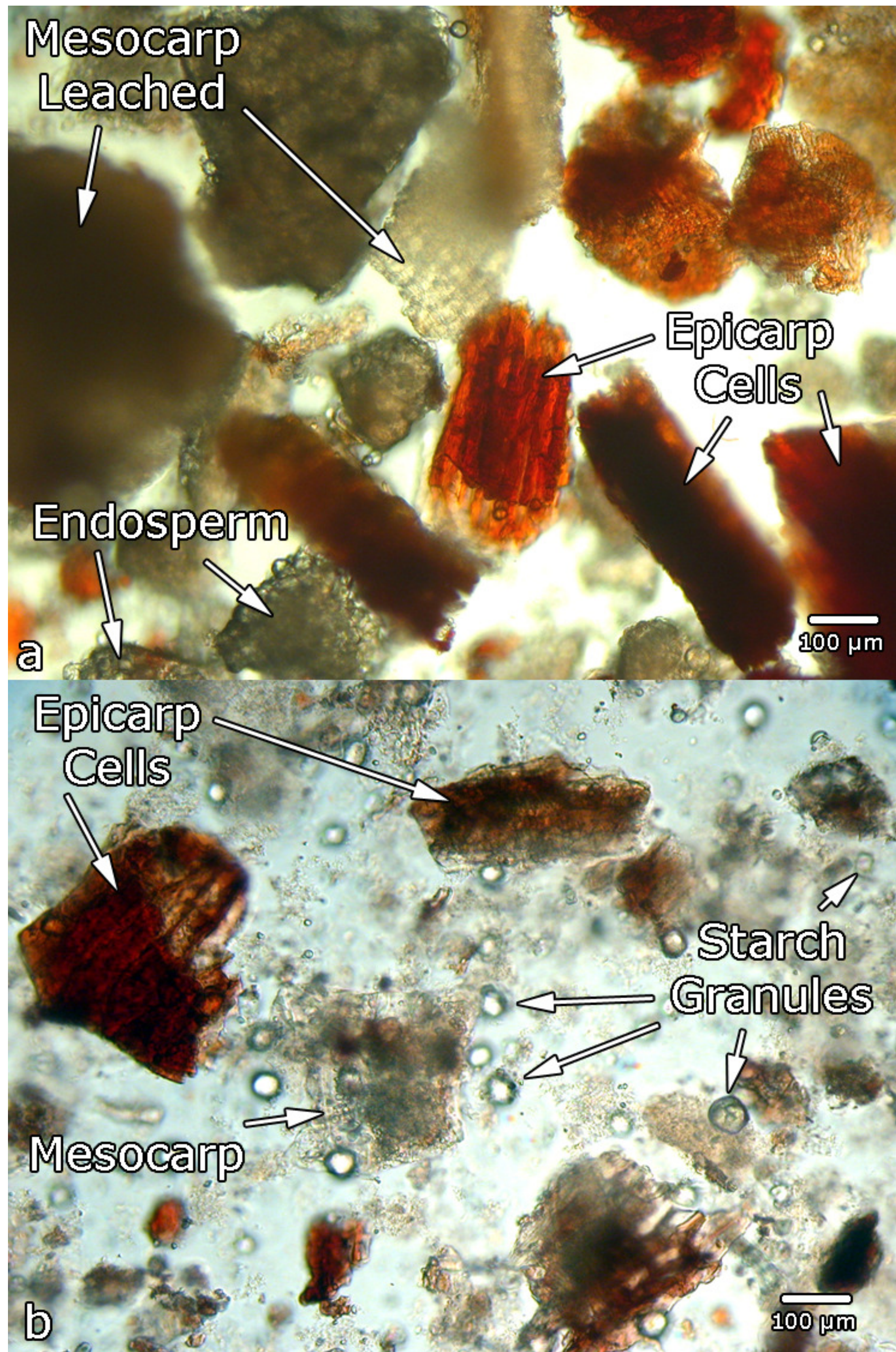

Fig. 79. AFP (Protease) 1.5 mL (10 enzyme units) [a] and BNP(Protease) 1.5 mL (26 enzyme units) [b], treated bran. 100X. Epicarp cells still retain pigments, while mesocarp has reduced pigment.

