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Sorghum phenolic extracts: Their storage stability and antioxidant activity in sunflower oil

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By

Fred E Sikwese

Submitted in partial fulfilment of the requirements for the degree

MSc (Agric) Food Science and Technology

in the

Department of Food Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

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August 2005

DECLARATION

I hereby declare that this dissertation submitted to the University of Pretoria for the award of MSc degree is my work and has not been submitted by me for a degree at any other University or institution of higher education.

Fred Edington Sikwese
August 2005

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ABSTRACT

Sorghum phenolic extracts: Their storage stability and antioxidant activity in sunflower oil

By

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Department: Food Science

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Whole grain and bran samples of two Malawian sorghums, *Phatafuli*, (a brown-coloured condensed tannin variety) and *Shabalala*, (a white-coloured condensed tannin-free variety) were analysed for their content of total phenols, condensed tannins and antioxidant activities. The effect of oxidizing conditions during extraction, and the storage stability of a freeze-dried crude phenolic extract (CPE) from the condensed tannin sorghum bran as influenced by packaging, storage temperature and length of storage, in relation to its content of total phenols, condensed tannins and antioxidant activity was also investigated. Antioxidant activity of the CPE, in comparison with tertiary butyl hydroquinone (TBHQ), was then evaluated in sunflower oil at concentrations of 1000, 1500 and 2000 ppm in the absence or presence of ferric ions at 2.2 and 4.4 ppm in the dark at 65^{o}C. Progression of oxidation was monitored by measurement of peroxide values (PV) and anisidine values (AV) during a 14-day storage period.

Phatafuli contained higher content of total phenols and antioxidant activity than *Shabalala* both in the whole grain and the bran, probably due to the presence of condensed tannins in *Phatafuli* sorghum, which were not detected in *Shabalala* sorghum. For both sorghum varieties, the bran contained higher levels of total phenols and antioxidant activity than the whole grain, confirming that phenolic compounds in sorghum are largely concentrated in the bran. Antioxidant activities of the sorghum varieties correlated highly with their total phenol and condensed tannin contents, suggesting that the phenolic compounds were largely responsible for the antioxidant activities of the sorghum grains.

Bubbling of oxygen into the liquid crude phenolic extract did not have any significant effect on the parameters tested. Similarly, vacuum-packed samples did not differ significantly in the parameters tested from the samples that were not vacuum-packed. CPE samples stored at -20^0C had significantly higher levels of total phenols, condensed tannins and antioxidant activity than those stored at 25^0C during some days of storage. Storage time was however the major factor influencing the levels of total phenols, condensed tannins and antioxidant activity of the CPE from *Phatafuli* sorghum during storage, which suggested that CPE from condensed tannin sorghum bran might need to be used shortly after extraction to ensure optimum antioxidant activity. There was an insignificant correlation between the antioxidant activities of the CPE and their phenolic contents during storage, which could have been due to the formation of new compounds with a lower antioxidant capacity.

The CPE inhibited oxidation of sunflower oil as shown by lower peroxide values and anisidine values compared to control samples. The CPE was however less effective in reducing peroxide values compared to TBHQ, but was similar to TBHQ in reducing anisidine values. In the presence of ferric ions, the CPE appeared to be less effective in reducing peroxide values compared to TBHQ, but appeared to be more effective than TBHQ in reducing anisidine values. The results showed that the tannin sorghum bran CPE appeared to act as both lipid radical scavengers and metal chelators. The CPE however imparted colour to the sunflower oil, which could limit its application as a natural antioxidant in edible oils.

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CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and statement of the problem

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop in the world in terms of production and area planted (FAO/ICRISAT, 1996). Due to its drought resistance, it forms one of the most important staple foods for millions of people in the semi-arid tropics of Africa and Asia (FAO, 1995). Sorghum is principally utilized as human food and animal feed. In the main sorghum production regions in Africa and Asia, more than 70% of the sorghum is consumed as food (FAO/ICRISAT, 1996), and is one of the principal sources of energy, protein, vitamins and minerals among the populations of these regions (FAO, 1995).

Sorghum contains phenolic compounds, which are considered secondary metabolites in that they are not directly involved in any plant metabolic processes. These phenolic compounds have been shown to have antioxidant properties (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003; Simontacchi, Sadovsky & Puntarulo, 2003), and are known to play a natural defensive role in the plant by protecting against pests and diseases as well as predatory attacks by herbivores (Butler, 1982a; Hahn, Faubion & Rooney, 1983; Hahn, Rooney & Earp, 1984; Jambunathan, Butler, Bandyopadhyay & Mughogho, 1986). In sorghum, these phenolic compounds are known to be concentrated in the outer layers of the grain (pericarp) (Rooney, Blakely, Miller & Rosenow, 1980; Hahn *et al*., 1984; Youssef, 1998; Beta, Rooney, Marovatsanga & Taylor, 2000).

The antioxidant properties of plant phenolic compounds provide potential benefits for the food industry and human health (Murkovic, 2003). In the edible oil processing industry, there is growing interest in finding suitable natural alternatives to synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) currently in use to prevent lipid oxidation, but are reportedly not safe (Ito, Hirose, Fukushuma, Tsuda, Shirai & Tatematsu, 1986; Lindenschimdit, Tryka, Goad & Witschi, 1986; Whysner, Wang, Zang, Iatropoulos & Williams, 1994; Frankel, 1996; Malecka, 2002). Lipid oxidation

is influenced by the presence of oxygen, prooxidant metals and conditions that activate oxygen such as light and high temperatures (Nawar, 1996). Though avoiding exposure of a fat or oil to these conditions could prevent or hinder oxidation, this has been found inadequate to control oxidation, hence the need to use antioxidants (Gunstone & Norris, 1983; Coultate, 1996). Phenolic compounds from some plant sources have been shown to be effective antioxidants in retarding lipid oxidation (Wanasundara & Shahidi, 1998; Abdalla & Roozen, 1999; Bonilla, Mayen, Merida & Medina, 1999; Malecka, 2002). Due to these reports surrounding the negative effects of synthetic antioxidants therefore, the exploration of natural sources of antioxidants continues to gather momentum.

Milling of sorghum grains into flour is one of the principal unit operations in sorghum processing. One of the primary objectives of milling is to separate the main anatomical components of the grain, namely the pericarp, endosperm and germ as cleanly as possible (Hoseney, 1994; Kent & Evers, 1994; FAO/ICRISAT, 1996). The bran (mainly pericarp and some of the germ) that results from the milling process is often discarded as a waste product. Though sorghum has not been studied extensively as a source of natural antioxidants, some black and brown sorghum brans have been shown to posses high antioxidant activity relative to fruits such as grapes, plums and strawberries (Awika & Rooney, 2004). Sorghum bran, which contains approximately 10% total phenols, could therefore be considered as a potential source of phenolic compounds for use as natural antioxidants in lipid foods such as refined sunflower oil. These phenolic compounds in sorghum are compartmentalized in the plant cell matrix, but once the cell matrix is broken during the extraction of phenolic compounds, they become prone to oxidation and polymerization reactions (Waterman & Mole, 1994), which could likely affect their antioxidant capacity. Storage temperature (Larrauri, Sanchez-Moreno & Saura-Calixto, 1998), light (Rodriguez de Sotillo, Hadley & Holm, 1994a; Mansour & Khalil, 2000) and exposure to oxygen (Waterman & Mole, 1994; Haslam, Lilley, Warminski, Liao, Cai, Martin, Gaffney, Goulding & Luck, 1992) are some factors that have been shown to affect the stability of phenolic compounds.

The successful application of phenolic extracts from sorghum as antioxidants in lipid food systems is therefore dependent on the antioxidant properties of such extracts and to what extent these can be preserved. The antioxidant properties of phenolic extracts

from sorghum (or any other plant source) are in turn dependent on the phenolic composition and stability of the phenolic compounds during extraction, handling and storage. It is of importance therefore to investigate the factors influencing antioxidant properties of sorghum phenolic extracts during storage and to what extent these extracts could be applied as natural antioxidants in lipid food systems such as sunflower oil.

1.2 Literature review

1.2.1 Sorghum

Sorghum is an important staple food crop for millions of people in the semi-arid tropics of Africa and Asia (Matz, 1991; Hoseney, 1994; Kent & Evers, 1994), where it is one of the principal sources of energy, protein, vitamins and minerals (FAO, 1995). It is a reliable crop in these areas due to its drought resistance, and is the second most important cereal after maize in sub-Saharan Africa (FAO, 1995). Provisional data for world sorghum production in 2004 was estimated at 60.2 million metric tonnes, of which 23.8 million metric tonnes and 6.5 million metric tonnes was produced in Africa and India, respectively. Africa accounted for about 40% of the 2004 sorghum global production, where the sorghum largely contributes to rural food security (FAO, 2004). Worldwide, 48% of the sorghum produced is used as animal feed, 42% is used as human food, while the remainder accounts for seed manufacturing purposes and waste (FAO/ICRISAT, 1996). In the main production regions in Africa and Asia however, more than 70% of the sorghum is consumed as food (FAO/ICRISAT, 1996).

Sorghum is consumed in different forms. The grain can be roasted while green (Dogget, 1988), can be boiled whole (Ensminger, A. H., Ensminger, M. E., Konlante & Robson, 1983; Dogget, 1988), popped, or milled into flour and used for making bread, and soft and thick porridges (Ensminger, *et al.,* 1983; Dogget, 1988; Matz, 1991; Ohiokpehai & Kebakile, 1996; Dicko, Hilhorst, Gruppen, Traore, Laane, van Berkel & Voragen, 2002). It is also used for the production of alcoholic beverages (clear and opaque beer) (FAO/ICRISAT, 1996; Dicko, *et al.*, 2002) and other non-fermented beverages (Beta, Rooney, Marovatsanga & Taylor, 1999; Dicko, *et al.*, 2002). Sorghum can also be processed for starch and oil (Dogget, 1988).

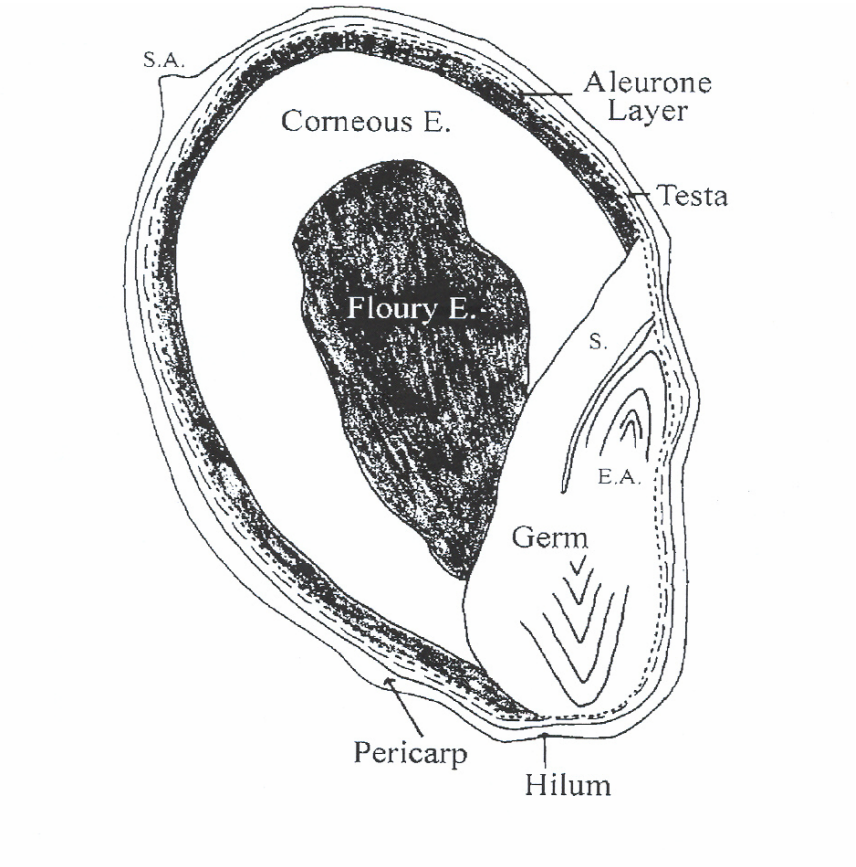


Figure 1.2.1. Sorghum kernel: S.A., stylar area; E., endosperm; S., scutellum; E.A., embryonic axis (Adapted from Hoseney, 1994).

Anatomically, a sorghum kernel consists of three major parts: the pericarp, endosperm and germ (Rooney, *et al.*, 1980; Matz, 1991; Waniska, 2000) (Figure 1.2.1). The pericarp forms 7.9% by weight of the sorghum kernel, the endosperm 82.3% and the germ 9.8% (Hoseney, 1994). The processing of sorghum involves the partial separation of these three major components (FAO/ICRISAT, 1996). The pericarp is removed to improve the palatability of the product (Hoseney, 1994). The germ is rich in oil, which could contribute to oxidative rancidity. The separation of the germ therefore helps ensure a longer shelf life of the flour or other products made from the decorticated grain (Hoseney, 1994). The decorticated grain may be eaten as such after cooking or frying in oil, or it is milled into flour from which various foods such as porridges, breads and beverages are made (Dogget, 1988; Matz, 1991; FAO/ICRISAT, 1996). The endosperm can then be milled into flour (Hoseney, 1994) for preparation of various foods, or used as an ingredient in other foods such as snacks (Matz, 1991). Sorghum bran may also be used as animal feed, and sometimes tannin from the bran of tannin sorghum is extracted and used for dyes, creams and cosmetics

(Sorghum Energy Management Company Limited, 2003). The bran is however mostly discarded as a waste product.

Another reason for the importance of sorghum bran is that it is a source of phenolic compounds of which tannins (in brown sorghums) are but one example. The phenolic compounds in sorghum, as is the case with other plants, have inherent agronomic advantages such as providing grain resistance to fungal attack (Hahn *et al*., 1983; Hahn & Rooney, 1986; Jambunathan *et al*, 1986; Waniska, Poe & Bandyopadhyay, 1989) and inhibition of mould growth (Hahn *et al*., 1984; Waniska, *et al.*, 1989). The astringency of the phenolic compounds imparts bird resistance to sorghum (Butler 1982a; Hahn *et al*., 1984), hence preventing pre-harvest losses due to bird predation, and also prevents post-harvest losses due to storage pests. The phenolic compounds in sorghum have, however, undesirable effects regarding the utilization of sorghum by man. They can cause off-colour and taste in products made from high tannin sorghum (Maxson & Rooney, 1972; Hahn, *et al.,* 1984), and they bind to proteins, hence reducing the nutritive quality of the grain (Strumeyer & Malin, 1975; Hahn, *et al.,* 1984). Sorghum bran is therefore removed to eliminate tannins in condensed tannin sorghums (Waniska, 2000), as well as to improve the palatability of the products made from sorghum (Hoseney, 1994).

Tannins from sorghum bran contribute colour and so may be extracted and used for dyes, creams and cosmetics (Sorghum Energy Management Company Limited, 2003). This may present an opportunity for the use of sorghum bran as a source of dyes and food colourants. The antioxidant properties of the phenolic compounds also provide a potential for their use as food antioxidants. They may also be used in medicinal products such as creams and cosmetics due to their pharmacological and antimicrobial properties (Awika *et al.*, 2003). These antioxidant properties of phenolic compounds from plant sources have led to a growing interest in their study by researchers.

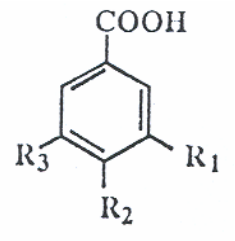
1.2.2 Sorghum phenolic compounds

Phenolic compounds are substances that possess an aromatic ring bearing one or more hydroxyl (OH) substituents (Waterman & Mole, 1994). As mentioned earlier, sorghum contains phenolic compounds that have antioxidant properties, and provide a protective role to the sorghum plant (Hahn *et al*., 1983; Hahn & Rooney, 1986; Hahn *et al*., 1984; Jambunathan *et al*., 1986; Waniska *et al*., 1989). Awika *et al.* (2003)

reported antioxidant activities of sorghums, their brans, and baked and extruded products due to phenolic compounds. These phenolic compounds in sorghum grain may be classified very broadly, into phenolic acids, flavonoids and condensed tannins (Waniska, 2000).

1.2.2.1 Phenolic acids

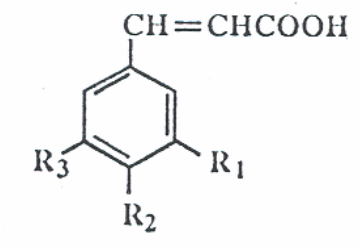
Phenolic acids are the simplest phenolic compounds and are derivatives of benzoic or cinnamic acids. They contain hydroxyl or methoxyl groups substituted at various positions on the aromatic ring (Hahn *et al.*, 1984). Waniska *et al.* (1989) identified protocatechuic, gentisic, caffeic, *p*-coumaric, salicylic, ferulic, sinapic and cinnamic acids as the major phenolic acids in sorghum grain, and *p*-hydroxybenzoic, vanillic and syringic as minor phenolic acids. On the other hand, Hahn *et al.*, (1983) separated and identified, by reverse phase high performance liquid chromatography (HPLC), eight main phenolic acids in sorghum grain extracts in both white and brown sorghum (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, *p*-coumaric, ferulic and cinnamic). These phenolic acids were found in both the free and bound form, with brown sorghum containing the highest amount of free phenolic acids. Bound phenolic acids are those that are complexed or form conjugates with sugars, proteins or cell wall polysaccharides (Regnier & Macheix, 1996; Francis, 2000). Waniska *et al.* (1989) also concluded that white sorghum varieties without pigmented testa contain the lowest amount of phenolic acids. Some of the phenolic acids found in sorghum are shown in Figure 1.2.2.



Benzoic acid

(a) Benzoic acid derivatives:

Name of acid	**Functional group position**			
	R_1	**R_2**	**R_3**	**R_4**
Gallic	H	OH	OH	OH
Protocatechuic	H	OH	OH	H
p-hydroxybenzoic	H	H	OH	H
Gentisic	OH	H	H	OH
Salicylic	OH	H	H	H



Cinnamic acid

(b) Cinnamic acid derivatives:

Name of acid	**Functional group position**		
	R_1	**R_2**	**R_3**
Caffeic	OH	OH	H
Ferulic	OCH_3	OH	H
p-coumaric	H	OH	H
Sinapic	OCH_3	OH	OCH_3

Figure 1.2.2. Phenolic acids reported in sorghum

1.2.2.2 Flavonoids

Flavonoids are the largest group of phenols in the plant kingdom (Hahn *et al.,* 1984), and are metabolites with a benzopyran nucleus having an aromatic substituent at carbon number 2 (C-2) of the C ring (Waterman & Mole, 1994). Figure 1.2.3 shows the basic flavonoid ring structure.

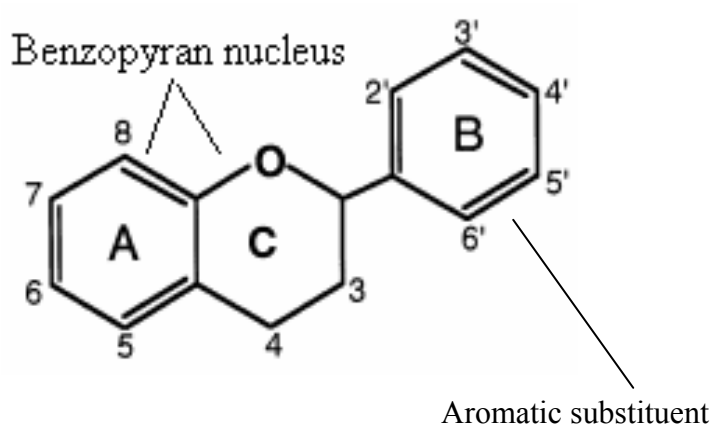
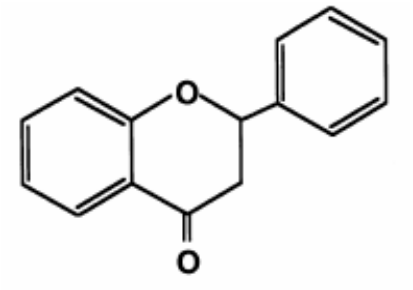
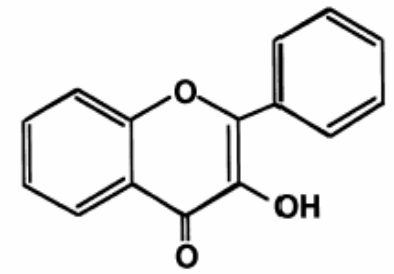


Figure 1.2.3. Basic flavonoid ring structure (Sugihara, Arakawa, Ohnishi & Furuno, 1999.)