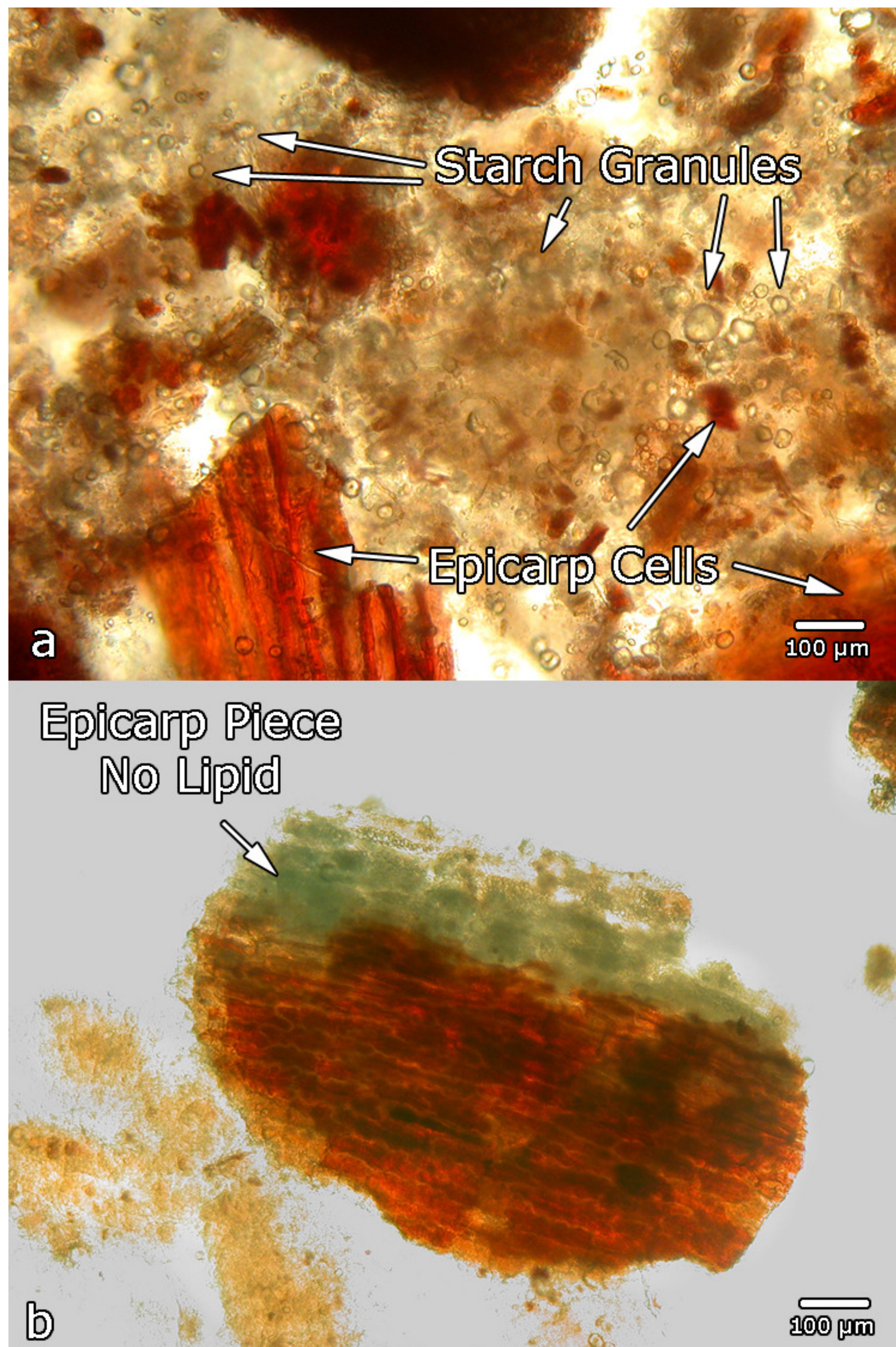

Fig. 80. BG (Beta Glucanase), 2mL (158 enzyme units) [a] and BG treated bran, Stained with Nile Blue A dye [b]. 100X; no lipids observed in epicarp.

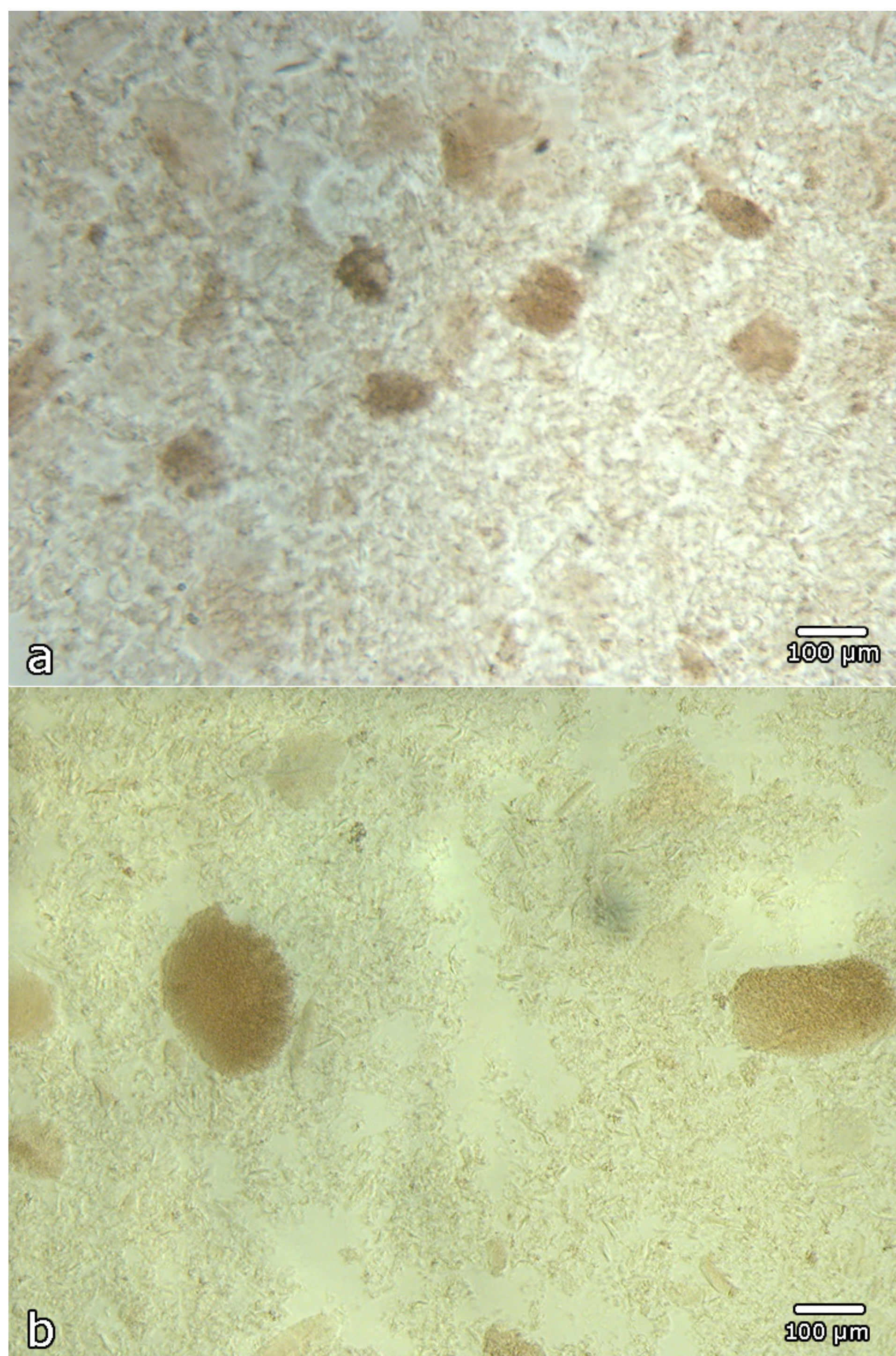

Fig . 81. AFP (Protease) (a); BG (Betalucanase) enzyme matrices (b) 100X.