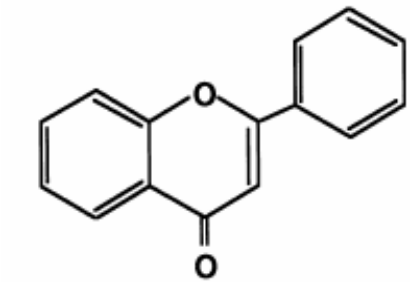
The most common types of flavonoids are flavanones, flavonols, flavones and flavans. As shown in Figure 1.2.4, characteristic features of these flavonoids are a carbonyl at C-4 (flavanones), carbonyl at C-4, double bond between C-2 and C-3, and hydroxyl at C-3 (flavonols), carbonyl at C-4 and a double bond between C-2 and C-3 (flavones), and no carbonyl at C-4 but with a hydroxyl at C-3 for the flavans (Hahn *et al.,* 1984).



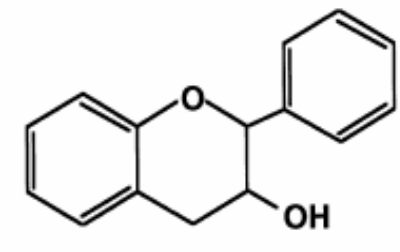
Flavanone



Flavonol



Flavone



Flavan

Figure 1.2.4. Basic structures of common flavonoid types (Sugihara *et al*., 1999)

Among the types of flavonoids that exist in nature, flavans are the major group found in sorghum (Hahn *et al.,* 1984; Waniska, 2000), and the major flavans are

leucoanthocyanidins (hydroxyl at C-3 and C-4), (+)-catechin (hydroxyl at C-3) and anthocyanidin (hydroxyl at C-3, double bond between C-3 and C-4) (Hahn *et al*., 1984). Anthocyanidins such as luteolinidin, cyanidin and apigeninidin have been reported in sorghum (Hahn, *et al.*, 1984). Awika, Rooney & Waniska (2004) identified apigeninidin and luteolinidin as the major 3-deoxyanthocyanidins (anthocyanidins that lack a hydroxyl group at the C-3 position) in black and brown sorghums. Blessin, Van Etten & Dimler (1963) found an anthocyanidin, fisetidin, in sorghum, and Yasumatsu, Nakayama & Chichester (1965) reported pelargonidin. Strumeyer & Malin (1975) however did not find any anthocyanidins in Leoti and Georgia 615 sorghums. The anthocyanin content of sorghum brans ranges from 1.6-9.8 mg/g (reviewed by Awika & Rooney, 2004). Other flavonoids reported in sorghum include naringenin and eriodictoyl (flavanones), taxifolin (flavonol) (Gujer, Magnolato & Self, (1986); apiforol (flavan-4-ol) and luteolin (flavone) (reviewed by Awika & Rooney, 2004). This shows that there is a wide variation in the flavonoid composition among sorghums. Some of these major flavonoids of sorghum are shown in Figure 1.2.5. Flavonoids may undergo modification such as loss of oxygen substituents, methylation, glycosylation, dimerization or polymerization, which may affect their properties (Waterman & Mole, 1994).

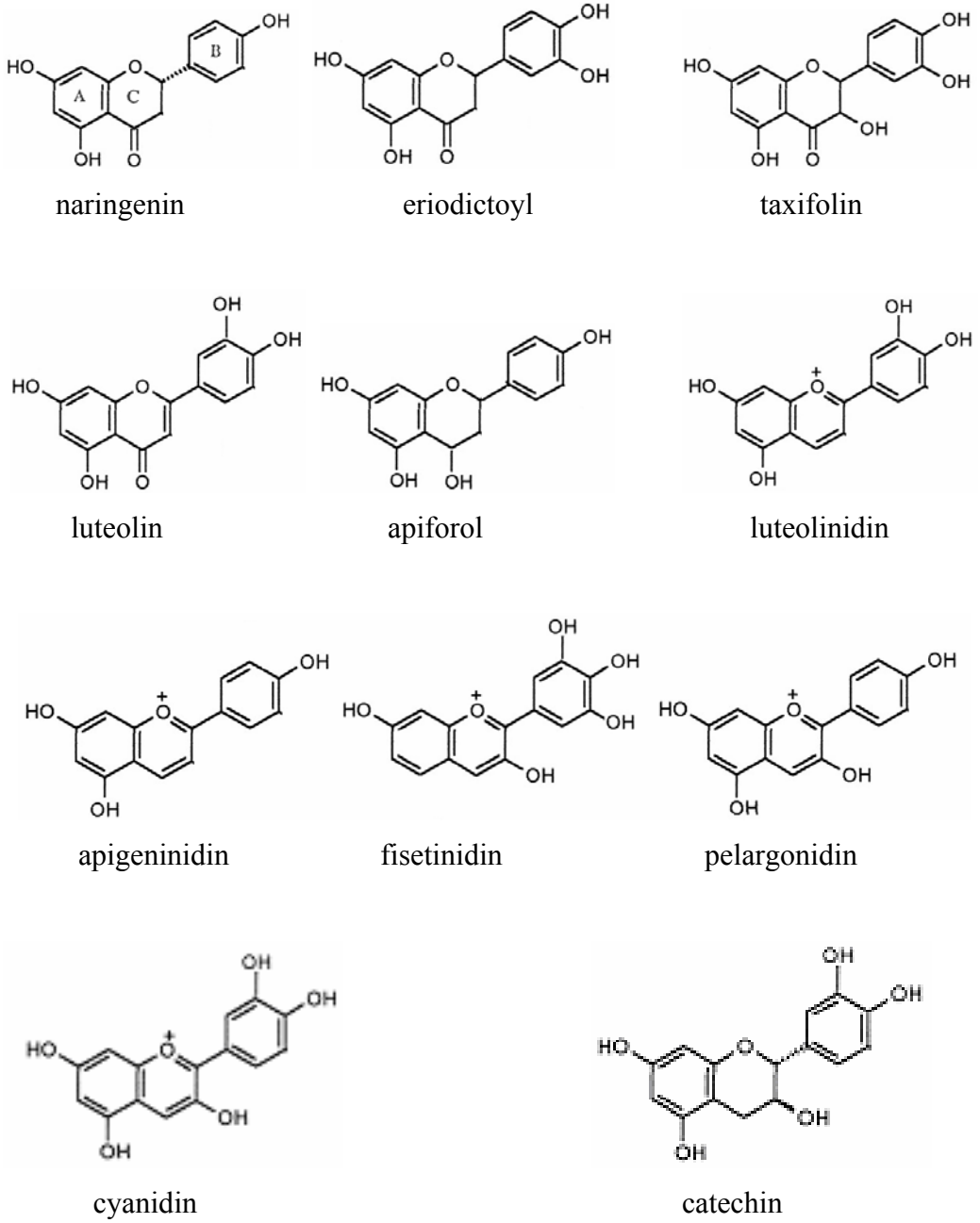


Figure 1.2.5. Some major flavonoids reported in sorghum (Adapted from Awika & Rooney, 2004).

1.2.2.3 Condensed tannins

Three distinct types of tannins have been identified in nature: phlorotannins, hydrolyzable tannins and condensed tannins, and each one of them originates from simpler phenols (Waterman & Mole, 1994). Condensed tannins are however the only tannin, which has been identified in sorghums (Awika & Rooney, 2004) and are the major phenolic compounds found in high tannin sorghum grain (Butler, 1982a; Hahn *et al.*, 1984).

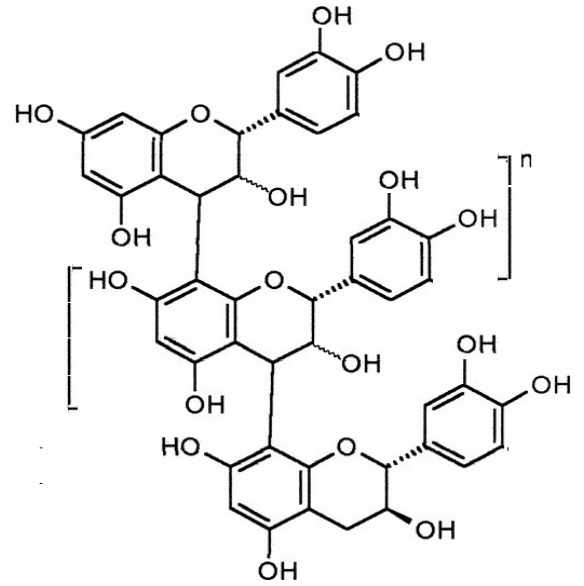


Figure 1.2.6. Structure of condensed tannin (procyanidin type), n = 1 to > 10 (Adapted from Awika & Rooney, 2004)

Condensed tannins are polymers of flavan-3-ol units (Butler, 1982a; Waterman & Mole, 1994; Schofield, Mbugua & Pell, 2001) linked by carbon-carbon bonds between the flavonol sub-units (Hahn, *et al.,* 1984; Schofield *et al*., 2001) (Figure 1.2.6). Condensed tannins are also referred to as proanthocyanidins because of the ability of the flavan-3-ol oligomers to depolymerize and yield monomeric anthocyanidin pigments (cyanidin) in strong acids (Butler, 1982a). Variations in condensed tannins can occur due to the number of monomers that become linked, the positions between which the linkage occurs, the oxygenation patterns on the A and B rings of the flavan-3-ol units, and the potential to add substituents to the ring hydroxyls, particularly the C-3 hydroxyl (Waterman & Mole, 1994).

Epicatechin and catechin are the units forming tannin of brown sorghum bran, with epicatechin forming the chain extension units and catechin as chain terminating units (Gupta & Haslam, 1978; Schofield, Hagerman & Harold, 1998). Catechin (Figure 1.2.5) is the most commonly reported monomer, while procyanidin B_1 (Figure 1.2.7) is the most common dimer found in sorghum (reviewed by Awika & Rooney, 2004).

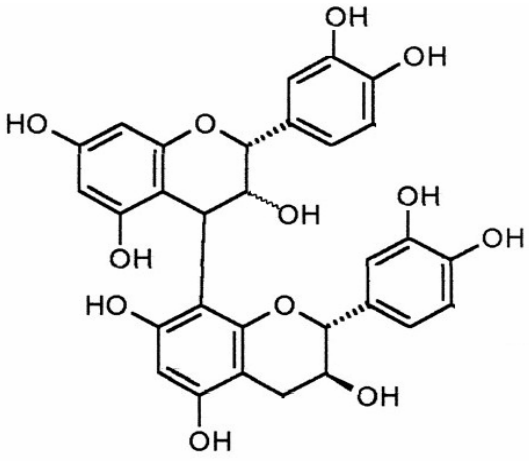


Figure 1.2.7. Structure of procyanidin B_1 (Awika & Rooney, 2004).

Tannins are responsible for astringency of some plants and are known to interact with, and precipitate proteins, hence they decrease nutritive value when present in the diet (Strumeyer & Malin, 1975; Hahn *et al.*, 1984). Due to their protein binding properties, tannins may also inactivate enzymes, and this could have negative implications in the sorghum beer brewing industry, in which enzyme activity is required for the brewing process. Tannins also contribute to the sour, bitter and astringent flavours of beer made from tannin sorghums (Bvochora, Danner, Miyafuji, Braun & Zvauya, 2005).

All sorghum varieties contain phenolic acids and most contain flavonoids, while only the high-tannin and bird-resistant varieties contain condensed tannins (Hahn *et al.*, 1984). Most of the phenolic compounds in the sorghum grain are concentrated in the outer layers (pericarp and testa) of the grain (Rooney *et al.*, 1980; Hahn *et al*., 1984; Youssef, 1998; Beta *et al.*, 2000). Higher amounts of phenolic compounds (10 to 100 times more) have been reported in the pericarp, glumes and leaf sheaths than in the endosperm (Waniska, 2000). Hahn & Rooney (1986) also reported insignificant amounts of extractable phenols in the endosperm.

1.2.3 Potential use of sorghum phenolic compounds as antioxidants

Phenolic extracts from several plant sources have been shown to have antioxidant activities. These include potato peels (Rodriguez de Sotillo *et al.*, 1994a & 1994b), tomatoes (Abushita, Hebshi, Daood & Biacs, 1997), garlic and ginger (Aruoma, Spencer, Warren, Jenner, Butler & Halliwell, 1997), sunflower seeds, wheat germ, fruits, vegetables and medicinal plants (Velioglu, Mazza, Gao & Oomah, 1998), rapeseed (Naczk, Amarowicz, Sullivan & Shahidi, 1998), tea (Matemu, 1998;

Wanasundara & Shahidi, 1998), sage and thyme (Abdalla & Roozen, 1999), plums (Chun, Kim, Moon, Kang & Lee, 2003), grape marc (Negro, Tommasi & Micelli, 2003), red clover (Kroyer, 2004), and tree spinach (Kuti & Konuru, 2004). Phenolic compounds from sorghum such as condensed tannin (Hagerman, Riedl, Alexander Jones, Sovik, Ritchard, Hartzfeld & Riechel, 1998; Awika *et al.* 2003) flavanones (such as naringenin) (Awika *et al.* 2003) and anthocyanins (Awika *et al.*, 2004) have also been reported to possess antioxidant activity. Sorghums, especially the phenol-rich sorghum bran, may therefore be considered potential sources of natural antioxidants for use in lipid foods such as edible oils to retard oxidation processes and increase shelf life.

1.2.4 Deterioration of fats and oils

Fats and oils deteriorate in quality with time. Changes that occur during deterioration include absorption of odours from surroundings (tainting), lipolysis and oxidation (Catsberg & Kempan-van Dommelen, 1990). Lipolysis is the enzymatic hydrolysis of ester linkages of lipids to release free fatty acids, resulting in the development of lipolytic or hydrolytic rancidity of the fats (Dugan, 1976; Nawar, 1996). Oxidative rancidity occurs when fats containing unsaturated fatty acids are oxidized, resulting in the breaking of the double bond of the unsaturated fatty acid molecules (McWilliams, 1997) and subsequent formation of various carbonyl compounds, hence a reduction in shelf life of the oil. Oxidative rancidity is therefore one of the major causes of lipid deterioration leading to the development of off-flavours and off-odours in edible oils and other lipid containing foods, which may also reduce the nutritional quality of foods (Madhavi, Deshpande & Salunkhe, 1996; Nawar, 1996).

Autoxidation (the reaction of lipids with molecular oxygen through a self-catalytic mechanism) is the main reaction involved in oxidative deterioration of lipids (Nawar, 1996). It is a free radical chain reaction that progresses in three stages: initiation reactions, propagation reactions and termination reactions (Gunstone & Norris, 1983; Gordon, 1990; Madhavi *et al.*, 1996; Stauffer, 1996; Coultate, 2002). During the initiation stage, highly reactive fatty acid molecules, namely free radicals ($R^{\bullet}$), are formed (reaction 1).

$$X^{\bullet} + RH \longrightarrow R^{\bullet} + XH \qquad (1)$$

These free radicals form after the reaction of an unsaturated fatty acid (RH) with a free radical species ($X^{\bullet}$) that initiates the autoxidation chain reaction sequence. This

initiator radical species ($X^{\bullet}$) is in most cases an alkoxyl radical ($RO^{\bullet}$) that is catalytically produced by the decomposition of hydroperoxides in the presence of trace metals (Gordon, 1990; Frankel, 1996; Nawar, 1996) or by exposure to light (Nawar, 1996). These hydroperoxides may exist in trace quantities before the oxidation reactions, and form when singlet oxygen ($^1O_2$) reacts with the double bond (C=C) of an unsaturated fatty acid (reaction 2, where RH is an unsaturated lipid and ROOH is hydroperoxide).

$$RH + {}^1O_2 \longrightarrow ROOH \qquad (2)$$

Singlet oxygen is a highly reactive, high-energy form of oxygen, which arises from reactions of low energy ground-state (triplet) oxygen ($^3O_2$) with pigments such as chlorophyll in the presence of light (Coultate, 2002).

Other decomposition products of the first hydroperoxides include hydroperoxyl ($HO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals (reactions 3 and 4, respectively), that are also reactive enough to abstract a hydrogen atom from monoenoic and polyenoic fatty acid residues (Coultate, 2002), hence may also initiate autoxidation.

$$ROOH \longrightarrow RO^{\bullet} + HO^{\bullet} \qquad (3)$$

$$2ROOH \longrightarrow RO^{\bullet} + ROO^{\bullet} + H_2O \qquad (4)$$

Trace metals, light (Dugan, 1976; Nawar, 1996), irradiation and heat are also known to initiate the formation of free radicals ($R^{\bullet}$).

In propagation reactions, atmospheric oxygen reacts with the highly reactive free radicals ($R^{\bullet}$) to form peroxyl radicals, $ROO^{\bullet}$ (reaction 5), or the peroxyl radicals ($ROO^{\bullet}$) formed in the initiation stage (decomposition of first hydroperoxides) react with the lipid to form hydroperoxides, ROOH, and $R^{\bullet}$ (reaction 6).

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \qquad (5)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \qquad (6)$$

The products $R^{\bullet}$ and $ROO^{\bullet}$ further react to propagate other free radical reactions, hence the chain reaction. As the peroxyl radicals ($ROO^{\bullet}$) react further, they produce an accumulation of hydroperoxides (ROOH). These hydroperoxides are the primary products of oxidation (Nawar, 1996; Fomuso, Corredig & Akoh, 2002), and are odourless and colourless (O'Brien, 2004). The hydroperoxides undergo decomposition (often catalyzed by transition metal ions or high temperatures) to form more free radicals that further propagate the chain reactions. Hydroperoxides may also decompose to form stable non-radical products in the form of volatiles such as aldehydes, ketones, alcohols and acids (Nawar, 1996; Coultate, 2002), which, due to their high volatility and low sensory threshold values give rise to signs of rancidity in the form of musty odours and undesirable flavours (Dugan, 1976; Nawar, 1996; Stauffer, 1996; McWilliams, 1997; Coultate, 2002; O'Brien, 2004).

In the final stage of autoxidation, namely termination, the free radicals arising from the reactions further react with each other to form stable non-radical products namely aldehydes, ketones, ethers, acids and other volatiles (reactions 7, 8, 9, 10, 11 and 12), as illustrated below (Dugan, 1976; Madhavi *et al.*, 1996):

$$R^{\bullet} + R^{\bullet} \longrightarrow R\text{-}R \qquad (7)$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \qquad (8)$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR \qquad (9)$$

$$RO^{\bullet} + R^{\bullet} \longrightarrow ROR \qquad (10)$$

$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR \qquad (11)$$

$$2RO^{\bullet} + 2ROO^{\bullet} \longrightarrow 2ROOR + O_2 \qquad (12)$$

1.2.5 Factors influencing the rate of oxidation of lipid foods

1.2.5.1 Fatty acid composition

Fats and oils with a high degree of unsaturation are more susceptible to lipid oxidation (Nawar, 1996; Tan, Che Man, Selemat & Yussof, 2002). According to the degree of

unsaturation of fatty acids, the relative rates of oxidation for oleic (C18: 1), linoleic (C18: 2), linolenic (C18: 3) and arachidonic acids (C18: 4) are 1, 12, 25 and 50, respectively (Stauffer, 1996). Chu & Kung (1998) found the oxidative stability of various oils (soybean, high oleic safflower, corn, olive and peanut) to be inversely related to the polyunsaturated fatty acid content, with linolenic acid (C18:3) having the most influence on the oxidative stability index, followed by linoleic acid (C18:2) and oleic acid (C18:1). Naz, Sheikh, Siddiqi & Sayeed (2004) reported increased peroxide and anisidine values of soybean, corn and olive oils in the order: soybean > corn > olive, due to their fatty acid composition. Soybean and corn oil have high relative reaction rates with oxygen due to their higher proportions of linoleic acid compared to olive oil, which has a high proportion of oleic acid. Neff, Selke, Mounts, Rinsch, Frankel & Zeitoun (1992) observed an increase in the formation of volatile compounds (pentane, propanal, pentanal, hexanal, *cis* and *trans* 2-heptenal, and 2,4-heptadienal), expressed as total volatiles, with increased concentration of linoleic and linolenic acids. Edible oils containing high proportions of linolenic or linoleic acid are hence more prone to oxidation than oils high in oleic acid.

1.2.5.2 Oxygen concentration and exposed surface area of the lipid

Availability of oxygen determines the rate of oxidation. This is true at low oxygen concentrations where the rate of oxidation increases with an increase in oxygen concentration, unlike when oxygen is abundant where the oxidation becomes independent of oxygen concentration (Nawar, 1996). Koelsch, Downes & Labuza (1991) showed that during the oxidation of soybean oil at 23°C, the formation of hexanal (an important volatile product of oxidation commonly used to determine the extent of lipid oxidation), as a function of time, increased with increasing oxygen concentrations. The rate of oxidation also increases with the surface area of the lipid that is exposed to air. Tan *et al.* (2002) observed that oils with a high surface-to-volume ratio had lower oxidative induction times than oils with a lower surface-to-volume ratio because of the fact that a larger surface area of the oil is exposed to oxygen. Minimizing exposure of the fats and oils to oxygen during processing (Schuler, 1990) and storage (Catsberg & Kempen-van Dommelen, 1990; Schuler, 1990), and reducing their surface-to-volume ratio can prevent their oxidation. Removal of residual oxygen by degassing, gas flushing with nitrogen or vacuum packaging may also be employed to reduce lipid oxidation (Koelsch *et al.*, 1991).