Enzyme treatment produced a gel like structure (Fig. 80). Staining of the structure showed it was neither a protein nor a starch gel (Fig. 81). The gel from the enzyme treatment was rich in fiber, because of the epicarp and mesocarp fractions present and could possibly have applications as a high energy beverage if further studies are done to establish its content.

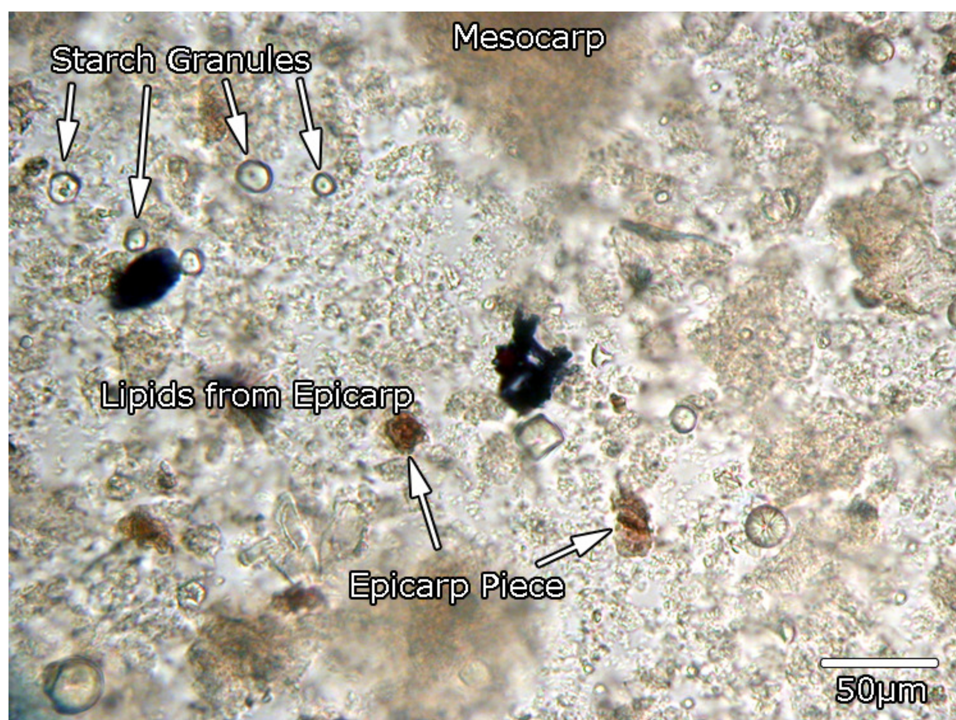

Fig. 82. Bran treated with BG 2mL (158 enzyme units), Nile Blue A dye stained, 250X.

Treating the bran with a combination of enzymes showed that the pigments from the epicarp are difficult to extract (Fig. 83). Different extraction solvents affected the bran differently (Fig. 84).