1.2.5.3 Temperature

Rate of lipid oxidation increases with an increase in temperature (Nawar, 1996). Suzuki, Yasui, Matsukura & Terao (1996) showed that lipid peroxides decomposed faster at higher temperatures (37^{o}C) than at lower temperatures (4^{o}C), which was confirmed by a corresponding increase in levels of carbonyls formed at higher temperatures. Naz *et al*. (2004) reported higher oxidative rates of soybean, corn and olive oil used for deep-frying at 180°C than oils exposed to air and light. Lowering the temperature could therefore reduce oxidation by slowing down the oxidative reactions; hence fats and oils kept at low temperatures would be less prone to oxidation.

1.2.5.4 Light

Edible fats and oils deteriorate faster when exposed to light due to its catalytic effect on lipid oxidation (Nawar, 1996; Eskin & Przbylski, 2001). Various studies have shown this catalytic effect of light on lipid oxidation (Abdalla, Tirzite, Tirzitis & Roozen, 1999; Gutierrez-Rosales, Garrido-Fernandez, Gallardo-Gurerrero, Gandul-Rojas & Minguez-Mosquera, 1992). The catalytic effect is due to the presence of natural pigments such as chlorophyll in a fat or oil, which act as photosensitizers and transfer energy (hv) from light to ground state (triplet) oxygen ($^{3}O_2$) to form singlet oxygen, $^{1}O_2$ (Gordon, 1990; Eskin & Przybylski, 2001) (reactions 13 and 14).

$$\text{Sensitizer}_{\text{Ground state}} + hv \longrightarrow \text{Sensitizer}_{\text{Excited}} \quad (13)$$

$$\text{Sensitizer}_{\text{Excited}} + {}^{3}O_2 \longrightarrow {}^{1}\text{Sensitizer}_{\text{Ground sate}} + {}^{1}O_2 \quad (14)$$

The singlet oxygen formed is highly reactive (Eskin & Przbylski 2001) and reacts with the double bond of an unsaturated fatty acid giving rise to hydroperoxides (as was previously shown in reaction 2), which can cleave and initiate a free radical chain reaction (Nawar, 1996). Oxidation of unsaturated fatty acids under the influence of light can therefore be prevented by using packaging that excludes light (Schuler, 1990; Talcott, Duncan, Del Pozo-Insfran & Gorbet, 2005), or storing the lipids in the dark (Catsberg & Kempen-van Dommelen, 1990).

1.2.5.5 Prooxidants

Prooxidants are substances that promote oxidation. Some known prooxidants include transition metals such as Fe, Cu, Mn and hematin compounds (Nawar, 1996) such as chlorophyll pigments. Various studies have reported increased oxidation of lipids in the presence of iron (Gordon & Weng, 1992; Luzia, Trugo, Da Paixao, Marcilio, De Maria & Quinteiro, 1998: Fomuso *et al.*, 2002). The transition metals catalyse oxidation by electron transfer (Jadhav, Nimbalkar, Kulkarmi & Madhavi, 1996) due to their oxidation-reduction potential (Nawar, 1996). They may:

(i) Catalyze decomposition of hydroperoxides (reactions 15 and 16)

$$M^{n+} + ROOH \longrightarrow M^{(n+1)+} + OH^- + RO^\bullet \quad (15)$$

or

$$M^{n+} + ROOH \longrightarrow M^{(n-1)+} + H^+ + ROO^\bullet \quad (16)$$

(ii) React with molecular oxygen to give singlet oxygen and peroxyl radical (17)

$$M^{n+} + O_2 \longrightarrow M^{(n+1)+} + O_2^- \quad \begin{matrix} \xrightarrow{-e^-} {}^1O_2 \\ \xrightarrow{+H^+} HO_2^\bullet \end{matrix} \quad (17)$$

(iii) React directly with an unoxidized, unsaturated fatty acid (reaction 18)

$$M^{n+} + RH \longrightarrow M^{(n-1)+} + H^+ + R^\bullet \quad (18)$$

The products of all the above reactions are more free radicals (e.g. $RO^\bullet$, $ROO^\bullet$, $R^\bullet$, $HO_2^\bullet$) or singlet oxygen ($^1O_2$), which may then promote further oxidation.

The presence of metals at concentrations as low as 0.1 mg/kg is known to increase the rate of oxidation (Nawar, 1996). Cu and Fe levels of less than 0.02 mg/kg and 0.1 mg/kg, respectively, are recommended for good oxidative stability of refined, bleached and deodorized oils in general (reviewed by Van der Merwe, 2003). The Codex Alimentarius Commission (2001) however allows higher tolerance levels of up to 0.1 mg/kg and 1.5 mg/kg for Cu and Fe respectively, in refined edible oils. Presence of the metals in vegetable oils arises due to their presence in the raw

materials originating from the soil in which the oil-bearing plant was grown, or from contact with metallic equipment used in manufacturing or storage (Garrido, Frias, Diaz & Hardison, 1994; Nawar, 1996). Chelating agents such as citric acid may be used to remove these metals and eliminate their prooxidant effect (O'Brien, 2004).

Chlorophyll pigments may also exert prooxidant effects. Green tea extracts were observed to exert a prooxidant effect in refined, bleached and deodorized seal blubber oil and menhaden oil (Wanasundara & Shahidi, 1998) due to the presence of chlorophyll pigments in the tea extracts.

1.2.5.6 Synthetic and natural antioxidants

Halliwell & Gutteridge (1989) defined an antioxidant as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". The primary purpose of adding antioxidants to lipids is to delay the onset of oxidation and accumulation of oxidative products (Wanasundara & Shahidi, 1998). Antioxidants may delay or inhibit oxidation, but cannot improve the quality of an already oxidized product (Kochhar & Rossell, 1990; Schuler, 1990). Antioxidants may act either as primary (chain breaking), or as secondary (preventative) antioxidants (Gordon, 1990). Some phenolic antioxidants are also classified as synergists due to their prominent effectiveness in the presence of other non-phenolic substances such as citric acid (Kikugawa, Kunugi & Kurechi, 1990).

Primary antioxidants such as BHA, BHT and TBHQ (Fig. 1.2.8) interfere with propagation reactions of autoxidation by converting propagating species to non-reactive ones to stop the chain propagation through the donation of a hydrogen atom to a free radical such as $ROO^{\bullet}$, as illustrated in reaction 19 (Frankel, 1996; Eskin & Przbylski, 2001). Primary antioxidants also react with alkoxyl radicals ($RO^{\bullet}$) to reduce the decomposition of hydroperoxides to degradation products (reaction 20).

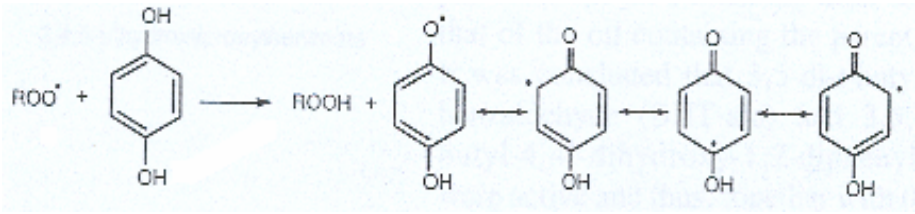
$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet} \quad (19)$$

$$RO^{\bullet} + AH \longrightarrow ROH + A^{\bullet} \quad (20)$$

(where AH is a phenolic antioxidant, $A^{\bullet}$ is antioxidant radical).

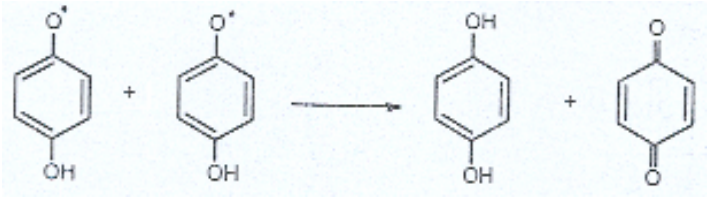
The antioxidant free radical formed ($A^{\bullet}$) is of low energy and is not reactive enough to continue the reaction sequence due to stabilization by resonance delocalization of

the unpaired electron around the aromatic ring of the antioxidant (Gordon, 1990; Nawar, 1996), and the lack of positions suitable for attack by molecular oxygen (Nawar, 1996). The phenolic nature of these primary antioxidants and the presence of the aromatic ring are of importance in their function of donation of hydrogen atoms to radicals and the stabilization of the unpaired electron by resonance, respectively. The resonance delocalization is further illustrated in reaction 21 by the reaction of a diphenolic antioxidant, such as TBHQ, with peroxyl radicals forming stable semiquinone resonance hybrids (Shahidi, & Wanasundara, 1992; Nawar, 1996).

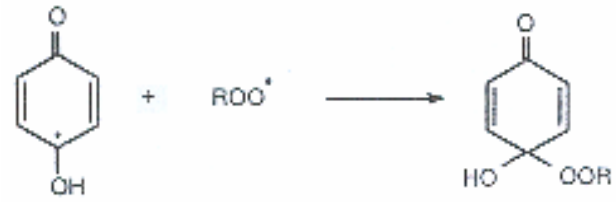


(21)

The antioxidant radicals (semiquinone radical intermediates) then undergo termination reactions (forming more stable products) by reacting with each other (reaction 22), or reacting with another peroxyl radical (reaction 23).



(22)

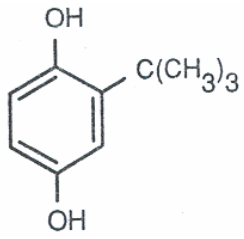


(23)

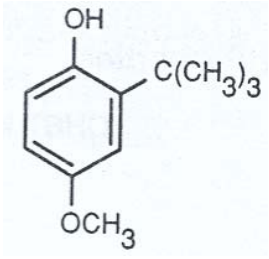
Secondary antioxidants prevent the formation of free radicals in the initiation step by scavenging oxygen, e.g. ascorbic acid, or chelating metal ions, e.g. citric acid (Gordon, 1990; Nawar, 1996) and flavonoids (Khokhar & Apenten, 2003). Some phenolic antioxidants have synergistic effects with ascorbic and citric acids (Kikugawa *et al.*, 1990). Ascorbic acid is primarily an effective oxygen scavenger (Gordon, 1990; Kikugawa *et al.*, 1990), but when used in combination with tocopherol gives rise to a stronger synergistic antioxidant effect. The synergistic effect of ascorbic acid with tocopherol is believed to be due to the ability of ascorbic acid to

regenerate tocopherol by reducing the tocopheroxyl radical resulting from the primary antioxidant effect of the tocopherol (Kikugawa *et al.*, 1990).

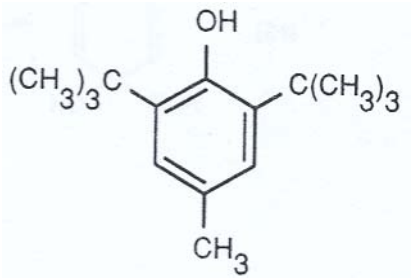
Synthetic antioxidants such as BHT, BHA, TBHQ and propyl gallate (Figure 1.2.8) are currently widely used in the food industry to increase the oxidative stability of fats and oils (Stauffer, 1996; Chu & Hsu, 1999; Moure, Cruz, Franco, J. M. Dominguez, Sineiro, H. Dominguez, Nunez & Parajo, 2001). The use of these synthetic antioxidants as food additives has however faced resistance in recent times due to accumulating evidence that they could be toxic, carcinogenic or mutagenic (Ito *et al.*, 1986; Lindenschimdit *et al.*, 1986; Whysner *et al.*, 1994; Frankel, 1996; Malecka, 2002). Due to these safety concerns, there has been a growing interest in the exploration of natural antioxidants. Naturally occurring antioxidants are presumed safe since they are found in food that has been used by man for centuries (Frankel, 1996). The natural antioxidants in food may also be beneficial because there is evidence that they prevent cancer development and that they inhibit oxidative reactions in the body system. For instance, the typical Mediterranean diet, which is rich in fruits and vegetables, has been linked to low occurrence of cardiovascular disease and cancer due in part to the high proportion of bioactive compounds such as flavonoids in the diet (Benavente-Garcia, Castillo, Lorente, Ortuno & Del Rio, 2000).



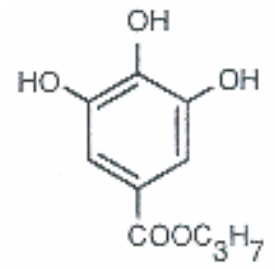
TBHQ



BHA



BHT



Propyl gallate

Figure 1.2.8. Chemical structures of some synthetic antioxidants used in food (Shahidi *et al.*, 1992)

Antioxidants for use in food however undergo strenuous evaluation to ensure that they satisfy economical, technological and toxicological requirements such as effectiveness at low concentrations, ease of incorporation in the food, should not impart flavour, odour or colour to the food, should be inexpensive, safe and non-toxic, and should not affect physical properties of the food (Dugan, 1976; Schuler, 1990). Antioxidants from natural sources would therefore have to satisfy these requirements for their acceptability and use in lipid foods.

1.2.6 Factors affecting antioxidant activity of phenolic compounds

1.2.6.1 Structure-activity relationships between phenolic compounds and antioxidant activity

Phenolic compounds have both primary and secondary antioxidant activities by acting both as chain breaking antioxidants by donating hydrogen atoms to lipid radicals (radical scavenging) or by preventing initiation reactions by such means as chelating metals (Rice-Evans *et al*., 1997; Luzia *et al*., 1998; Sugihara *et al*., 1999; Khokhar & Apenten, 2003). Both the chain breaking (radical scavenging) and metal chelating properties are known to be possible due to the structural features of the phenolic compounds.

The key structural features that have been shown to enable phenolic compounds exert radical scavenging activity are as follows:

(i) The number of phenolic hydroxyl groups on the phenolic compound molecule. Studies have shown that generally, the more the number of hydroxyl groups on a phenolic structure, the higher the radical scavenging capacity (Hudson & Lewis, 1983; Shahidi *et al.*, 1992; Hagerman *et al*., 1998; Milic, Djilas & Canadanovic-Brunet, 1998; Yamaguchi, Yoshimura, Nakazawa & Ariga, 1999; Van den Berg, Haenen, Van den Berg, van der Vijgh, & Bast, 2000). More hydroxyl groups on a phenolic compound provide more sites for radical scavenging, hence the increased antioxidant activity of compounds with multiple hydroxyl groups.

(ii) Presence of hydroxyl groups on carbon number 3 and 4 in the B ring (3', 4'-dihydroxyl catechol configuration) (Rice-Evans, Miller & Paganga, 1996 & 1997; Benavente-Garcia *et al*., 2000; Pietta, Simonetti, Gardana & Mauri, 2000). The strong radical scavenging activity by the 3, 4'-

dihydroxyl group in the B ring is due to its ability to provide highly stable phenoxyl radicals after the donation of a hydrogen atom (Bors, Heller, Michel & Saran, 1990). Some of the sorghum flavonoids i.e. luteolinidin, fisetinidin, luteolin, catechin, cyanidin and taxifolin (Figure 1.2.5) contain this configuration and therefore could contribute significantly to the antioxidant activity of sorghum phenolic extracts containing these flavonoids.

(iii) Presence of a hydroxyl group at C-3 in combination with a carbonyl (keto) group on C-4 of the C ring (R. van den Berg *et al*., 2000) such as in taxifolin (Figure 1.2.5). This may be due to maximal radical scavenging potential and strong radical absorption due to hydrogen bonding between the OH group at C-3 and the carbonyl group at C-4 (Bors *et al*., 1990).

(iv) The unsaturation of the C ring such as a double bond between C-2 and C-3 in conjunction with the 4-carbonyl group on the C ring (such as in luteolin – Figure 1.2.5), or in combination with a 3-hydroxy group on the C ring (Rice-Evans *et al*., 1996). This structural feature allows the delocalization of an electron from phenoxyl radicals on the B ring to the C ring. A 2,3 double bond in combination with a 3-hydroxy group on the C ring also results in increased antioxidant activity because the resonance stabilization for electron delocalization across the molecule is increased (Bors *et al*., 1990; Benavente-Garcia *et al*., 2000; R. van den Berg *et al*., 2000).

On the contrary, methoxylation of hydroxyl groups (introduction of a $–OCH_3$ group into the phenolic structure, replacing the OH group) decreases antioxidant activity (Pietta *et al*., 2000). This reduction in the antioxidant activity may be attributed to differences in redox potential of the OH groups and the substituted groups (such as the $–OCH_3$ group) on the phenyl ring. Hydroxyl groups have lower redox potential, which makes them good electron-donating groups (i.e. good reducing agents), hence increasing the efficacy of phenolic antioxidants. Substituted groups such as $–OCH_3$ however increase the redox potential of phenols, hence reducing their antioxidant potential due to a reduction in their ability to donate electrons (Pietta *et al*., 2000).

Phenolic compounds, flavonoids in particular, are able to chelate metals due to the presence of hydroxyl groups attached to their ring structures (Rice-Evans *et al*., 1997; Luzia *et al*., 1998; Sugihara *et al*., 1999; Khokhar & Apenten, 2003). The complexed

oxidized forms of metal are more stable and become more difficult to reduce resulting in a decrease in its tendency to take on electrons (Christian, 1986; Halliwell, Aeschbach, Loringer & Aruoma, 1995). Three possible metal complexing positions on a flavonoid molecule that can interact with metal ions by hydrogen bonding have been proposed: (i) between the 3', 4'-dihydroxy group located on the B ring (as in luteolinidin, fisetinidin and luteolin – Figure 1.2.5); (ii) between the 3-hydroxy and 4-carbonyl group in the C ring (as in taxifolin – Figure 1.2.5); and (iii) between the 5-hydroxy of the A ring and 4-carbonyl group in the C ring (as in naringenin, taxifolin, eriodictoyl and luteolin – Figure 1.2.5) (Rice-Evans *et al*., 1997; Luzia *et al*., 1998; Sugihara *et al*., 1999; Khokhar & Apenten, 2003). Various flavonoids found in sorghum may therefore be able to chelate metal ions due to the possible metal complexing positions.

1.2.6.2 Concentration of phenolic compounds and antioxidant activity

Some antioxidants provide increased protection with increasing concentration, while others have optimal levels after which higher levels exert prooxidant effects (Dugan, 1976). Moure *et al*. (2001) reported that there is a general trend for antioxidant activity to increase with the concentration of the antioxidant up to a certain limit, depending on the antioxidant and the test used, and that most natural extracts and tests have shown a maximum antioxidant activity at a 0.05% concentration. Various phenolic extracts from plant sources have been shown to increase in antioxidant activity with increasing concentration of the extract (Gordon & Weng, 1992; Onyeneho & Hettiarachchy, 1992; Negro *et al*., 2003). Gordon & Weng (1992) showed that induction periods of lard samples increased as concentration of hexane extracts of powdered tanshen (*Salvia miltiorrhiza Bunge*). Onyeneho & Hettiarachchy (1992) also found out that increasing the concentration of freeze-dried extracts from the bran of durum wheat led to increased antioxidant activity shown by a decrease in peroxide values of soy oil. Similarly, ethanolic extracts from red grape marc, peels and seeds showed increased antioxidant activity with increased extract concentration (Negro *et al*., 2003). The increase in antioxidant activity of phenolic compounds due to the increase in their concentration could be due to an increase in the concentration of potential sites for radical scavenging. On the other hand, at high concentrations, prooxidant effects could arise due to the involvement of the phenolic compounds in initiation reactions (formation of radicals) as shown in reactions 24 and 25 (Gordon, 1990).

$$AH + O_2 \longrightarrow A^{\bullet} + HOO^{\bullet} \quad (24)$$

$$AH + ROOH \longrightarrow RO^{\bullet} + H_2O + A^{\bullet} \quad (25)$$

(where AH is phenolic antioxidant, $A^{\bullet}$ is antioxidant radical, ROOH is hydroperoxide and $RO^{\bullet}$ is alkoxy radical).

The antioxidant radical ($A^{\bullet}$) formed during the antioxidant action of the phenolic antioxidant may also react with a hydroperoxide to form peroxyl radicals ($ROO^{\bullet}$) (reaction 26).

$$A^{\bullet} + ROOH \longrightarrow ROO^{\bullet} + AH \quad (26)$$

1.2.6.3 Stability of phenolic compounds after extraction

In order to use sorghum as a source of natural antioxidants, the phenolic compounds have to be extracted and converted into a dry and relatively stable form by, for example, freeze-drying. Stability of the extracts during storage is therefore of significance.

Phenolic compounds are compartmentalized in the cell matrix, but once the cell matrix is broken during the extraction of phenolic compounds, the compounds become prone to degradation. It has been shown that temperature, light (Rodriguez *et al*., 1994a), oxygen (Waterman & Mole, 1994; Haslam *et al*., 1992), enzymes (Murkovic, 2003) and pH (Friedman & Jurgens, 2000) affect stability of phenolic compounds. The changes that may occur may in turn affect the antioxidant properties of the phenolic compounds.

Phenolic compounds can be degraded through enzymatic or chemical oxidation that may occur during the extraction process. Plant tissues contain the enzyme polyphenol oxidase, which when in contact with phenolic compounds in the presence of oxygen as the plant tissues disintegrate, may catalyze the oxidation of the phenols to quinones (Murkovic, 2003). The phenolic compounds can also be oxidized non-enzymatically in which the phenol group can be transformed into a quinone upon exposure to air (Waterman & Mole, 1994; Haslam *et al.,* 1992). The quinones formed can subsequently undergo rapid non-enzymatic polymerisation that would change the

original nature of the phenolic compound, hence causing changes in its chemical properties. For instance, the disintegration of plant tissues in potatoes releases polyphenol oxidase, which reacts with phenolic compounds in the presence of oxygen resulting in the formation of polymerized brown compounds (enzymatic browning) (Kaaber, Martinsen, Brathen & Shomer, 2002). The non-enzymatic oxidation of phenolic compounds would however be the more likely mechanism in sorghum since polyphenol oxidase is not important in sorghum. For sorghum, the possible polymerization of extracted phenolic compounds due to exposure to oxygen may therefore be of significance if the phenolics are intended for use as antioxidants.

It would appear that liquid phenolic extracts and freeze-dried phenolic extracts both have good storage stability at a temperature range of 4 – 37°C when stored in the dark, while significant degradation occurs when these are stored exposed to light. For instance, Rodriguez de Sotillo *et al.* (1994a) reported no major changes in concentration of phenolic acids in liquid potato peel liquid extracts stored at 4°C and 37°C in the dark, but the extracts stored at 25°C exposed to light showed degradation of chlorogenic acid to caffeic acid after 7 days of storage. The concentration of caffeic acid also started decreasing from day 9 of storage, and none was detected after 20 days of storage. Similar results on the effect of light on phenol content and antioxidant activity of extracts from plant sources have been reported (Rodriguez de Sotillo *et al.*, 1994b; Mansour & Khalil, 2000). Degradation of the phenolic compounds in the presence of light is generally accompanied by a reduction in antioxidant potency (Mansour & Khalil, 2000). Based on this observed effect of light on phenolic acids, it could hence also be expected that flavonoids may similarly be degraded under the influence of light.

Stability of phenolic compounds however also appears to be greatly affected at temperatures higher than 50°C and may lead to a decrease in antioxidant activity (Azizah, Ruslawati & Swee Tee, 1999; Larrauri *et al.*, 1998). Rates of chemical reactions increase as temperature increases; the reduction in the antioxidant activity of phenolic extracts at these high temperatures may be due to increased oxidation of the phenolic compounds at the high temperatures. Handling or storing the phenolic compounds at low temperatures may therefore prevent their oxidation.

1.2.7 Assessment of quality of oils

One of the most important indicators of the quality of oils is their oxidative stability (Tan *et al*., 2002), which is the resistance of oil to oxidation (Guillen & Cabo, 2002). Methods used to determine the extent of oxidation of oils are based on the measurement of the concentration of the primary or secondary products of oxidation. Various methods such as oxidative stability index (OSI) (Chu & Hsu, 1999; Tan *et al*., 2002), Differential Scanning Calorimetry (Rudnik, Szcucinska, Gwardiac, Szuk & Winiarska, 2001; Tan *et al*., 2002), Rancimat method (Rudnik *et al*., 2001), the measurement of conjugated diene hydroperoxides, volatile compounds such as aldehydes, anisidine values (Abou-Gharbia, Shehata & Shahidi, 2000; Guillen & Cabo, 2002) and the measurement of peroxide values (Abdalla & Roozen, 1999; Rudnik *et al*., 2001) have been used for assessing the extent of oxidation of edible oils. These methods employ accelerated oxidation conditions such as exposing the oil at a determined constant temperature, flow of air or the presence or absence of light and catalysts (Guillen & Cabo, 2002) and assessing the extent of oxidation. The measurement of peroxide values (PV) and anisidine values (AV) can hence be used to monitor the formation of primary and secondary oxidation products respectively, as a means of determining the quality of oils.

1.2.7.1 Peroxide value

Since hydroperoxides are the primary products of lipid oxidation, PV (a measure of hydroperoxides concentration) is in most cases used as an indicator of the initial stages of oxidation (O'Brien, 2004). PV gives a good guide to the quality of a fat or oil (Rossell, 1986), and is based on an iodometric titration of iodine (I_2), which is produced when an excess of potassium iodide (KI) is added to oil. The peroxides are reacted with potassium iodide in the presence of acetic acid, liberating iodine (reactions 27 and 28), which is produced in equivalent amount to the peroxides present in fat or oil. The iodine is then titrated with standard sodium thiosulfate, $Na_2S_2O_3$ (Christian, 1986) (reaction 29).

$$2KI + 2CH_3COOH \longrightarrow 2HI + 2CH_3COO^{\bullet}K^{+} \quad (27)$$

$$ROOH + 2HI \longrightarrow ROH + H_2O + I_2 \quad (28)$$

$$I_2 + 2Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2NaI \quad (29)$$

A wide range of PVs have been reported which could lead to a fat or oil developing signs of rancidity resulting from the decomposition of the hydroperoxides. Rossell (1986) indicated that a freshly refined fat should have a PV of less than 1 milli-equivalent/kg (meq/kg) of the fat, and fats that have been stored for some time after refining could have a PV of up to 10 meq/kg before off-flavours are encountered. The Codex Alimentarius Commission (2001) similarly allows up to a maximum PV of 10 meq/kg for refined oils in general. O'Brien (2004) on the other hand outlined the following classification specifically for soybean oils: a PV of 1 or less indicates freshness; 1 to 5, low oxidation; 5 to 10, moderate oxidation; greater than 10, poor flavour.

However, it appears that storage temperature affects the perception of rancidity or off-flavour in relation to PV. At lower storage temperatures oils develop rancidity (or are perceived to be rancid) at higher levels of PV. For instance, Semwal & Arya (1992) (according to Van der Merwe, 2003) did not detect off-flavours in sunflower oil stored for two years at room temperature even when the PV increased from 8.5 to 22.6. Yousuf Ali Khan, Lakshminarayana, Azeemoddin, Atchyuta Ramayya & Thirumala (1979) (according to Van der Merwe, 2003) only noted development of detectable off-odours in sunflower oil stored at ambient temperature when PV reached 25 meq/kg oil. On the other hand, at higher storage temperatures rancidity is detected at lower PVs. The average PV of soybean, low erucic acid rapeseed and sunflower oils after eight days of storage at 60°C when they were perceived rancid were 8.3, 11.0 and 13.6 respectively (Warner, Frankel & Mounts, 1989). In general, it appears that at a higher storage temperature rate of decomposition of hydroperoxides is much faster, which could lead to early development of rancidity than at lower temperatures (Warner *et al*., 1989).

Another consideration worth noting when interpreting PVs and development of rancidity in oil samples is that a low PV may not always be an indication of good quality oil. It is well known that during oxidation of fats and oils, the initial rates of formation of hydroperoxides exceeds the rate of their decomposition, and the relative rates of these reactions are reversed during the later stages of oxidation (Yang, Quiang, Morehouse, Rosenthal, Ku & Yurawecz, 1991). Fomuso *et al.* (2002) also showed that an increasing incubation time brought about a decrease in the PV, which they attributed to hydroperoxide decomposition rates being greater than formation

rates. Measurement of hydroperoxides alone may therefore not always provide an accurate evaluation of the oxidative stability of fats and oils because peroxides are not stable and decompose to volatile products. According to Dugan (1976) and O'Brien (2004), measurement of PV may only be useful during the initial stages of oxidation before extensive decomposition of hydroperoxides begins.

1.2.7.2 Anisidine value

The anisidine test determines the level of secondary oxidation products, i.e. aldehydes (principally 2-alkenals), present in oils (Rossell, 1986; O'Brien, 2004), hence it is a useful test in the study of oxidative deterioration of oils. The method is based on the reaction of the volatile aldehydic compounds with *p*-anisidine reagent in an iso-octane solution and the spectrophotometric determination of the reaction products at 350 nm (IUPAC, 1979). Hexanal is one of the important volatile products that is used as a marker for the oxidative decomposition of oils (Abdalla & Roozen, 1999). According to Rossell (1986), the AV of good oil should be less than 10.

1.2.8 Methods for the determination of total phenols, condensed tannins and antioxidant activity of sorghum extracts

1.2.8.1 Determination of total phenols

Measurement of total phenol content quantifies the total concentration of phenolic hydroxyl groups present in an extract being assayed, regardless of the specific molecules in which the hydroxyl groups occur (Waterman & Mole, 1994). The assays used to measure total phenols do not therefore provide information on the particular phenolic compounds present in a sample. Some common methods for the measurement of total phenols include the colorimetric Prussian blue, Folin-Denis (Waterman & Mole, 1994; Deshpande, Cheryan & Salunkhe, 1986), ferric ammonium citrate, or Folin-Ciocalteu methods (Waterman & Mole, 1994).

The Folin-Ciocalteu method however appears to be the method widely used for the determination of total phenols (Waterman & Mole, 1994). The method is based on the reducing power of the phenolic hydroxyl groups (Hahn *et al*., 1984), which react with the Folin-Ciocalteu phenol reagent (an oxidizing agent comprised of heteropolyphosphotungstate-molybdate) under basic conditions to form chromogens that can be detected spectrophotometrically at 760 nm. The reagent is more sensitive

to reduction by phenolics, and the addition of lithium sulfate to the reagent makes it less prone to precipitation and interference by non-phenolics (Waterman & Mole, 1994).

1.2.8.2 Determination of condensed tannins

The methods used for the determination of total phenols such as the Prussian blue and Folin-Denis methods have also been employed in the analysis of tannins but are not specific for condensed tannins because they detect all phenolic hydroxyl groups regardless of the type of phenolic compound (Hahn *et al*., 1984; Deshpande *et al*., 1986). Other methods used include protein precipitation (Hahn *et al*., 1984; Deshpande *et al*., 1986), vanillin-HCl (Price, van Scoyoc & Butler, 1978; Hahn *et al*., 1984; Waterman & Mole, 1994), and proanthocyanidin (butanol-HCl) method (Waterman & Mole, 1994). The protein precipitation methods are biochemical techniques that are based on the ability of the tannins to form insoluble complexes with proteins. For screening purposes however, chemical assays are preferred (Butler, 1982b). The widely used assay for sorghum tannins is the vanillin-HCl method (Price *et al*., 1978), and is specific for condensed tannins (Waterman & Mole, 1994). The method is based on the ability of the condensed tannin units to react with the vanillin reagent in the presence of mineral acids to produce a red colour that is measured spectrophotometrically. The vanillin reagent reacts with flavanols with a single bond between C-2 and C-3 of the C ring (Earp, Akingbala, Ring & Rooney, 1981), and also with the free meta-oriented OH groups on the B ring, and since condensed tannins are condensation products of flavan-3-ols and flavan-3,4-diols, they give a positive reaction with vanillin (Gupta & Haslam, 1980). A blank subtraction is done to correct for non-tannin compounds that give a positive vanillin reaction. Some authors (Earp *et al.,* 1981) have however shown that the use of the vanillin-HCl method with or without blanks is both useful for estimating tannin levels in sorghum.

1.2.8.3 Determination of antioxidant activity

Several methods have been described to assess antioxidant capacity of compounds both *in vivo* and *in vitro,* such as the measurement of prevention of oxidative damage to biomolecules such as lipids or DNA, and methods assessing radical scavenging ability (Halliwell *et al*., 1995). The following *in vitro* methods have been described: oxygen radical absorbance capacity (ORAC) method (Cao, Alessio & Culter, 1993), thin layer chromatography (Duve & White, 1991; Onyeneho & Hettiarachchy, 1992), Trolox Equivalent Antioxidant Capacity (TEAC) assay (Re, Pellegrini, Proteggente,

Pannala, Yang & Rice-Evans, 1999; Van den Berg, Haenen, Van den Berg & Bast, 1999), β-carotene-linoleate model system (Wanasundara, Amarowic & Shahidi, 1996; Negro *et al*., 2003) and free radical scavenging using 2,2-diphenyl-1picryhydrazyl (DPPH) (Brand-Williams, Cuvelier & Berset, 1995).

Much attention however is being focused on determination of antioxidant capacity of compounds using the TEAC assay (Van den Berg *et al*., 1999; Van den Berg *et al*., 2000; Awika *et al.,* 2003). The assay measures the relative ability of an antioxidant to scavenge the 2,2'-azinobis (3-ethyl-benzothiazoline-6 sulfonic acid) radical cation ($ABTS^{\bullet+}$) generated in aqueous phase, as compared with Trolox (water soluble vitamin E analogue) standard. Reacting a strong oxidizing agent such as potassium permanganate or potassium persulfate with the ABTS salt generates the $ABTS^{\bullet+}$. The extent of the decolorization of the blue-green $ABTS^{\bullet+}$ radical cation by hydrogen donating antioxidant is measured spectrophotometrically at 734 nm. The assay is applicable for assessing antioxidant capacity of single compounds, food components, and food extracts as well as biological systems (R. van den Berg *et al*., 1999), and is applicable to both aqueous and lipophilic systems (Arnao, Cano & Acosta, 2001). It is also a relatively low cost and easy to use method (Awika *et al.,* 2003).

1.2.9 Gaps in knowledge

It has been shown from the preceding sections that sorghum (in particular, its bran) contains phenolic compounds with antioxidant properties (Hagerman *et al*., 1998; Awika *et al*., 2003; Awika & Rooney, 2004). With the growing interest in natural sources of antioxidants to replace synthetic ones currently used in the food industry to retard lipid oxidation of fats and oils and other lipid containing foods, numerous studies have been done and reported on the potential of plants as sources of natural antioxidants in lipid foods (Gordon & Weng, 1992; Wanasundara & Shahidi, 1998; Medina, Tombo, Satue-Gracia, German & Frankel, 2002; Naz *et al*., 2004). However, the use of sorghum as a source of natural antioxidants in lipid foods such as edible oils has not been studied extensively. Some brown and black sorghum brans have however been shown to have better antioxidant properties than some plants sources such as fruits (Awika & Rooney, 2004). During the utilization of sorghum the bran is removed from the kernel and often disposed as a waste product. Sorghum bran may therefore be a potentially cheap source of phenolics for use as natural antioxidants.

The antioxidant properties of phenolic compounds are due to their ability to scavenge lipid radicals as well as chelate prooxidant metal ions. Information regarding the effect of sorghum phenolic compounds on the oxidation of edible oils in the presence or absence of prooxidant metal ions appears to be lacking.

In order to assess the effectiveness of phenolic compounds as antioxidants, the compounds have to be extracted from the plant matrix. Once separated from the plant matrix, the phenolic compounds are prone to degradation (Haslam *et al*., 1992; Rodriguez de Sotillo, *et al*., 1994a; Waterman & Mole, 1994; Murkovic, 2003), which may lead to the reduction in the phenolic content as well as the antioxidant properties. Therefore, proper handling of the phenolic compounds during extraction, storage and use is important. Temperature, light (Rodriguez de Sotillo, 1994a) and oxygen (Haslam *et al*., 1992; Waterman & Mole, 1994) are some of the factors that affect the stability and antioxidant activity of phenolic compounds. A number of studies reported in literature on the stability of phenolic compounds during storage have either been done on liquid phenolic extracts (Rodriguez de Sotillo *et al*., 1994a; Mansour & Khalil, 2000) or on model solutions (Nicoli, Calligaris & Manzocco *et al*., 2000; Pinello, Manzocco, Nunez & Nicoli, 2004). Information on the stability of freeze-dried phenolic extracts from sorghum under various storage conditions is however scarce.

1.3 Hypotheses

1.3.1 Condensed tannin sorghum (*Phatafuli) would* contain higher total phenol contents and have higher antioxidant activity than condensed tannin-free sorghum (*Shabalala*). Condensed tannins contain multiple phenolic hydroxyl groups (the reactive functional group for total phenol assays such as the Folin-Ciocalteu method) compared to simple phenolics, and would therefore give higher contents of total phenols. More hydroxyl groups on a phenolic compound provide more sites for radical scavenging; therefore higher antioxidant activity.

1.3.2 Oxidizing conditions brought about by the bubbling of oxygen in the extracts and packaging of the freeze-dried extracts, as well as storage of the freeze-dried extracts at higher temperature would make the extracts from condensed tannin sorghum bran unstable in terms of phenolic content and antioxidant activity. The presence of oxygen could lead to oxidation of phenolic

compounds and reduction in antioxidant activity. Higher temperature may increase the rate of oxidation.

1.3.3 Freeze-dried phenolic extracts from condensed tannin sorghum bran will retard oxidation of sunflower oil due to the ability of the phenolic compounds to scavenge free radicals by donation of hydrogen atoms, accompanied by resonance stabilization of phenolic antioxidant radicals, and by chelation of ferric ions.

1.4 Objectives

1.4.1 To determine the phenolic contents and antioxidant activities of extracts from condensed tannin and condensed tannin-free sorghum varieties, and to determine if these two parameters are correlated.

1.4.2 To determine the effect of storage temperature, time of storage and oxidizing conditions on the phenolic contents and antioxidant activity of freeze-dried phenolic extracts from the bran of condensed tannin sorghum.

1.4.3 To determine the antioxidative effect of freeze-dried phenolic extracts from the bran of condensed tannin sorghum on the oxidative stability of sunflower oil in the presence or absence of added ferric ions.

CHAPTER 2

2 RESEARCH

2.1 Phenolic contents and antioxidant activity of Malawian condensed tannin and condensed tannin-free sorghums

2.1.1 Abstract

Whole grain and bran samples of two Malawian sorghums, *Phatafuli*, (a brown-coloured condensed tannin variety) and *Shabalala*, (a white-coloured condensed tannin-free variety) were analysed for their content of total phenols, condensed tannins and antioxidant activities using the Folin-Ciocalteu, Vanillin-HCl and the Trolox Equivalent Antioxidant Capacity (TEAC) assays, respectively. A Chlorox bleach test revealed the presence of a pigmented testa in *Phatafuli* while no pigmented testa was detected in *Shabalala* grain. The *Phatafuli* variety had higher total phenols and antioxidant activity than the *Shabalala* variety both in the whole grain and the bran. For both sorghum varieties, the bran contained higher levels of total phenols and antioxidant activity than the whole grain. *Phatafuli* bran contained higher levels of condensed tannins than the whole grain. No condensed tannins were detectable in *Shabalala* bran and whole grain. Antioxidant activities of the sorghum varieties correlated highly with their total phenol and condensed tannin contents, suggesting that these phenolic compounds may be largely responsible for the observed antioxidant activities of the sorghum grains.

Key words: Sorghum; Bran; Whole grain; Antioxidant activity; Total phenols; Condensed tannins

2.1.2 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is a major cereal crop grown mainly in the semi-arid regions of the world, and widely used as a food crop in Africa and Asia (Strumeyer & Malin, 1975; Hahn, Rooney & Earp, 1984; Waniska, 2000). Sorghum contains phenolic compounds, which have both beneficial and undesirable properties. The phenolic compounds in sorghum are associated with a number of agronomic advantages such as bird resistance due to astringency, hence preventing pre-harvest losses due to bird predation, and also post-harvest losses due to storage pests (Hahn *et al*., 1984; Waniska, Poe & Bandyopadhyay, 1989). On the other hand, phenolic compounds in sorghum, especially tannins, are known to decrease the nutrient value of sorghum due to their ability to bind proteins, rendering them unavailable in the diet (Butler, 1981; Francis, 2000). The phenolic compounds also affect the colour and appearance of the grain and sorghum products (Hahn *et al*., 1984).

Recently there has been much interest in research on phenolic compounds from plant sources in the fields of medicine and food science due to their antioxidant properties. Phenolic compounds can be used as antioxidants in the food industry to increase shelf life of lipid-containing food by retarding lipid oxidation, and they are also known to have long-term health benefits in reducing some chronic degenerative diseases, cardiovascular disease and cancer (Murkovic, 2003). Several plant sources contain natural antioxidants in the form of phenolics (Rodriguez de Sotillo, Hadley & Holm, 1994; Tsaliki, Lagouri & Doxastakis, 1999; Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003; Chun, Kim, Moon, Kang & Lee, 2003; Kroyer, 2004; Kuti & Konuru, 2004) some of which are as effective as synthetic ones (Marinova & Yanishlieva, 1997; Abdalla & Roozen, 1999). Antioxidant activity of these phenolics is mainly due to their reducing properties, which allows them to act as hydrogen donors and singlet oxygen quenchers (Rice-Evans, Miller, & Paganga, 1996).

The interest in phenolic compounds as antioxidants in food has grown due to the limitations of synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ), which are currently in use. There is accumulating evidence that these synthetic antioxidants could be toxic, carcinogenic or mutagenic (Ito, Hirose, Fukushuma, Tsuda, Shirai & Tatematsu, 1986; Lindenschimdit, Tryka, Goad & Witschi, 1986; Whysner, Wang,

Zang, Iatropoulos & Williams, 1994), resulting in increasing interest in antioxidants from natural sources. Natural antioxidants are presumed safe since they are found in food that has been used by man for centuries (Frankel, 1996).