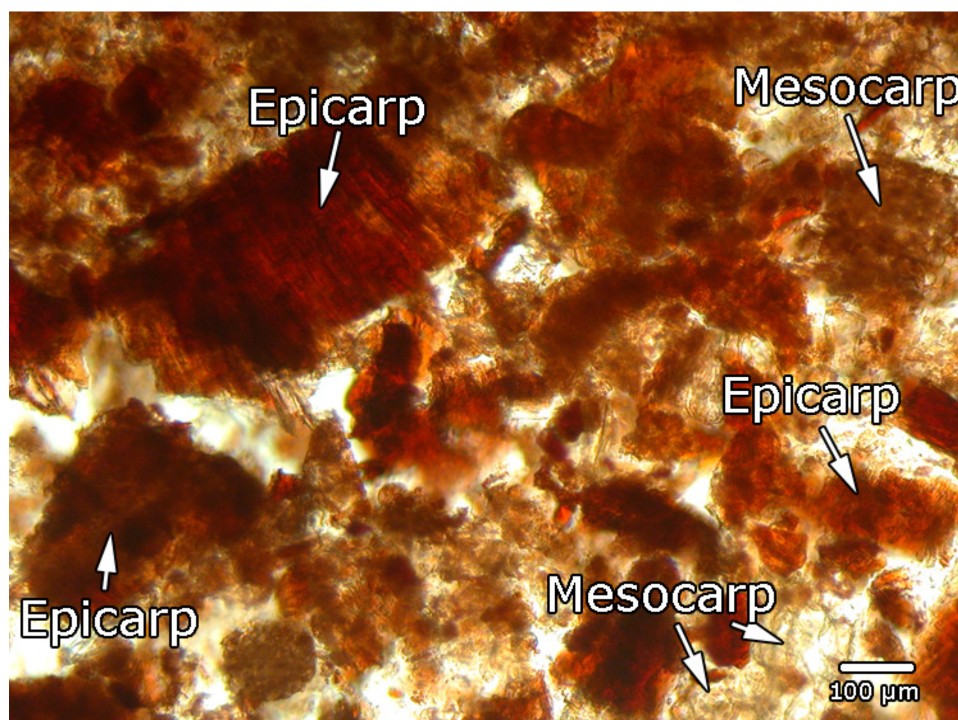

Fig. 83. ANC (Cellulase/Hemicellulase) + AFP (Protease) and α-amylase, treated bran.100X. No starch gel revealed, pigments in mesocarp leached out more than epicarp which still retains pigment.

For example, 1% HCl/methanol does not remove pigments from epicarp completely. However, it breaks down epicarp into smaller pieces. The mesocarp loses pigments, while starch remains in large endosperm fractions. 1% HCl/ethanol reduces starchy endosperm cells into separate starch granules. Mesocarp loses pigments, while starch remains in large endosperm cells. Pigment appears in all layers. As seen under the light microscope, pigments might be present in the aleurone layer wall, but not in the aleurone cell content (picture not shown).