Phenolic compounds in sorghum may be broadly categorised into phenolic acids, flavonoids and condensed tannins (Waniska, 2000). All sorghum varieties contain phenolic acids and most contain flavonoids, while only the high-tannin and bird-resistant varieties contain condensed tannins (Hahn *et al*., 1984). White sorghum varieties without pigmented testa contain the lowest amount of phenolic acids (Waniska *et al.*, 1989). These phenolic compounds in sorghum are largely concentrated in the outer layers of the grain (pericarp and testa) (Rooney, Blakely, Miller & Rosenow, 1980; Hahn *et al*., 1984; Hahn & Rooney, 1986; Youssef, 1998; Beta, Rooney, Marovatsanga & Taylor, 1999). The pericarp and testa are often removed and disposed of as bran during utilization of the sorghum; hence sorghum bran could potentially be a cheap source of phenolic compounds for use as antioxidants.

The objectives of this study were therefore to determine the phenolic contents and antioxidant activities of two Malawian sorghum varieties (condensed tannin and condensed tannin-free), and also to determine if there was any relationship between phenolic contents and antioxidant activities.

2.1.3 Materials and methods

2.1.3.1 Materials

Approximately 50 kg of each of a brown-coloured condensed tannin sorghum variety (*Phatafuli*) and a white-coloured condensed tannin-free sorghum variety (*Shabalala*) (Figure 2.1.1) grown during the 2002/2003 season on a local farm in the Chikwawa district of Malawi were obtained through Kasinthula Research Station, Ministry of Agriculture and Irrigation, Malawi.

Phatafuli

Shabalala

Figure 2.1.1. Condensed tannin sorghum (*Phatafuli*) and condensed tannin-free sorghum (*Shabalala*) used in the study.

Folin-Ciocalteu's reagent, sodium carbonate (anhydrous), catechin, potassium persulfate, 2, 2'–Azinobis- (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and 6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylicacid (trolox) were obtained from Sigma-Aldrich (Steinheim, Germany), tannic acid was obtained from Merck (Johannesburg, South Africa), di-sodium hydrogen orthophosphate (anhydrous) was obtained from Saarchem – Holpro Analytic (Pty) Ltd (Johannesburg, South Africa), while vanillin and sodium dihydrogen orthophosphate (anhydrous) were obtained from Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa). Distilled, deionized water was used in all analyses, and all other chemicals and solvents used were of analytical grade.

2.1.3.2 Methods

2.1.3.2.1 Sample preparation

The sorghum samples were hand cleaned and sorted to remove broken, diseased or insect-infested grain, glumes and other foreign material. Sorghum grain of each variety (10 kg) was then decorticated using a sorghum dehuller (Rural Industries Innovation Centre, Kanye, Botswana). The bran obtained (approximately 10% decorticated bran yield) and portions of whole grain for the two varieties were separately ground to pass a 0.5 mm sieve using a Laboratory Mill 3100 (Falling Number, Huddinge, Sweden). The sorghum whole grain and bran samples were vacuum packed and stored at –20°C until analyzed (Awika *et al*., 2003).

2.1.3.2.2 Chlorox bleach test

The chlorox bleach test was administered to determine presence or absence of a pigmented testa in the sorghum grains using a simplified method of Waniska, Hugo & Rooney (1992). Sorghum kernels (100) were placed in a glass beaker and soaked with Chlorox reagent (5% NaOH in commercial household bleach, 3.5% sodium hypochlorite). The beaker was left to stand for 20 min at room temperature, with swirling of the contents at 5-min intervals. The bleaching reagent was discarded and the sorghum kernels rinsed with tap water, then dried on a paper towel. The bleaching causes constituents in the pericarp and testa to oxidize and yield black pigments on the surface of the caryopsis (Waniska *et al*., 1992). Presence of a black-pigmented testa layer indicated presence of condensed tannins, while absence of a black-pigmented testa indicated absence of condensed tannins. Numbers of kernels with pigmented testa were counted and results were expressed as a percentage. Condensed tannin (DC 99) and condensed tannin-free (NK 283) sorghums were used as standards.

2.1.3.2.3 Determination of total phenols

Aqueous acetone (75%) was used as the extracting solvent for the determination of total phenols (Kaluza, McGrath, Roberts & Schroeder, 1980). Duplicate 0.25 g whole grain and bran samples of condensed tannin and condensed tannin-free sorghum varieties were extracted using 15 ml of the solvent as follows: 5 ml of solvent was added to 0.25 g sample in glass centrifuge tubes and the samples were vortex mixed every 10 min for 2 h to improve extraction efficiency. The samples were then centrifuged at 3,500 rpm for 10 min (25°C) using a Medifriger centrifuge (J. P. Selecta, s. a.) and decanted. Each sample residue was rinsed twice with 5 ml of solvent, vortex mixed for 5 min, centrifuged as above and decanted. The three supernatants were then combined and used for analysis.

The Folin-Ciocalteu method (Singleton & Rossi, 1965) as modified by Waterman & Mole (1994) was used to determine total phenols in the sorghum grain and bran extracts. This method is based on the reducing power of the phenolic hydroxyl groups (Hahn *et al*., 1984), which react with the Folin-Ciocalteu phenol reagent to form chromogens that can be detected spectrophotometrically. The 75% aqueous acetone phenolic extract (0.5 ml) was added to a 50 ml volumetric flask containing distilled water and mixed. Folin-Ciocalteu phenol reagent (2.5 ml) was then added and mixed, followed by 7.5 ml sodium carbonate solution (20 g/100 ml) within one to eight

minutes after addition of the Folin-Ciocalteu phenol reagent. The contents were mixed and the flask made up to volume with distilled water, stoppered and thoroughly mixed. Absorbance of the reactants was read after 2 h (from addition of the sodium carbonate solution) at 760 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). Tannic acid was used as a standard and results were expressed as mg tannic acid equivalents/100 mg of sample, dry weight basis.

2.1.3.2.4 Determination of condensed tannins

The vanillin-HCl method of Price, Van Scoyoc & Butler (1978) was used for the determination of condensed tannins. This method is based on the ability of flavanols to react with vanillin in the presence of mineral acids to produce a red colour that is measured spectrophotometrically. Duplicate 0.2 g whole grain and bran samples were extracted with 10 ml of 100% methanol step-wise as follows: 5 ml solvent was added to the sample in a centrifuge tube and vortex mixed every 5 min for 20 min, followed by centrifuging at 3,500 rpm for 10 min (25°C) using a Medifriger centrifuge (J. P. Selecta, s. a.), and decanting. The sample residue was rinsed with 5 ml of solvent, vortex mixed and centrifuged as above and decanted. The two supernatants were combined and used for analysis.

The extracts and reagents were maintained at 30°C in a thermostat-controlled water bath before mixing the reactants. The methanolic extract (1 ml) was added to 5 ml vanillin reagent (4% HCl in methanol and 0.5% vanillin in methanol) and mixed. Sample blanks were done with 4% HCl in methanol replacing the vanillin reagent. The reactants were maintained at 30°C and absorbance was read at 500 nm after 20 min. Absorbance readings of the blanks were subtracted from those of the samples. Catechin was used as a standard and the results were expressed as mg catechin equivalents/100 mg sample, dry weight basis.

2.1.3.2.5 Determination of antioxidant activity

Antioxidant activity of the extracts was determined using the Trolox Equivalent Antioxidant Capacity (TEAC) assay as described by Awika *et al*. (2003). TEAC is a spectrophotometric technique that measures the relative ability of hydrogen-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation chromogen in relation to that of Trolox, the water-soluble Vitamin E analogue, which is used as an antioxidant standard. The ABTS radical cations ($ABTS^{\bullet+}$) were produced by mixing equal

volumes of 8 mM ABTS with 3 mM potassium persulfate prepared in distilled water and allowed to react in the dark for at least 12 h at room temperature before use. The $ABTS^{\bullet+}$ solution was diluted with a phosphate buffer solution (pH 7.4) prepared by mixing 0.2M of NaH_2PO_4, 0.2M of Na_2HPO_4 and 150mM NaCl in 1 litre of distilled water, with pH adjustment using NaOH where necessary. This solution was made fresh for each analysis. The $ABTS^{\bullet+}$ solution (2900 µl) was added to the 75% aqueous acetone sorghum bran and grain extracts (100 µl) or Trolox (100 µl) in a test tube and mixed. Absorbance readings (at 734 nm) were taken after 30 min (for the samples) and 15 min (for the standard) of the initial mixing of the samples and standard, respectively, using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The results were expressed as µM Trolox equivalents/ g of sample, dry weight basis.

2.1.3.3 Statistical analysis

The experiment was replicated twice and all assays were performed in duplicate. Results reported are means of four determinations. Data was analyzed by analysis of variance (ANOVA), and the Fisher's least significant difference test was used to identify differences among the means.

2.1.4 Results and discussion

2.1.4.1 Chlorox bleach test

Table 2.1.1 shows the Chlorox bleach test results for *Phatafuli* (brown) and *Shabalala* (white) sorghum grain varieties.

Table 2.1.1 Chlorox bleach test results for *Phatafuli* and *Shabalala* sorghum grain varieties[1]

Variety	Grain with pigmented testa (%)
Phatafuli	99
Shabalala	0
DC 99∗	100
NK 283∗∗	0

[1] Results are means of four determinations

∗Condensed tannin sorghum grain standard

∗∗Condensed tannin-free sorghum grain standard

A positive result for the presence of a black-pigmented testa layer was obtained for *Phatafuli*, an indication of the presence of condensed tannins (Waniska *et al.*, 1992). On the other hand, none of the *Shabalala* sorghum grains tested showed a black-pigmented testa, an indication of the absence of condensed tannins.

2.1.4.2 Phenolic contents and antioxidant activities of sorghum grain and bran

Total phenol and condensed tannin contents and antioxidant activities of *Phatafuli* and *Shabalala* sorghum grain and bran are shown in Table 2.1.2. For both bran and whole grain, *Phatafuli* sorghum had significantly ($p < 0.001$) higher total phenols than *Shabalala* (Table 2.1.2). This result may be attributed to the presence of condensed tannins in *Phatafuli*, which are absent in *Shabalala* grain and bran (Table 2.1.2). Condensed tannin molecules are polymers of flavan-3-ol units (Butler, 1981; Waterman & Mole, 1994; Schofield, Mbugua & Pell, 2001) which posses multiple phenolic hydroxyl groups. The Folin-Ciocalteu method is based on the reducing power of phenolic hydroxyl groups (Hahn *et al.*, 1984). The greater the levels of phenolic hydroxyl groups (as with the condensed tannins in *Phatafuli*) the higher the levels of total phenols assayed using the Folin-Ciocalteu method. This indicates the higher amounts of total phenols found in the condensed tannin *Phatafuli* bran and whole grain compared to the condensed tannin-free *Shabalala*.

Table 2.1.2 Total phenols, condensed tannins and antioxidant activity of whole grain and bran of *Phatafuli* and *Shabalala* sorghum[1]

Sample	Total phenols (mg TAE/100 mg)	Condensed tannins (mg CE/100 mg)	Antioxidant activity (μM TE/ g)
Phatafuli			
Bran	10.12 a[2] (0.60)[3]	45.16 a (4.39)	605.01 a (47.63)
Whole grain	3.09 b (0.40)	6.12 b (0.52)	195.40 b (14.41)
Shabalala			
Bran	0.18 c (0.05)	ND[4]	11.44 c (1.70)
Whole grain	0.02 d (0.01)	ND	1.93 d (0.10)

[1]Results are means of four determinations expressed on a dry basis.

[2]For each variety, means within each column followed by a different letter are significantly different ($p < 0.001$)

[3]Standard deviations are given in parentheses.

[4]Not detected

TAE, Tannic acid equivalents; CE, Catechin equivalents; TE, Trolox equivalents

The trend observed (higher total phenol content in coloured sorghums than that in white sorghums) is in agreement with previous findings (Beta *et al.,* 1999*;* Dicko, Hilhorst, Gruppen, Traore, Laane, Van Berkel & Voragen, 2002*;* Awika *et al.,* 2003). It is however difficult to do a direct comparison of the phenolic contents of the sorghums observed in this study to others in literature due to differences in extracting solvents, test methods and standards used. There is also a wide variation in the phenolic composition and content of sorghums due to both the genetic make-up of the sorghums and the environmental conditions in which the sorghum is grown (Francis, 2000; Awika & Rooney, 2004).

The level of condensed tannins obtained in *Phatafuli* whole grain is comparable to levels reported elsewhere for condensed tannin sorghums using the vanillin-HCl assay. Beta *et al*. (1999) reported condensed tannin level of 5.48 mg catechin equivalents/100 mg sample for DC-75, a condensed tannin sorghum variety. Earp, Akingbala, Ring & Rooney (1981) reported a condensed tannin range of 0.09 – 6.70 mg catechin equivalents/100 mg sample in Type II sorghums. The inability of the vanillin-HCl assay to detect any condensed tannins in *Shabalala* grain and bran lends further support to the results of the Chlorox bleach test. Generally, for condensed tannin-free sorghums, levels of condensed tannins obtained using the vanillin-HCl method are either very low (mostly less than 1.0 mg catechin equivalents/100 mg) (Earp *et al*., 1981; Beta *et al*., 1999) or none at all (Beta *et al*., 1999).

The antioxidant activities for *Phatafuli* bran and grain in this study are comparable to 512 µM TE/ g, dry weight basis and 108 µM TE/ g (dry weight basis) for Hi tannin bran and grain, respectively, and 768 µM TE/ g (dry weight basis) and 226 µM TE/ g (dry weight basis) for sumac 99 bran and grain, respectively, as reported by Awika *et al.* (2003). On the other hand, the antioxidant activities for *Shabalala* bran and grain for this study are comparatively lower than those for white sorghum bran (28 µM TE/ g, dry weight basis) and grain (6 µM TE/ g, dry weight basis) reported by the same authors. Awika *et al.* (2003) however reported relatively lower antioxidant activity values but higher total phenol values for condensed tannin sorghum bran, and relatively higher antioxidant activity and total phenol values for white sorghum bran compared to results of this study. It is however difficult to do a direct comparison of the total phenol values between results of this study and that of Awika *et al.* (2003) because different standards were used for the determination of total phenols content:

Awika *et al.* (2003) used gallic acid, while tannic acid was used in this study. However, the trend observed in both studies show that the lower the total phenol content, the lower the antioxidant activity.

For both whole grain and bran, *Phatafuli* had significantly higher antioxidant activity than *Shabalala*. Higher molecular weight phenolic compounds such as the condensed tannins have been shown to have higher antioxidant activities than lower molecular weight phenolics (Hagerman, Riedl, Alexander Jones, Sovik, Ritchard, Hartzfeld, & Riechel, 1998). It has also been reported that phenolic acids generally have lower antioxidant activities than flavonoids (Guo, Cao, Sofic, & Prior, 1997). The higher antioxidant activities of *Phatafuli* whole grain and bran (compared to *Shabalala*) may therefore be attributed to their higher total phenol contents mainly due to the presence of condensed tannins. The availability of multiple free phenolic hydroxyl groups (Guo *et al.*, 1997) and other significant areas of charge delocalization on the condensed tannin molecule may enable the scavenging and stabilization of the $ABTS^{\bullet+}$ radical cations. This radical scavenging power is lower with simple, lower molecular weight phenolics due to their limited phenolic hydroxyl groups (Hageman *et al.*, 1998).

For both sorghum varieties, the bran had higher levels of total phenols and antioxidant activity than the whole grain. This may be attributed to the fact that phenolic compounds, which are known to be largely responsible for antioxidant activities, are highly concentrated in outer layers of the sorghum grain (Rooney *et al.*, 1980; Hahn *et al.*, 1984; Hahn & Rooney, 1986; Beta, Rooney, Marovatsanga & Taylor, 2000). For *Phatafuli*, the bran had 3 times more antioxidant activity than the whole grain, while *Shabalala* bran had 6 times more antioxidant activity than the whole grain. Awika *et al*. (2003) reported 3-5 times more antioxidant activity in the bran of red sorghum than the whole grain.

2.1.4.3 Relationship between phenolic contents and antioxidant activities

Correlations were done to evaluate the relationship between the antioxidant activities of the sorghum grain and bran and their phenolic contents, and results are shown in (Table 2.1.3).

Table 2.1.3 Correlation coefficients for antioxidant activity and phenolic contents of *Phatafuli* and *Shabalala* sorghum varieties

Relationship	Correlation coefficient (R^2)
AA – TP	
Phatafuli	0.99
Shabalala	0.88
AA – CT	
Phatafuli	0.99

AA, Antioxidant activity; TP, Total phenol content; CT, Condensed tannin content

Significantly high ($p < 0.01$) correlations were found between the antioxidant activities of *Phatafuli* and *Shabalala* sorghum samples and their total phenol contents, as well as between antioxidant activity and condensed tannin content for *Phatafuli* sorghum This suggests that phenolics could be largely responsible for the antioxidant activity of the sorghum. Awika *et al*. (2003) found similar correlations ($R^2 = 0.97$) between antioxidant activity of sorghums and their phenolic contents using the TEAC and Folin-Ciocalteu methods respectively. Several studies have also reported similar high correlations between antioxidant activities and phenolic contents in plants (Guo *et al*., 1997; Velioglu, Mazza, Gao & Oomah, 1998; Chun *et al*., 2003; Kroyer, 2004; Kuti & Konuru, 2004). This indicates that phenolic compounds are a major group of compounds that contribute to the antioxidant activities of plants.

2.1.5 Conclusions

Extracts prepared from bran and grain of *Phatafuli* sorghum variety have significantly higher total phenol contents and antioxidant activities than *Shabalala* bran and grain extracts. The presence of condensed tannins in *Phatafuli* sorghum appears to be a major contributing factor to its higher total phenol contents and antioxidant activity in the bran and grain compared to *Shabalala*. Both sorghum varieties have higher total phenol contents (and antioxidant activities) in the bran than the grain, an indication that phenolic compounds are highly concentrated in the outer layers of the grain. There is a high correlation between the antioxidant activities of the sorghums and their phenolic contents, suggesting that the antioxidant activities of sorghum may be largely due to their phenolic contents.

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2.2 Storage stability of a crude phenolic extract from condensed tannin sorghum bran in relation to its phenolic content and antioxidant activity

2.2.1 Abstract

Storage stability of a freeze-dried crude phenolic extract (CPE) from condensed tannin sorghum bran as influenced by packaging (vacuum packing or not), storage temperature (-20°C or 25°C) and length of storage (20 days), in relation to its content of total phenols, condensed tannins and antioxidant activity was investigated. The effect of oxidizing conditions was also investigated by bubbling a stream of oxygen in the liquid extracts prior to freeze-drying. Total phenols, condensed tannins and antioxidant activity of the samples were determined on selected days (0, 5, 10, 15 and 20) of storage. Bubbling of oxygen in the liquid extract did not have any significant ($p > 0.05$) effect on the parameters tested. Similarly, vacuum-packed samples did not differ significantly ($p > 0.05$) in the parameters tested from the samples that were not vacuum-packed. Samples stored at –20°C had significantly higher levels of total phenols, condensed tannins and antioxidant activity than those stored at 25°C during some days of the storage period. Storage time however was the major factor influencing the levels of total phenols, condensed tannins and antioxidant activity during storage. Overall (for all the treatments), there was an initial increase in the three parameters tested during the first five days of storage, followed by a subsequent decrease during the remaining days of storage. To ensure optimum antioxidant activity, crude phenolic extracts from condensed tannin sorghum bran may therefore need to be used shortly after extraction, since longer storage could result in a reduction in antioxidant properties.

Key words: Sorghum bran; Phenolic extract; Storage stability; Total phenols; Condensed tannins; Antioxidant activity

2.2.2 Introduction

Phenolic compounds from plant sources have become a subject of interest for researchers due to their antioxidant properties. These compounds are reported to have health benefits such as the reduction of some degenerative diseases, cardiovascular disease and cancer (Murkovic, 2003). They are also reported to possess the potential for use as food antioxidants for shelf life extension of lipid foods (Wanasundara & Shahidi, 1998; Abdalla & Roozen, 1999; Bonilla, Mayen, Merida & Medina, 1999; Malecka, 2002), to replace synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) which use is being discouraged due to safety concerns (Frankel, 1996; Malecka, 2002). Several plant sources contain natural antioxidants in the form of phenolics (Rodriguez de Sotillo, Hadley & Holm, 1994a; Tsaliki, Lagouri & Doxastakis, 1999; Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003; Chun, Kim, Moon, Kang & Lee, 2003; Kroyer, 2004; Kuti & Konuru, 2004), some of which have been shown to be as effective as synthetic ones (Abdalla & Roozen, 1999).

Sorghum (*Sorghum bicolor* (L.) Moench), a major cereal food crop mainly in the semi-arid regions of the world (FAO, 1995), contains phenolic compounds that possess antioxidant activity (Awika *et al*., 2003; Simontacchi, Sadovsky & Puntarulo, 2003; Dicko, Gruppen, Traore, van Berkel & Voragen, 2005). Sorghum may therefore be considered a potential source of natural antioxidants that can be used for extending the shelf life of oils and fats and other lipid-containing foods through the retardation of lipid oxidation.

Phenolic compounds are compartmentalized in the plant cell matrix, but once the cell matrix is broken during extraction of the compounds, they become prone to degradation. Temperature, light (Rodriguez de Sotillo *et al*., 1994a), oxygen (Waterman & Mole, 1984; Haslam, Lilley, Warminski, Liao, Cai, Martin, Gaffney, Goulding & Luck, 1992; Manzocco, Anese & Nicoli, 1998; Nicoli, Calligaris & Manzocco, 2000) and pH (Friedman & Jurgens, 2000) affect stability of phenolic compounds. The changes that may occur to phenolic compounds due to the degradation may affect their antioxidant properties, in view of the fact that phenolic compounds are known to be largely responsible for the antioxidant activity of plant extracts from sources such as sorghum (Awika *et al*., 2003), fruits and vegetables

(Guo, Cao, Sofic & Prior, 1997; Velioglu, Mazza, Gao & Oomah, 1998), plums (Chun *et al*., 2003), red clover (Kroyer, 2004) and tree spinach (Kuti & Konuru, 2004). It is therefore important to investigate factors affecting the storage stability of phenolic extracts from plant sources in order to ensure the preservation of their antioxidant properties. It is also important to gain an understanding of how oxidizing conditions during extraction may affect antioxidant properties of plant phenolic extracts.

Studies have been conducted on the storage stability of phenolic compounds in liquid extracts (Rodriguez de Sotillo *et al*., 1994a; Larrauri, Sanchez-Moreno & Saura-Calixto, 1998; Azizah, Ruslawati & Swee Tee, 1999; Mansour & Khalil, 2000), model solutions (Nicoli *et al*., 2000; Pinelo, Manzocco, Nunez & Nicoli, 2004) or in whole food products (Gomez-Plaza, Gil-Munoz, Lopez-Roca & Martinez, 2000; Del Carlo, Piga, Pinna, Fenu & Agabbio, 2004; Goncalves, Landbo, Knudsen, Silva, Moutinho-Pereira, Rosa & Meyer, 2004; Talcott, Duncan, Del-Pozo-Insfran & Gorbet, 2005). In the presence of oxygen, phenolic compounds may be enzymatically oxidized when in contact with polyphenol oxidase (Murkovi, 2003), or may be oxidized non-enzymatically (Haslam *et* al., 1992; Waterman & Mole, 1994). Light (Rodriguez de Sotillo *et al*., 1994b) and temperature (Aziza *et al*., 1999) have also been shown to significantly reduce the concentration of phenolic compounds. However, information regarding the storage stability of freeze-dried phenolic extracts from sorghum bran is lacking.

The aim of this study therefore, was to determine the effect of mode of packaging, storage temperature and the length of storage on the levels of phenolic compounds and antioxidant properties of a phenolic extract from sorghum bran. In addition, the effect of oxidizing conditions during extraction was determined.

2.2.3 Materials and methods

2.2.3.1 Materials

A brown-coloured condensed tannin sorghum variety (*Phatafuli*) (section 2.1.3.1, Figure 2.1.1) grown during the 2002/2003 season on a local farm in Chikwawa district of Malawi and obtained through Kasinthula Research Station, Ministry of Agriculture and Irrigation, was used in this study.

2.2.3.2 Methods

2.2.3.2.1 Preparation of freeze-dried crude phenolic extracts (CPE) from sorghum bran

The tannin sorghum grain was decorticated using a sorghum dehuller (Rural Industries Innovation Centre, Kanye, Botswana). The bran yield was approximately 10%. The bran obtained was ground to pass a 0.5 mm sieve using a Laboratory Mill 3100 (Falling Number, Huddinge, Sweden). Duplicate bran samples were extracted using 75% aqueous acetone (Kaluza, McGrath, Roberts & Schroeder, 1980). The bran (40 g) was extracted in 450 ml of solvent as follows: 150 ml solvent was added to 40 g bran in plastic centrifuge tubes and vortex mixed every 10 min for 2 h to improve extraction efficiency. The samples were then centrifuged at 3,500 rpm for 10 min (25°C) using a Medifriger centrifuge (J. P. Selecta, s. a.) and decanted. The sample residue was rinsed twice with 150 ml solvent, mixed for 5 min, centrifuged as above and decanted. The three supernatants were then pooled. To evaluate the effect of oxidizing conditions on the phenolic content and antioxidant activity of the sorghum extracts, a stream of oxygen was bubbled through a set of the duplicate sample extracts for 10 min at a pressure of 30 kPa, prior to freeze-drying. All extracts were concentrated in a Buchi Rotavapor RE 120 rotary evaporator (Laboratoriums Technik AG, Switzerland) at 35°C, freeze-dried and thoroughly mixed to obtain a fine powder.

2.2.3.2.2 Packaging and storage of the CPE

Both sets of the CPE (with or without oxygen bubbling) were stored in polyethylene vacuum bags (composite thickness 80 µm) with oxygen permeability of 25.7 cc/m^2/24 h and water permeability of 0 g/m^2/24 h at 25°C. For each set of the CPE, some samples were either vacuum sealed or sealed with some air in the bag, wrapped in aluminium foil and either stored in the dark in a cold room maintained at –20°C or stored in the dark in a Labcon forced circulation oven Type FSOE 16 (Labcon Pty Limited, Rooderport, South Africa) maintained at 25°C (see Figure 2.2.1 for the experimental design).

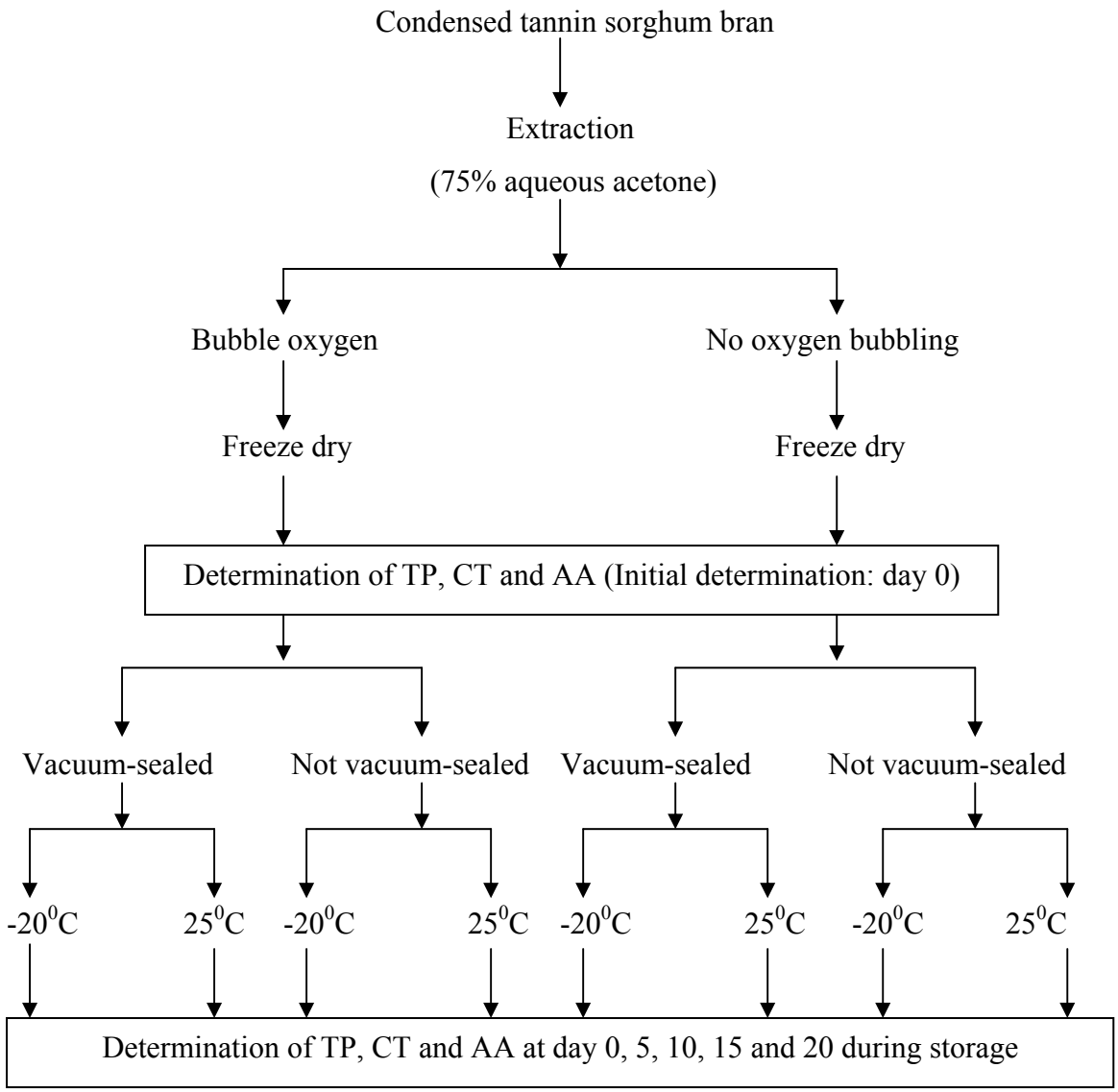


Figure 2.2.1. Experimental design (TP, total phenolics; CT, condensed tannins; AA, antioxidant activity).

2.2.3.2.3 Determination of storage stability of the CPE

A 0.02 g sample was analysed from each polyethylene bag before storage (t = 0 days), and periodically thereafter on day 5, 10, 15 and 20 of storage, after thorough mixing of the contents, for content of total phenols, condensed tannins and antioxidant activity. Samples were appropriately resealed after each sampling.

2.2.3.2.4 Determination of total phenols

Total phenol content of the CPE was determined according to the Folin-Ciocalteu method (Singleton & Rossi, 1965) as modified by Waterman & Mole (1994). This method is based on the reducing power of phenolic hydroxyl groups (Hahn, Rooney & Earp, 1984), which react with the Folin-Ciocalteu phenol reagent to form

chromogens that can be detected spectrophotometrically. The CPE (0.02 g) was reconstituted in 15 ml solvent (75% aqueous acetone), and an aliquot (0.5 ml) of the reconstituted sample was used to determine total phenols as described in section 2.1.3.2.3.

2.2.3.2.5 Determination of condensed tannins

The vanillin-HCl method of Price, Van Scoyoc & Butler (1978) was used for the determination of condensed tannins. This method is based on the ability of flavonols to react with vanillin in the presence of mineral acids to produce a red colour that is measured spectrophotometrically. The CPE (0.02 g) was reconstituted in 15 ml methanol and a 1 ml aliquot was used for the determination of condensed tannins as previously described in section 2.1.3.2.4.

2.2.3.2.6 Determination of antioxidant activity

Antioxidant activity of the CPE was determined using the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Awika *et al*., 2003). TEAC is a spectrophotometric technique that measures the relative ability of hydrogen-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation chromogen in relation to that of Trolox, the water-soluble Vitamin E analogue, which is used as an antioxidant standard. The CPE (0.02 g) was reconstituted in 15 ml solvent (75% aqueous acetone), and further diluted (1:20) in the same solvent for analysis. The diluted CPE was used for determination of AA as described in section 2.1.3.2.5.

2.2.3.3 Statistical analysis

The experiment was replicated twice and all measurements were done in duplicate. Data was subjected to analysis of variance (ANOVA), computed using the SAS Generalized Linear Model (GLM) procedure (SAS Institute, Inc., 2000). F-tests were conducted to determine differences among means at $p < 0.05$.

2.2.4 Results and discussion

2.2.4.1 Yield of extract

The yield of the freeze-dried extract (soluble solids) obtained from the bran of condensed tannin sorghum (expressed as percent by weight of the bran) was approximately 10%. This result is comparable to 9.7% for durum wheat bran (Onyeneho & Hettiarachchy, 1992).

2.2.4.2 Effect of oxidizing conditions

Table 2.2.1 shows the effect of oxidizing conditions (induced by bubbling oxygen through the liquid phenolic extract) on phenolic content and antioxidant activity of the CPE from sorghum bran during storage.

Table 2.2.1. Effect of oxidizing conditions on content of total phenols, condensed tannins and antioxidant activity during storage of crude phenolic extract from condensed tannin sorghum bran

Parameter tested	Treatment	Storage time (days)				
		0	5	10	15	20
TP	BO[1]	56.86[3]a[4]	57.45a	56.01a	56.42a	53.27a
	NOB[2]	58.35a	58.46a	56.65a	57.13a	53.28a
SEM[5]		0.06	0.52	0.39	0.57	0.48
CT	BO	478.62a	509.22a	544.96a	502.36a	430.76a
	NOB	491.80a	504.12a	558.07a	489.35a	432.38a
SEM		0.65	12.62	9.59	5.51	4.63
AA	BO	4415.45a	4954.73a	3390.20a	3095.94a	3215.09a
	NOB	4598.40a	5021.23a	3515.05a	3073.48a	3229.68a
SEM		16.72	106.27	57.76	58.49	54.47

TP, total phenols (mg TAE/100 mg); CT, condensed tannins (mg CE/100 mg); AA, antioxidant activity (μM TE/g).

[1] Bubble oxygen

[2] No oxygen bubbling

[3] Results are means of four determinations

[4] For each parameter tested and for any particular day, means within each column followed by a different letter are significantly different ($p < 0.05$)

[5] Standard error of the mean

Over the whole storage period, though levels for total phenols, condensed tannins and antioxidant activity for the CPE that was bubbled with oxygen were generally lower on any particular day (except in a few isolated cases) than that for the extract which was not bubbled with oxygen, the differences were not significant, showing that bubbling of oxygen did not have a significant effect on the contents of total phenols, condensed tannins and antioxidant activity. Phenolic compounds are known to be largely responsible for the antioxidant activities of sorghums (Awika *et al*., 2003) and many other plants (Velioglu *et al*., 1998; Kroyer, 2004; Kuti & Konuru, 2004). The absence of significant changes in the levels of phenolics (total phenols and condensed tannins) of the extract after bubbling with oxygen was also reflected in the absence of significant changes in antioxidant activities. Oxygen may bring about oxidation of phenolic compounds and formation of new compounds such as quinones (Haslam *et al*., 1992; Waterman & Mole, 1994), which may subsequently undergo rapid polymerization. The net result is changes in the original nature of the phenolic compounds and in their chemical and functional properties such as antioxidant activity. The lack of any significant influence of the bubbling of oxygen on the levels of phenolics and antioxidant properties of the tannin sorghum bran CPE may probably be due to insufficient time of exposure of the extract to oxygen, since oxygen was bubbled into the extract for only 10 min after which the extract was concentrated and then freeze-dried.

2.2.4.3 Effect of packaging

Table 2.2.2 shows the effect of packaging on phenolic content and antioxidant activity of the CPE from condensed tannin sorghum bran during storage. Throughout the storage period, the content of total phenols, condensed tannins and antioxidant activity of the CPE that were vacuum packed did not differ significantly from that of the CPE that were not vacuum packed, except for day 20, where the vacuum packed extract had a significantly higher antioxidant activity than the extract which was not vacuum packed. Though the differences were generally not significant during most of the storage period, vacuum-packed samples generally had a higher content of total phenols, condensed tannin and antioxidant activity than the samples that were not vacuum-packed. Vacuum packing was aimed at excluding air from the samples so that the effect of the availability of air during the storage of the CPE could be investigated. The results suggest that the presence or absence of air in the package did not cause any significant changes in the total phenol and condensed tannin content

that could have significantly influenced the antioxidant activity of the freeze-dried extracts. This could probably be due to enhanced stability of the extract in the dry state in which it was stored, which would be difficult for major changes in phenol content and antioxidant activity to occur compared to liquid state. Gonzalez, Cruz, Dominguez & Parajo (2004) also did not find changes in the radical scavenging capacity of a freeze-dried extract of *Eucalyptus globulus* wood samples stored for 70 days at 4°C in the dark both in the presence and absence of air.

Table 2.2.2. Effect of packaging (presence or absence of air) on content of total phenols, condensed tannins and antioxidant activity during storage of crude phenolic extract from condensed tannin sorghum bran

Parameter tested	Treatment	Storage time (days)				
		0	5	10	15	20
TP	V[1]	57.60[3]a[4]	57.63a	56.62a	56.95a	53.32a
	NV[2]	57.60a	58.28a	56.03a	56.60a	53.23a
SEM[5]		0.06	0.52	0.39	0.57	0.48
CT	V	485.21a	513.95a	562.44a	501.54a	431.70a
	NV	485.21a	499.39a	540.58a	490.16a	431.43a
SEM		0.65	12.62	9.59	5.51	4.63
AA	V	4506.92a	4927.88a	3443.43a	3101.12a	3296.55a
	NV	4506.92a	5048.08a	3461.82a	3068.30a	3148.22b
SEM		16.72	102.27	57.76	58.49	54.47

TP, total phenols (mg TAE/100 mg); CT, condensed tannins (mg CE/100 mg); AA, antioxidant activity (μM TE/ g).

[1] Vacuum packed

[2] Not vacuum packed

[3] Results are means of four determinations

[4] For each parameter tested and for any particular day, means within each column followed by a different letter are significantly different ($p < 0.05$)

4 Standard error of the mean

2.2.4.4 Effect of storage temperature

Table 2.2.3 shows the effect of storage temperature on phenolic content and antioxidant activity of the CPE from condensed tannin sorghum bran.

Table 2.2.3. Effect of storage temperature on content of total phenols, condensed tannins and antioxidant activity during storage of crude phenolic extract from condensed tannin sorghum bran

Parameter tested	Treatment	Storage time (days)				
		0	5	10	15	20
TP	-20°C	57.60[1]a[2]	58.14a	56.94a	57.37a	53.54a
	25°C	57.60a	57.77a	55.71b	56.18a	53.01a
SEM[3]		0.06	0.52	0.39	0.57	0.48
CT	-20°C	485.21a	513.13a	549.64a	515.79a	446.29a
	25°C	485.21a	500.21a	553.38a	475.92b	416.84b
SEM		0.65	12.62	9.59	5.51	4.63
AA	-20°C	4506.92a	5158.05a	3531.51a	3156.04a	3277.10a
	25°C	4506.92a	4817.91b	3373.74b	3013.38a	3167.67b
SEM		16.72	106.27	57.76	58.49	54.47

TP, total phenols (mg TAE/100 mg); CT, condensed tannins (mg CE/100 mg); AA, antioxidant activity (μM TE/ g).

[1] Results are means of four determinations

[2] For each parameter tested and for any particular day, means within each column followed by a different letter are significantly different ($p < 0.05$)

[3] Standard error of the mean.

There was generally higher content of total phenols, condensed tannins and antioxidant activity during the storage period for the CPE stored at –20°C compared to that stored at 25°C, with these differences being significant during some of the days of storage. The differences in total phenol content were only significant during day 10 of storage, while for condensed tannin content the differences were significant during day 15 and 20. For antioxidant activity, the samples stored at –20°C had significantly higher levels than samples stored at 25°C during day 5, 10 and 20. High temperatures are known to influence the oxidation of phenolic compounds (Palma, Pineiro & Baroso, 2001). Lower total phenols, condensed tannins and antioxidant activity of the CPE stored at 25°C compared to those stored at –20°C during some of the days may therefore be attributed to oxidation of the phenolic compounds due to the higher storage temperature.

Contrary to findings of this study, Rodriguez de Sotillo, Hadley & Holm (1994b) did not find significant differences in the phenolic acid content and composition of potato peel freeze-dried extracts stored at 4°C and 25°C for 15 days. The results of this study may be different from those reported by Rodriguez de Sotillo *et al*. (1994b) due to the different parameters tested and the methods used. Storage stability of the sorghum CPE in this study was monitored by measuring the content of total phenols, condensed tannins and antioxidant activity using the Folin-Ciocalteu, vanillin-HCl and TEAC assay, respectively, while Rodriguez de Sotillo *et al*. (1994b) studied the storage stability of the potato peel freeze-dried extracts by monitoring phenolic acid contents using high performance liquid chromatography (HPLC). The wider temperature range used in this study (-20 and 25°C), compared to the range used by Rodriguez de Sotillo *et al*. (1994b) (4 and 25°C) may also explain the differences in the results.

2.2.4.5 Effect of storage time

For the oxidized samples, there were variations in the parameters tested on different days over the storage period (Table 2.2.1). There was an initial increase in contents of total phenols, condensed tannins and antioxidant activity on day 5, and also a further increase for condensed tannin content on day 10, followed by a general decreasing trend with the progression of time. On each particular day there were no significant differences on total phenols, condensed tannins and antioxidant activity between

samples bubbled with oxygen and those not bubbled with oxygen. At the end of the 20-day storage period there was a reduction of 6.3% and 8.7% in total phenols for the CPE that was bubbled with oxygen and the one not bubbled, respectively. Condensed tannin content on the other hand was reduced by 10.0% for the extract bubbled with oxygen, while the one not bubbled with oxygen was reduced by 12.1%. The antioxidant activity of the extract that was bubbled with oxygen was reduced by 27.2% while the one not bubbled with oxygen was reduced by 29.7%. This is an indication that storage time rather than bubbling of oxygen was the more important factor influencing variations in levels of phenolics and antioxidant activity over the storage period. The gradual decrease in antioxidant activity could probably be due to oxidation of the phenolic compounds over time.