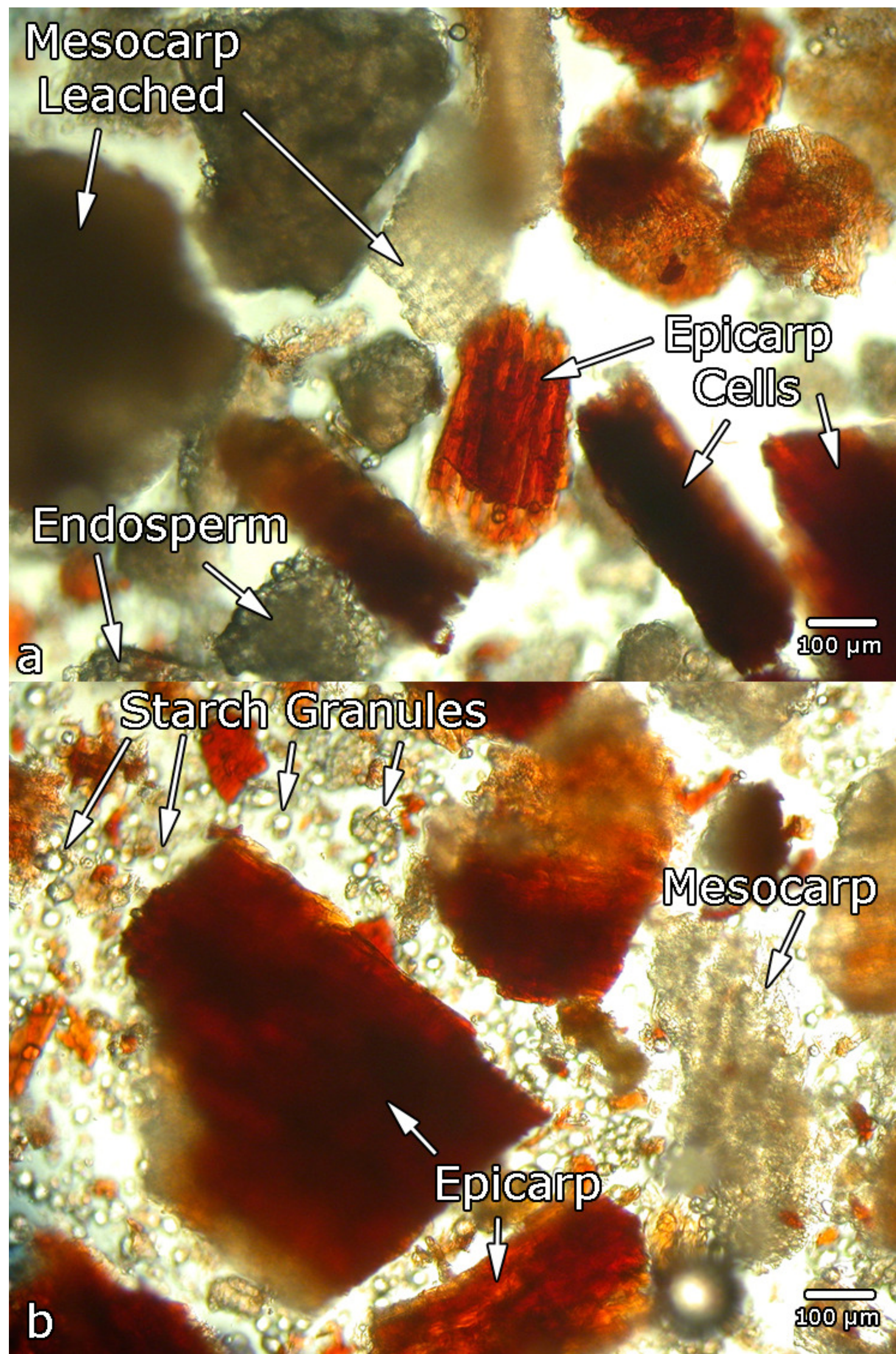

Fig. 84. l % HCl/MetOH (a), and 1%HCl/EtOH (b), treated bran. 100X. Different solvents break down bran particles differently. 1% HCl/methanol does not leach pigment from epicarp completely, but breaks epicarp into smaller pieces.

In most cases, enzyme treatment left intact cross and tube cells (pictures not shown), in which pigments were trapped. The colored compounds (3-deoxyanthocyanins) were trapped in the gel matrix as seen under the microscope. Microscopic examination of the residues from enzyme extraction revealed a break-down of cell wall components and leaching of pigments which was not detected by HPLC analysis.

A gel-like material resulting from the enzyme treatments was neither protein nor starch gel as revealed by the staining characteristics. It is probable the phenolic compounds were trapped in the gel-like structure. The structure of the 3-deoxyanthocyanins might be altered as a result of the enzyme activities. Appropriate enzyme preparation increased anthocyanins yields in berries and formed unknown components as well as caused changes in the anthocyanin profiles of the juices (Buchert et al 2005). Beta-glucanase did not have much effect on sorghum bran probably because sorghum has limited beta-glucans.

Although the study created more questions than answers, there is a potential to develop the gel-like material rich in fiber and phenolic compounds into antioxidant capsules or some nutritional beverage. More research is required to test this idea. Further studies are needed to understand the fate of phenolic compounds after enzyme extraction. It is probable that with careful understanding of enzyme activities, enzyme extraction of 3-deoxyanthocyanins from sorghum can be achieved.

CHAPTER VI

SUMMARY AND CONCLUSION

Analysis of sorghum varieties with and without pigmented testa confirmed that pigmented testa can be used as a predictor of polymeric flavonoids (tannins) in sorghum but not of simple flavonoids such as flavan-4-ols, 3-deoxyanthocyanins, flavones and flavanones. Non-tannin sorghums with the “red turning into black" pericarp had increased phenol levels and antioxidant activities as measured by ABTS, DPPH and ORAC methods. These high values were due to high levels of flavan-4-ols and 3-deoxyanthocyanins. Further studies are needed to understand the factors contributing to the unusually high antioxidant ORAC values in these special sorghums and the role of the “red turning black gene”.