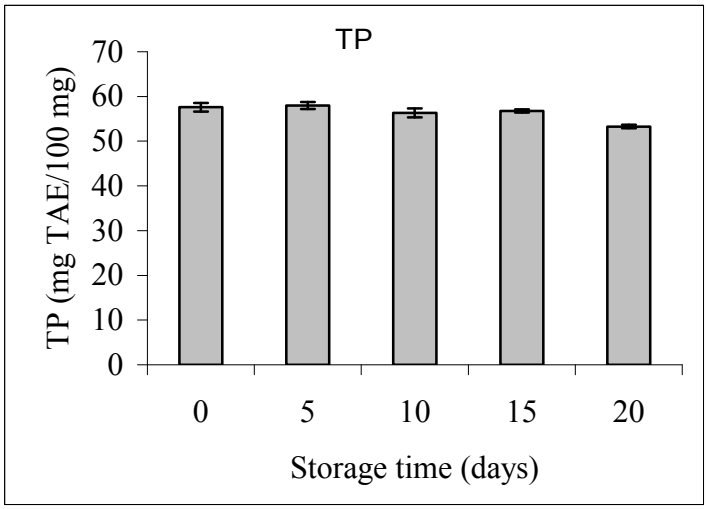
Similar to samples exposed to oxidizing conditions, storage time affected the parameters to some extent in the samples that were packaged under different conditions (Table 2.2.2). For both treatments (vacuum packaged or not vacuum packaged), there was an initial increase in the content of total phenols, condensed tannins and antioxidant activity on day 5 of storage, followed by a subsequent decline from day 10 for total phenol content and antioxidant activity, while condensed tannin content started declining from day 15.

The trends observed in total phenols, condensed tannins and antioxidant activity over the whole storage period for samples stored at -20 or 25°C (Table 2.2.3) were similar to those observed in Tables 2.2.1 and 2.2.2. By the end of the storage period there was an overall decrease of 7.0% and 8.0% in total phenol content for the extracts stored at –20°C and 25°C, respectively, while there was a reduction of 8.0% and 14.1% in condensed tannins content for the extracts stored at –20°C and 25°C, respectively. There was however a higher reduction in antioxidant activity of 27.3% and 29.7% for the samples stored at –20°C and 25°C, respectively.

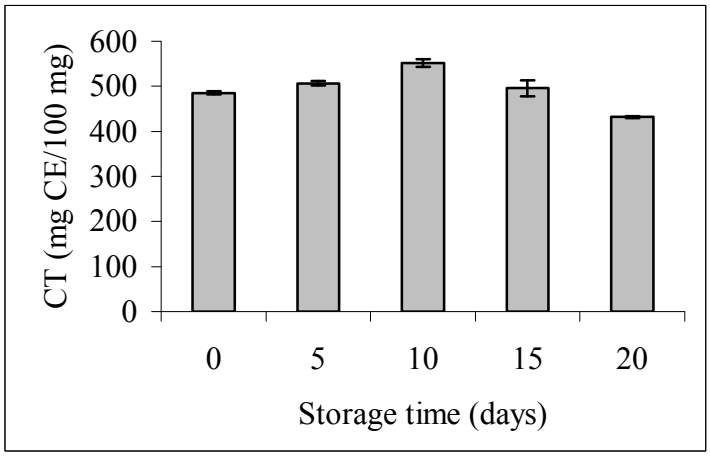
The overall effect (on all treatments) of storage time on the total phenols, condensed tannins and antioxidant activity of the CPE from condensed tannin sorghum bran is shown in Fig. 2.2.2. Time of storage had a significant effect on all the parameters tested, particularly on condensed tannins and antioxidant activity. There was an overall initial increase of 0.6%, 4.4% and 10.7% in total phenols, condensed tannins and antioxidant activity, respectively, during day 5 of storage, with the increase for

condensed tannins (Figure 2.2.2B) and antioxidant activity (Figure 2.2.2C) being significant compared to that at day 0. The increase in condensed tannin content during day 10 was also significantly higher than that at day 0. This initial increase was then followed by a decreasing trend in total phenol content and antioxidant activity from day 10 onwards, while condensed tannin content started declining from day 15.

(A)



(B)



(C)

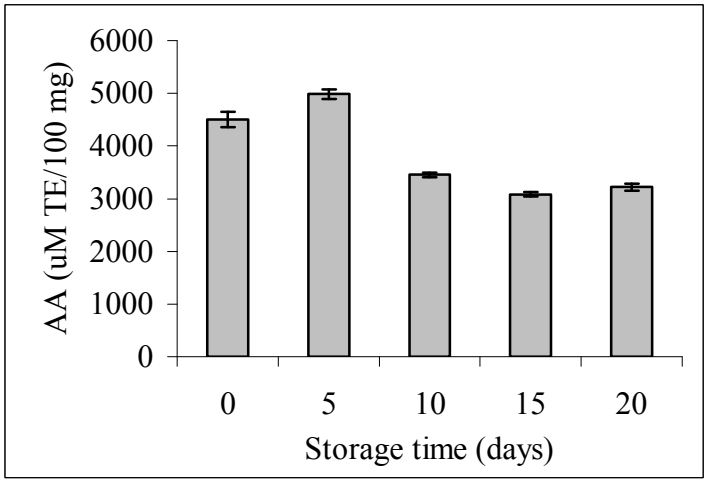


Figure 2.2.2. Effect of storage time on the total phenol content (TP) **(A)**, condensed tannins (CT) **(B)** and antioxidant activity (AA) **(C)** of the CPE from condensed tannin sorghum bran. Error bars represent standard error of the mean.

Phenolic compounds may undergo various reactions during storage that could affect their antioxidant activity. Nicoli *et al*. (2000) observed an increase higher than 50% in the antiradical activity of enzymatically and chemically oxidized pure catechin solutions during the first 2 days of storage at 25°C, after which the antiradical activity declined. This initial increase in antiradical activity was attributed to the formation of procyanidins with larger aromatic structure, due to polymerization, which exhibited more antioxidant activity than the catechin monomers. Guyot, Vercauteren & Cheynier (1996) showed that predominant products of early stages of oxidation are dimers, which are more potent than original monomers. Plumb, De Pascual-Teresa, Santos-Buelga, Cheynier & Williamson (1998) also showed that procyanidin B_1 (a dimmer) is more effective than catechin or epicatechin (monomers). This increased antioxidant effectiveness of dimmers compared to monomers could be due to the increased capacity of their aromatic structure to support unpaired electrons and their delocalization around the aromatic ring (Nicoli *et al*., 2000). Pinelo *et al*. (2004) also reported a similar initial increase followed by a decrease in antioxidant activity of ethanol solutions containing a single phenol or a mixture of catechin, resveratrol and quercetin due to polymerization. They noted that though polymerization of phenolic compounds might initially result in an increase in antioxidant activity, when the degree of polymerization reaches a critical value the increased molecular complexity results in a decreased antioxidant capacity, probably due to the steric hindrance, which reduces the availability of the hydroxyl groups to scavenge radicals. Nicoli *et al*. (2000) also showed a similar trend of initial increase followed by a decrease in the chain breaking activity during the storage of both an apple puree which was stirred in air to favour enzymatic oxidation, and a blanched apple puree which was taken as a non-oxidized control. Results of this study followed the same trend as the catechin solutions reported by Nicoli *et al*. (2000) and Pinelo *et al*. (2004) and the apple puree (Nicoli, *et al*., 2000). It may therefore be suggested that the phenolic compounds in the CPE may have undergone oxidation reactions that resulted in polymerization, which initially resulted in an increase followed by decreasing antioxidant activity (Pinelo *et al*., 2004).

Phenolic compounds have widely been reported to be largely responsible for the antioxidant activity of plant extracts (Guo *et al*., 1997; Velioglu *et al*., 1998; Awika *et al*., 2003; Kuti & Konuru, 2004). Some studies have however shown low correlations between phenolic compounds and antioxidant activity, an indication that phenolic

compounds may not be the only compounds responsible for antioxidant activity (Fombang, Taylor, Mbofung & Minnaar, 2005). Over the storage period of this study, there were positive, but insignificant correlations between total phenol content and antioxidant activity (R^2 = 0.68), as well as between condensed tannin content and antioxidant activity (R^2 = 0.16). The low correlation between condensed tannin content and antioxidant activity is demonstrated during day 5 and 10 (Figure 2.2.2); where it was observed that while the condensed tannin content increased from day 5 to day 10, the antioxidant activity rather decreased significantly. This shows that phenolic content and antioxidant activity may not always show a high positive correlation. Two possible reasons may explain this observation: (1) antioxidant activity may depend on the type of phenolic compounds, and in the case of the CPE, perhaps the compounds formed during the storage period may not necessarily support free radical scavenging because of their structure; (2) phenolic compounds may not be the only components in the CPE responsible for the antioxidant activity. Some cereal grains contain tocopherols and tocotrienols that are present in the lipid fraction of the germ, and these posses antioxidant activity (MacEvilly, 2003). The bran comprises the pericarp and some of the germ; therefore the bran crude phenolic extract may have contained other components (such as tocopherols and tocotrienols) with antioxidant activity. It is possible that the bran used in this study could have contained some lipid germ components since the bran samples were not defatted prior to extraction. It may also be possible that the assays used were not sensitive enough to detect changes in phenols and condensed tannins.

On average, at the end of the storage period the total phenol content, condensed tannin and antioxidant activity significantly decreased by 7.5%, 11.0% and 28.5%, respectively. The significant reduction in the antioxidant activity of the tannin sorghum bran freeze-dried extract from day 10 of storage could therefore suggest that tannin sorghum bran freeze-dried extracts may need to be used shortly after extraction, since storing it for longer times would result in a faster decline in antioxidant activity.

2.2.5 Conclusions

Bubbling of oxygen in the extracts appear to have no significant effect on the content of total phenols (TP), condensed tannins (CT) and antioxidant activity (AA) of the condensed tannin sorghum bran crude phenolic extracts. Vacuum packaging of the

condensed tannin sorghum freeze-dried crude phenolic extracts (CPE) during storage similarly appears not to significantly affect the levels of TP, CT and AA of the CPE. Storage temperature shows some significant effect on the levels of TP, CT and AA during some days of storage. Storage time however appears to be the major factor influencing the variations in levels of TP, CT and AA of the CPE during storage. There is a general trend in all the treatments of an initial increase in TP, CT and AA during the first 5 days, followed by a subsequent decrease during the remaining days of storage. Crude phenolic extracts from condensed tannin sorghum bran may therefore need to be used shortly after extraction to ensure optimum antioxidant activity since storing it for longer times could result in a decline in their antioxidant activity. The reduced potency of the crude phenolic extracts from the condensed tannin sorghum due to storage time may therefore pose practical challenges in their utilization as a source of natural antioxidants. The insignificant correlation between the antioxidant activities of the CPE and their phenolic contents during storage may suggest the formation of new phenolic compounds with a lower antioxidant capacity.

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2.3 Antioxidant activity of a crude phenolic extract from sorghum bran in sunflower oil in the presence of ferric ions

2.3.1 Abstract

Antioxidant activity of a freeze-dried crude phenolic extract (CPE) from the bran of condensed tannin sorghum, in comparison with tertiary butyl hydroquinone (TBHQ), was evaluated in sunflower oil at concentrations of 1000, 1500 and 2000 ppm in the absence or presence of ferric ions at 2.2 and 4.4 ppm in the dark at 65^{o}C. Progression of oxidation was monitored by measurement of peroxide values (PV) and anisidine values (AV) during a 14-day storage period. The CPE inhibited lipid oxidation in the sunflower oil as shown by lower PVs and AVs compared to the control samples without additives. The CPE was however less effective in controlling PVs compared to TBHQ, but AVs of the samples containing the CPE were comparable to that of samples containing TBHQ. In the presence of ferric ions, the CPE appeared to be less effective in reducing PVs compared to TBHQ, but appeared to be more effective than TBHQ in reducing AVs. The possibility of ferric ion chelation by phenols of the sorghum extract is suggested. The CPE imparted colour to the sunflower oil, which could limit its application as a natural antioxidant in edible oils.

Keywords: Sorghum; Phenolic extract; Lipid oxidation; Antioxidant activity; Oxidative stability; Ferric ions; Peroxide value; Anisidine value

2.3.2 Introduction

Oxidation is one of the major causes of deterioration of fats and oils leading to the development of rancid odours and taste (Moure, Cruz, Franco, Siniero, Dominguez, Nunez & Lema, 2001), hence causing a reduction in the shelf life of the fat or oil. Oxidation can also decrease the nutritional quality and safety of lipids through the formation of toxic products in foods after cooking and processing (Madhavi, Deshpande & Salunkhe, 1996; Frankel, 1996). Oxidation of the lipids has been shown to take place through a chain reaction that involves three stages: initiation, propagation and termination (Gunstone & Norris, 1983; Gordon, 1990; Madhavi *et al*., 1996; Stauffer, 1996; Hamilton, Kalu, Prisk, Padley & Pirce, 1997; Coultate, 2002). During the initiation stage, highly reactive free radicals are formed. In the subsequent propagation stage, free radicals may react with oxygen or other fatty acids. More free radicals and hydroperoxides are the main products of the propagation stage. During the termination stage, the chain reaction ends, during which stable deterioration products, mainly carbonyl compounds are formed. These carbonyl compounds (especially the aldehydes) are responsible for the perception of rancidity in oxidized oils.

Factors influencing lipid oxidation include the presence of high amounts of polyunsaturated fatty acids such as linoleic and linolenic acids in oils (Nawar, 1996; Chu & Kung, 1998; Tan, Che Man, Selemat & Yussof, 2002), exposure to oxygen (Nawar, 1996; Tan *et al*., 2002), light (Nawar, 1996; Abdalla, Tirzite, Tirzitis & Roozen, 1999; Eskin & Przbylski, 2001), high temperatures (Nawar, 1996; Suzuki, Yasui, Matsukura & Terao, 1996), and trace metals (mainly transition metals such as Fe and Cu) (Gordon & Weng, 1992; Luzia, Trugo, Da Paixao, Marcilio, De Maria & Quinteiro, 1998; Satue-Gracia, Frankel, Rangavajhyala & German, 2000; Fomuso, Corredig & Akoh, 2002; Medina, Tombo, Satue-Gracia, German & Frankel, 2002). Oxidation of fats and oils may therefore be prevented by minimizing their exposure to oxygen during processing (Schuler, 1990) and storage (Catsberg & Kempen-van Dommelen, 1990; Schuler, 1990), packaging the lipid to exclude light (Schuler, 1990; Talcott, Duncan, Del Pozo-Insfran & Gorbet, 2005), and storing them at low temperatures (Catsberg *et al*., 1990). These methods are however found inadequate to prevent the deterioration of fats and oils due to oxidation; hence to a large extent this

is achieved by the use of antioxidants (Coultate, 2002; Gunstone & Norris, 1983; Naz, Sheikh, Siddiqi & Sayeed, 2004).

Synthetic phenolic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are commonly added at 200 ppm (Shahidi & Wanasundara, 1992; Stauffer, 1996) to lipid foods to stabilize the lipids against oxidation (Stauffer, 1996; Chu & Hsu, 1999; Moure *et al*., 2001). There is however growing concern about the use of the synthetic antioxidants due to their negative effects that have been reported such as carcinogenicity (Lindenschimdit, Tryka, Goad & Witschi, 1986; Whysner, Wang, Zang, Iatropoulos & Williams, 1994) and toxicity (Wanasundara & Shahidi, 1998; Moure *et al.,* 2001). This has led to an increasing interest in the search for naturally occurring antioxidants (Duve & White, 1991; Malecka, 2002).

Sorghum (*Sorghum bicolor* (L.) Moench) contains phenolic compounds in the form of phenolic acids, flavonoids and condensed tannins (Waniska, 2000), which have been shown to have antioxidant activity (Hagerman, Riedl, Alexander Jones, Sovik, Ritchard, Hartzfeld & Riechel, 1988; Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003; Awika, Rooney & Waniska, 2004). These phenolic compounds, which are concentrated in the outer layers of the sorghum grain (pericarp and testa) (Rooney, Blakely, Miller & Rosenow, 1980; Hahn, Rooney & Earp, 1984; Youssef, 1998; Beta, Rooney, Marovatsanga & Taylor, 2000), possess structural features favourable for radical scavenging and/or metal chelation, which would enable them to be effective antioxidants. The pericarp and testa are often removed and disposed as bran during utilization of the sorghum. A potential therefore exists to use sorghum bran as a cheap source of natural antioxidants to prevent the development of oxidative rancidity in edible oils and other lipid food systems.

The aim of this study was therefore to investigate the antioxidant activity of a crude phenolic extract from the bran of condensed tannin sorghum in sunflower oil in the presence or absence of ferric ions in comparison with TBHQ, a commonly used synthetic antioxidant.

2.3.3 Materials and methods

2.3.3.1 Materials

2.3.3.1.1 Samples and reagents

Refined, bleached and deodorized (RBD) sunflower oil without added antioxidants was supplied by Continental Oil Mills (Johannesburg, South Africa). A brown-coloured condensed tannin sorghum variety (*Phatafuli*) (section 2.1.3.1, Figure 2.1.1) grown during the 2002/2003 season on a local farm in Chikwawa district of Malawi was obtained through Kasinthula Research Station, Ministry of Agriculture and Irrigation, Malawi. TBHQ was obtained from Merck (Schuchardt, Germany).

2.3.3.1.2 Preparation of freeze-dried crude phenolic extract (CPE) from sorghum bran

The tannin sorghum grain was decorticated, milled and extracted as described in section 2.2.3.2.1. The three supernatants from the extraction were combined and concentrated in a Buchi Rotavapor RE 120 rotary evaporator (Laboratoriums Technik AG, Switzerland) under vacuum at 35°C. This process enabled the evaporation of the solvent (acetone) and allowed the extract to be easily frozen before the freeze-drying process. The concentrated extracts were then freeze-dried and thoroughly mixed to obtain a fine powder. The freeze-dried crude phenolic extract (CPE) was stored at –20°C until used.

2.3.3.1.3 Preparation of ferric palmitate

Ferric palmitate, a lipid soluble compound, was added to fresh, refined sunflower oil to investigate the effect of the CPE from condensed tannin sorghum bran on the oxidative stability of sunflower oil in the presence of ferric irons. The ferric palmitate was prepared as previously described by Gordon & Weng (1992). Sodium hydroxide (4.2 g) was dissolved in 100 ml distilled water and palmitic acid (25.6 g) was added. The mixture was stirred for 5 min, and then diluted to 400 ml with distilled water followed by heating with stirring to 90°C. The sodium palmitate solution was then poured into a beaker containing ferric chloride hexahydrate (20.0 g), and the mixture was heated to 80°C with stirring for 20 min. The brown precipitate formed was removed by filtration and washed three times with hot distilled water at 80°C. The solid was dried in a vacuum oven for 8 h. When analyzed by atomic absorption

spectrophotometry, the ferric palmitate prepared contained approximately 6.8% iron, the theoretical value of ferric palmitate.

2.3.3.2 Methods

2.3.3.2.1 Evaluation of the oxidative stability of sunflower oil

CPE from the condensed tannin sorghum bran was added to 80 g sunflower oil sample aliquots at concentrations of 1000, 1500 and 2000 ppm (mg extract per kg oil) in screw-capped 100 ml Schott glass bottles externally covered with aluminium foil to exclude light. The CPE were introduced into the oil with the aid of absolute methanol to facilitate dispersion in the oil. The synthetic antioxidant, TBHQ, was also added to an 80 g sunflower oil sample at the legal limit of 200 ppm (Shahidi & Wanasundara, 1992; Stauffer, 1996), and another oil sample without any additive as a control. To assess the effect of the CPE on the oxidative stability of the oil in the presence of ferric ions, a similar set of the above samples were prepared with the addition of 2.2 ppm ferric ions (32.35 mg ferric palmitate per kg oil) or 4.4 ppm ferric ions (64.70 mg ferric palmitate per kg oil) to the oil. Control samples containing 2.2 ppm or 4.4 ppm ferric ions without addition of CPE or TBHQ, but with the addition of equal quantities of absolute methanol were prepared. The contents were thoroughly mixed for 5 min. All samples were stored in the dark in a Labcon forced circulation oven Type FSOE 16 (Labcon Pty Limited, Rooderport, South Africa) at 65°C. Oxidative stability was evaluated by determining the peroxide values (PV) and anisidine values (AV) of the oil during 14 days of storage at 2-day intervals. The measurements for day zero were taken 2 h after incubation of the samples at 65°C to allow for evaporation of the solvent. The contents of each bottle were thoroughly mixed for 2 min every day, and before removing the aliquot for determinations.