Flavan-4-ols were higher in sorghum with black pericarp followed by those with red pericarp, but were not dependent on the presence or absence of a pigmented testa (Fig. 14; p. 62). Flavan-4-ols are associated with mold reduction in sorghums (Jambunathan et al 1991; Menkir et al 1996). Total phenols were highly correlated with ABTS (0.97) and DPPH (0.87) suggesting that, in addition to tannins, other phenolic compounds such as flavan-4-ols and 3-deoxyanthocyanins contribute to antioxidant activity.

Pericarp color affected 3-deoxyanthocyanins, flavones and flavanones differently. For example, black pericarp sorghums had elevated levels of 3-

deoxyanthocyanins; however the levels decreased when grains are severely weathered. The presence of a pigmented testa and pericarp color did not affect flavones. Flavones are concentrated in the pericarp and reported as phytoalexins (Seitz 2004), but may not contribute to pericarp color.

Pericarp color affected the flavanones but pigmented testa did not (Fig. 25: p. 87). In general, the red and black Shawaya had higher levels of eriodictyol than naringenin. There were no detectable flavanones in white pericarp sorghums. The sorghum varieties used in this study were grown in 2005 and suffered severe weathering. Thus, the phenols were low compared to non-weathered sorghums from other crop years (Awika 2003; Boren & Waniska 1992; Dykes 2008). The brighter the black color of a sorghum grain, the higher the concentration of the 3-deoxyanthocyanins and the brighter the lemon-yellow, the higher the levels of the flavanones present. Genetics and environment have major roles in determining the phenolic content of sorghum. Pigmented testa does not affect the composition of simple flavonoid.

The non-grain materials of sorghum, with the exception of the stalks, are potential sources of sorghum bioactive compounds for large scale applications especially when sorghum biomass for alcohol production is developed. The glumes of R-07007 had almost 600 folds more 3-deoxyanthocyanins than the grains while the glumes of ATx631xRTx436 and the leaves of Tx430Black x Sumac had 161 folds and 77 folds more flavones respectively than their grains (Table 6; p. 112).

Color intensity of the non-grain tissues had an effect on 3-deoxyanthocyanin but not on flavones and flavanones. Intensely pigmented leaves sheaths and glumes had high levels of 3-deoxyanthocyanins. For example, the leaves of Tx430 Black x Sumac and the sheaths and glumes of Tx2911 were the most intensely colored and had the highest levels of 3-deoxyanthocyanins among the hybrid sorghums. The leaves of an intensely purple colored biomass sorghum "*collier*" variety, contained higher levels of 3-deoxyanthocyanins (6199 µg/g dry weight) than all hybrid sorghum leaves; 3.4 times higher than in the leaves of Tx430 Black x Sumac. Biomass sorghum has a potential as a source of sorghum bioactive phenols. Among the traditional biomass sorghum cultivars, grains make up about 2.2-4.8% and the rest is biomass (Unpublished data). This suggests that with the interest in sorghum biomass for alcohol production, the by-products of this technology are potential sources for bioactive phenols. Tx430 Black and SC748-5 sheaths, glumes leaves and grains are good sources of the bright orange luteolinidin and its methoxylated derivative. The glumes, sheaths, leaves and grains, in descending order, for R-07007, Tx2911 and Tx430 Black x Sumac, are good sources of the yellow apigeninidin and its methoxylated derivative (Table 6; 113).

Luteolin and apigenin are major dietary flavones commonly found in aromatic herbs (parsley, rosemary and thyme). In general the sheaths, glumes and leaves of red plant sorghum varieties were higher in apigenin than luteolin. The grains were higher in luteolin, except for Tx2911 which did not have

detectable levels of luteolin. Clearly, the grains of sorghum are low in flavanoids in general compared to their non-grain components. Probably because flavonoids are produce as a defense mechanism and the parts of the sorghum plant that are more prone to biotic and abiotic stresses will have more of these compounds than the parts that are protected like the stalks and the grains.

Non-grain sorghum components compared favorably with common sources of flavones (Fig. 47; p. 130). The non-grain tissues are viable sources of large quantities of flavones compared to the grains. The glumes of Tx2911 had high levels of apigenin, while the sheaths of SC748-5 had high luteolin among all the components evaluated (Fig. 48; p. 132 & Fig. 49; p. 133).

The flavone-glycosides (Table 8; p. 136) were identified based on their spectral and mass spectrometric characteristics, showing distinctive fragmentation. The fragmentation yielded product ion signals at $[M\text{-}H\text{-}60]^-$, $[M\text{-}H\text{-}90]^-$, and $[M\text{-}H\text{-}120]^-$ in the negative ion mode MS^n analyses (Gattuso et al, 2007; Caristi et al, 2006; Pereira et al, 2005; Ferreres et al, 2003; Voirin et al, 2000). The grains and non-grain components of selected sorghum varieties are excellent sources of flavanones (Fig. 57; p. 145 and Fig. 58; p. 146). R-07007 grains are rich in eriodictyol, while the glumes of Tx2911 are a viable source of naringenin. The lemon-yellow pericarp sorghums are potent sources of flavanones as reported by (Dykes, 2008). The non-grain sorghum materials are excellent sources of bioactive phenols with potential application in food, nutraceutical, pharmaceutical and cosmetic industries. Overall, the glumes

sheaths and leaves all provide large concentrations of flavonoids; however, specific classes of flavonoids may be obtained from specific sorghum tissues. For example, 3-Deoxyanthocyanins are predominant in the glumes>sheaths>leaves; flavones in the glumes>leaves>sheaths while flavanones are predominant in the glumes>grains>leaves>sheaths in varieties in which they are present. Sorghum grains with lemon-yellow pericarp are high in flavanones. Given the diversity in sorghum germplasm, more studies are required to evaluate the phenolic compounds and profile in a wide variety of sorghum.

0.5% Citric acid in 70% aqueous ethanol was the most suitable food friendly solvent for the extraction of phenolic compounds from black sorghum. This solvent gives the same flavonoid profile as the commonly used 1 % HCl/methanol although the levels are reduced by 50%. O.5% CA/70% ethanol was more effective in extracting phenolic compounds from black tannin sorghum than the 1%HCl/methanol (Fig. 65; p. 162 & Fig. 66; p. 163) which extracted more phenols from Tx430 non-tannin black sorghum. However, using food grade HCl at 1% in ethanol might be a better option since it yields more phenols and 3-deoxyanthocyanins than the food acids.

Storage of bran leads to reduction in levels of phenolic compounds. Phenols were 47 % higher in freshly prepared sorghum bran compared to stored bran (Fig. 69; p. 167 & Fig. 70; p. 168). Decortication may release phenol catabolizing enzymes like polyphenol oxidases (PPO) and peroxidases (POX).

Extraction of phenols from sorghum using enzymes was not successful. The enzymes succeeded in breaking down the cell wall components and releasing some phenols but which were trapped in a gel-like matrix rich in fiber and high in antioxidant capacity. Perhaps this matrix can be processed into a high fiber, high antioxidant supplement or ingredient with potential food and nutraceutical application. Use of enzyme for extraction of sorghum phenols is possible if the chemistry is better understood.

The conversion of sorghum biomass into alcohol will likely produce by-products that will concentrate the phenols and other potentially useful bioactives. Some sorghum can be valuable sources of unique compounds such as 3-deoxyanthocyanins, flavones, flavanones and flavan-4-ols. Further studies are however needed to understand the fate of these compounds released after enzyme treatment. With appropriate processing technology this might be a potential source for bioactive phenolic compounds.

Sorghum leaves, sheaths and glumes in addition to the grains are viable sources of various bioactive compounds. Further studies are required to evaluate the antioxidant, anticarcinogenic and anti-inflamatory properties of these compounds as well as the bioavailability of sorghum phenols in biological systems as well as the effect of processing on these compounds.

The increased levels of phenols and antioxidant activities in sorghum with the “red turning into black gene” is a promising finding that plant breeders can use to select for special traits aimed at developing sorghums with enhanced and

desired functionality such as antioxidant potential and other healthy attributes. The leaves, sheaths and glumes of sorghum are excellent sources of unique phenolic compounds with *in vitro* antioxidant properties. Extraction of bioactive phenols from by-products of the alcohol production will adds value to the market potential of sorghum and provides an additional dietary source of phenols with potentials for commercialization.

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