2.3.3.2.2 Determination of peroxide value

PV was determined according to the AOAC Official Method 965.33 (AOAC, 2000). The method is based on iodometric titration, which measures the iodine produced from potassium iodide by the peroxides present in the oil. The PV was expressed as milli-equivalents peroxides per kilogram of oil sample (meq/kg oil).

2.3.3.2.3 Determination of anisidine value

AV was determined by the International Union of Pure and Applied Chemistry (IUPAC) standard method 2.504 (IUPAC, 1979). The method is based on the reaction

of *p*-anisidine reagent with aldehyde compounds, mainly α and β-alkenals (Guillen & Cabo, 2002), in an oil sample in an acetic acid solution, producing yellowish reaction products that are determined spectrophotometrically. The intensity of the yellow colour depends on the amount of the aldehyde compounds present in a sample. The AV was expressed as absorbance at 350 nm.

2.3.3.3 Statistical analysis

The experiment was replicated three times, and all measurements were done in duplicate. Data was subjected to analysis of variance (ANOVA), computed using the SAS Generalized Linear Model (GLM) (SAS Institute, Inc., 2000). F-tests were conducted to determine differences among means at $p < 0.05$.

2.3.4 Results and discussion

2.3.4.1 Initial quality of sunflower oil

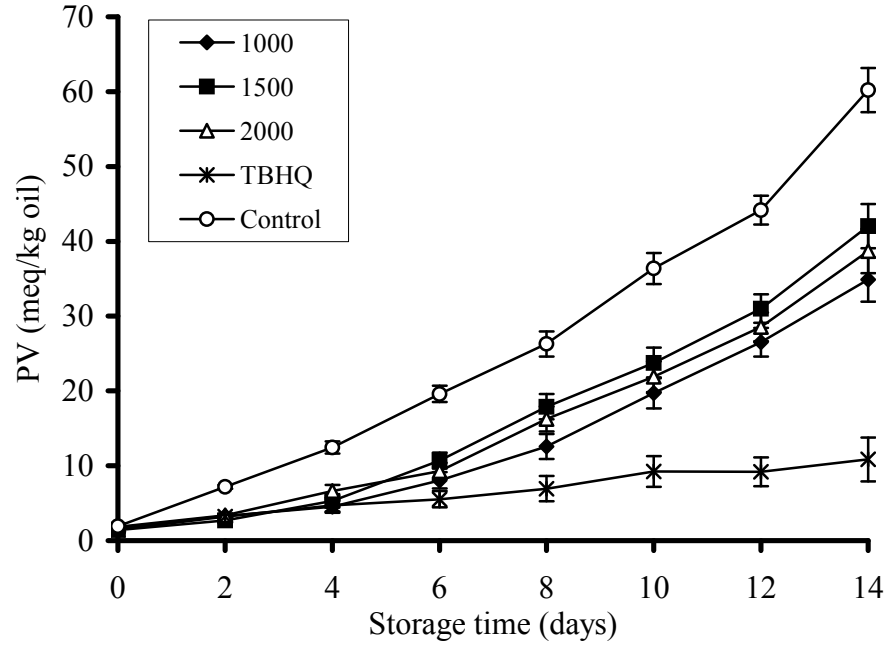
The initial quality of the oil was checked by determining the PV and AV, and was found to be 1.29 meq/kg oil and 2.59 (absorbance at 350 nm), respectively. According to Rossell (1986), freshly refined oil should have a PV of less than 1 meq/kg, and oils that have been stored for sometime after refining could have a PV of up to 10 meq/kg, and that an AV of less than 10 indicates good oil. The PV and AV levels of the sunflower oil used in this study were therefore within acceptable limits for good quality oil. The initial PV and AV in this study were even lower than those reported by Guillen & Cabo (2002) for non-oxidized sunflower oil samples (3.7 and 7.3 for PV and AV, respectively).

2.3.4.2 Effect of CPE from condensed tannin sorghum bran on the oxidative stability of sunflower oil

The oxidative stability of the sunflower oil was monitored by the measurement of PVs and AVs. PV is a measure of hydroperoxides (primary oxidation products) and AV measures aldehydic compounds (secondary oxidation products), hence the measurement of PVs and AVs may be used as indicators of primary and secondary oxidation products, respectively. At all the three concentrations studied (1000, 1500 and 2000 ppm), the CPE reduced formation of primary oxidation products (hydroperoxides) as shown by lower PVs (Figure 2.3.1A) and secondary oxidation products (aldehydes) as shown by lower AVs (Figure 2.3.1B) compared to the control

oil sample without any additive. In all the samples, PVs generally increased from the beginning of the storage period to the last day, showing the progression of primary oxidation. As expected, the lowest rate of increase of PVs was in the samples containing TBHQ. For the control oil sample without any additive however, the progressive increase in PV was significantly ($p < 0.05$) higher compared to the samples containing the CPE or TBHQ throughout the storage period. Though the PVs of the oil samples containing CPE at 1000 pm were lower, they did not appear to be significantly ($p < 0.05$) different from the PVs of oil samples containing the CPE at 1500 and 2000 ppm during most of the storage period, showing a comparable protective effect by the extracts at the three concentrations studied against the production of hydroperoxides.

(A)



(B)

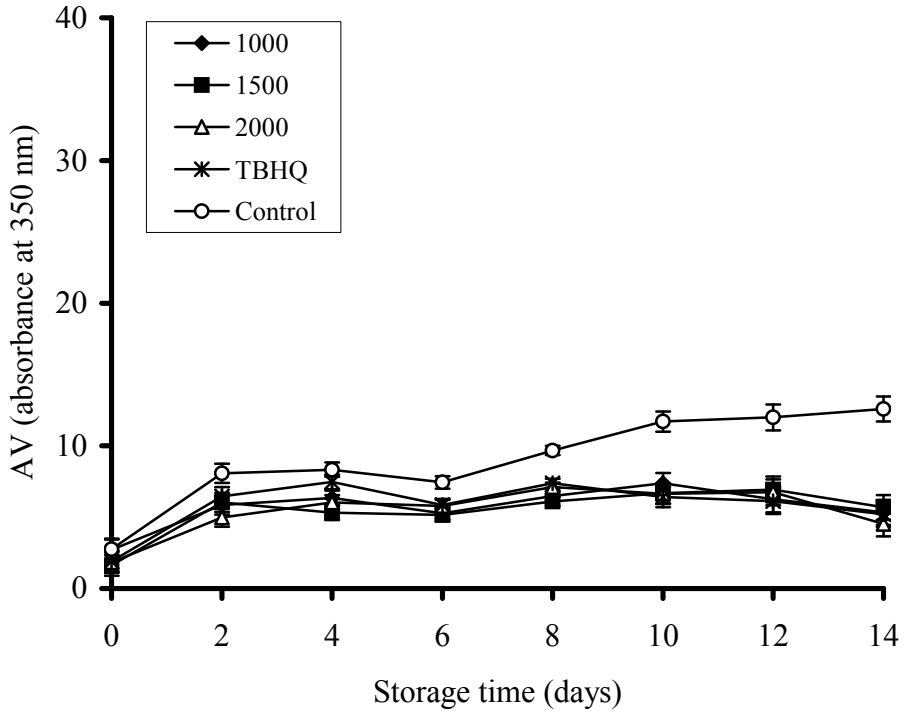


Figure 2.3.1. Effect of sorghum crude phenolic extract (CPE) and TBHQ on peroxide value (PV) **(A)** and anisidine value (AV) **(B)** of sunflower oil over a 14-day storage period. Each point on the graphs is the average value of six determinations obtained in three independent experiments. Bars represent standard error of the mean.

From day 4 to the end of the storage period, AVs for the control oil sample were significantly ($p < 0.05$) higher than the oil samples containing the CPE or TBHQ, showing the ability of the CPE and TBHQ to inhibit the formation of secondary oxidation products. Over the 14-day storage period, oil samples containing CPE at the three concentrations, and the samples containing TBHQ had similar AVs (Figure 2.3.1B). This indicates that the sorghum CPE (at the three concentrations) had a similar ability to inhibit formation of secondary oxidation products in the oil as TBHQ over the storage period.

Comparing Figure 2.3.1A and 2.3.1B, it can be observed that a high rate of hydroperoxide formation (Fig. 2.3.1A) did not necessarily lead to a high rate of generation of secondary oxidation products (Fig. 2.3.1B) in all samples. While the PVs for all samples showed a steady increase during the storage period, AVs for oil samples containing CPE and TBHQ were almost constant with only the control oil sample showing a gradual increase. Lipid oxidation is a complex multi-step process (Huang, Frankel & German, 1994), and antioxidants may have differing abilities in interfering with the various steps of the oxidation process. The above results suggest that though the sorghum CPE may not be as effective as TBHQ in preventing formation of hydroperoxides (primary oxidation products), they are as effective in preventing the decomposition of these hydroperoxides into secondary oxidation products. This may be due to the ability of the CPE to scavenge the radicals (perhaps alkoxyl radicals especially) arising from the decomposition of the hydroperoxides, hence preventing the radicals from further reaction to form volatile compounds such as aldehydes and ketones (Eskin & Przybylski, 2001). Similar results were obtained by Abdalla & Roozen (1999) who reported better inhibition of hexanal (secondary oxidation product) generation than formation of conjugated dienes (primary oxidation products) by thyme and lemon balm extracts in sunflower oil. Since secondary oxidation products (mainly aldehydes) are the substances that contribute to off-flavours in oxidized oils (Wanasundara & Shahidi, 1998), it may be suggested that at the concentrations studied and under the conditions of the oxidation process, the CPE from condensed tannin sorghum bran could be as effective as TBHQ at 200 ppm in preventing the development of oxidative rancidity or rancid odours in sunflower oil.

The antioxidant effect of the sorghum CPE may be attributed to the presence of phenolic compounds. The CPE was found to have total phenol content of 58.35 mg

tannic acid equivalents/100 mg, condensed tannin content of 491.80 mg catechin equivalents/100 mg, and an antioxidant activity of 4598.40 µM trolox equivalents/g (section 2.2.4.1, Table 2.2.1). Phenolic compounds are able to scavenge and stabilize lipid radicals by donating hydrogen atoms (Frankel, 1996; Eskin & Przbylski, 2001), hence reducing the rate of chain propagation of the oxidation process. Effectiveness of plant extracts to inhibit lipid oxidation due to their phenolic content has been previously demonstrated (Gordon & Weng, 1992; Onyeneho & Hittiarachchy, 1992; Wanasundara & Shahidi, 1998).

2.3.4.3 Effect of ferric ions on the oxidative stability of sunflower oil

Transition metals may promote lipid oxidation through their ability to decompose lipid hydroperoxides to free radicals (Gordon & Weng, 1992; Satue-Gracia *et al*., 2000; Fomuso *et al*. 2002), which then promote free radical reactions and the formation of volatile breakdown products (Satue-Gracia *et al.,* 2000). Previous studies have reported higher PVs in oil containing ferric ions (Gordon & Weng, 1992; Luzia *et al*., 1998) indicative of the influence of ferric ions on lipid oxidation. Results of this study however show significantly lower PVs for oil with added ferric ions compared to the control without added ferric ions (Figure 2.3.2A).

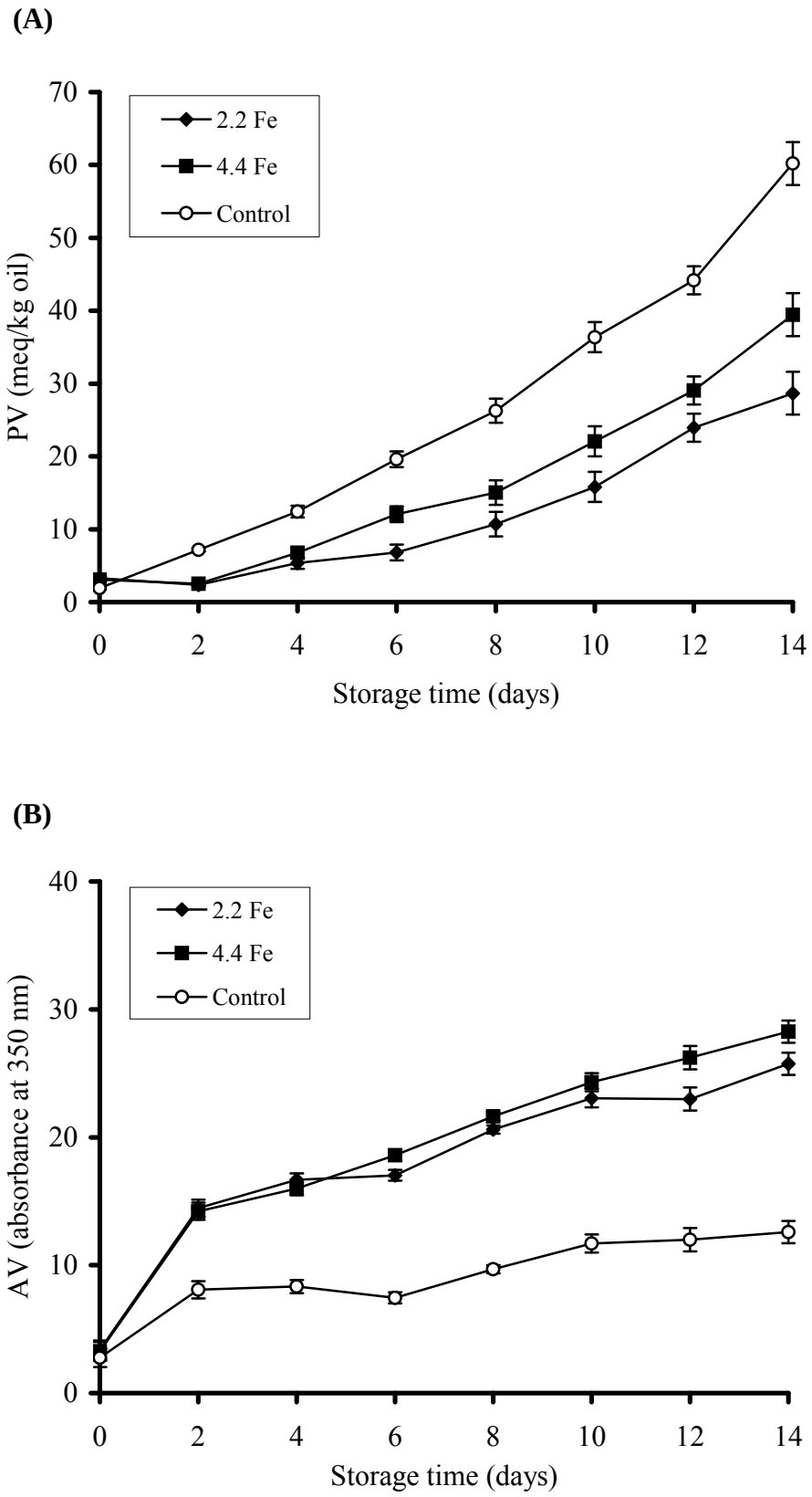


Figure 2.3.2. Effect of ferric ions on peroxide value (PV) **(A)** and anisidine value (AV) **(B)** of sunflower oil. Each point on the graphs is an average of six determinations obtained from three independent experiments. Bars represent standard error of the mean.

The lower PVs for oil with added ferric ions may however not be due to lower lipid oxidation rates. Iron has been shown to have an increased ability to decompose lipid peroxides, hence preventing their accumulation, which leads to lower PVs (Mancuso, McClements, & Decker, 1999). It may therefore be suggested that in this study, ferric ions accelerated the breakdown of hydroperoxides to secondary oxidation products (Satue-Gracia *et al*., 2000), and prevented their accumulation in the oil samples containing ferric ions. This means that oil samples containing ferric ions would be expected to have higher AVs compared to control samples due to an increase in secondary oxidation products, and this can be observed in Figure 2.3.2B. Van der Merwe (2003) reported similar findings for palm-olein where the PVs for oil samples containing copper were found to be lower than the control oil without added copper, whereas the AVs for the samples containing copper were higher (double as much) than that of the control oil. The author attributed the low PVs to the ability of copper to decompose the peroxides to secondary oxidation products. Fomuso *et al.* (2002) also reported a similar effect of iron in a model oil-in-water emulsion in which increased iron concentrations increased the PV, but an increasing incubation time brought about a decrease in the PV, which they attributed to hydroperoxide decomposition rates being greater than formation rates. Halliwell & Gutteridge (1990) also reported accelerated decomposition of pure lipid hydroperoxides in the presence of metal complexes, especially iron salts.

In general, from day 4, the oil containing a higher concentration of ferric ions (4.4 ppm) had higher PVs and AVs than the oil containing 2.2 ppm ferric ions. This suggests that the rate of production of hydroperoxides (as shown by PV) as well as the decomposition of hydroperoxides to aldehydes (as shown by AV) is proportional to the concentration of the ferric ions in the oil samples, in agreement with previous studies (Satue-Gracia *et al*., 2000; Fomuso *et al.*, 2002).

2.3.4.4 Effect of CPE from condensed tannin sorghum bran on the oxidative stability of sunflower oil in the presence of ferric ions

The PVs of sunflower oil samples containing CPE and ferric ions at various concentrations over the 14-day storage period are presented in Table 2.3.1. On the average, while PVs of oil samples containing CPE at 1000 ppm were similar to control oil samples (i.e. oil samples containing ferric ions but without extract or TBHQ) at both ferric ion concentrations, oil samples containing CPE at 1500 and

2000 ppm generally had higher PVs compared to the control samples. It could have been expected that the control oil samples would have higher PVs than the samples containing the extracts (at both concentrations of ferric ions), due to the known prooxidant effect of ferric ions (Gordon & Weng, 1992; Luzia *et al.,* 1998). Hydroperoxides are unstable and easily break down to secondary oxidation products under the influence of metal ions (Nawar, 1996). It may be suggested therefore that the lower PVs in the control samples compared to the oil samples containing CPE and ferric ions may not necessarily be an indication of lower oxidation rates, but that the peroxides formed did not accumulate in the oil samples due to their further decomposition by the ferric ions to secondary oxidation products (Mancuso *et al*, 2000; Fomuso *et al*., 2002). The ferric ions did not have the same influence on the oil samples containing extracts probably due to the chelation of the ferric ions by the CPE. There was no CPE in the control samples to chelate the ferric ions; therefore the ions were freely available to bring about the decomposition of the peroxides to secondary oxidation products. Chelation of metal ions by polyphenols reduces the metals' ability to catalyze reactions of lipid oxidation (Sugihara, Arakawa, Ohnishi & Furuno, 1999; Fernandez, Mira, Florencio & Jennings, 2002; Medina *et al*., 2002). Condensed tannins have been shown to form metal complexes with ferric ions (Porter, 1992; Slabbert, 1992). The suggested metal chelation by the CPE may therefore partly be attributed to the presence of condensed tannins in the CPE (section 2.2.4.1) among other phenolics present in the extract, which could also chelate the ferric ions.

Table 2.3.1. Effect of storage time on peroxide values of sunflower oil samples containing either sorghum crude phenolic extract (at different concentrations) or TBHQ, both in the presence of ferric ions

Treatment	Storage time (days)							
	0	2	4	6	8	10	12	14
Control (+2.2ppm Fe)	3.29[1] d[2]	2.37 a	5.37 b	6.82 bc	10.69 bc	15.81 ab	23.94 bc	28.68 a
Control (+4.4ppm Fe)	3.08 cd	2.51 a	6.79 bc	12.08 de	15.03 cd	22.07 cd	29.07 cd	39.47 b
TBHQ+2.2ppm Fe	2.62 bc	1.09 a	1.60 a	2.44 a	5.32 a	10.92 a	14.08 a	21.90 a
TBHQ+4.4ppm Fe	2.61 b	1.14 a	3.59 ab	4.77 ab	9.62 ab	13.17 a	19.57 ab	26.25 a
[3]1000E+2.2ppm Fe	1.59 a	3.79 ab	5.66 b	7.57 bc	11.51 bc	15.98 abc	21.61 b	28.84 a
1000E+4.4ppm Fe	1.74 a	3.44 a	7.09 bc	10.68 cd	17.19 cd	24.60 de	28.37 cd	38.32 b
1500E+2.2ppm Fe	2.09 ab	3.06 a	6.85 bc	11.75 de	17.93 de	25.16 de	30.84 de	41.47 b
1500E+4.4ppm Fe	1.89 a	3.78 ab	9.54 d	14.82 ef	23.09 f	31.44 f	39.41 f	52.92 d
2000E+2.2ppm Fe	1.80 a	3.63 ab	6.06 bc	9.49 cd	15.18 cd	21.46 bcd	28.06 cd	39.38 b
2000E+4.4ppm Fe	1.96 a	4.31 b	9.89 d	15.15 f	22.24 ef	29.62 ef	36.52 ef	51.04 c
SEM[4]	0.22	0.24	0.8	1.09	1.68	2.07	1.92	1.92

[1] Results are means of six determinations obtained in three independent experiments

[2] Means within each column followed by a different letter are significantly different ($p < 0.05$)

[3]1000E = 1000 ppm crude phenolic extract from sorghum bran in sunflower oil (similar interpretation for 1500E and 2000E)

[4] Standard error of the mean.

The proposed accelerated decomposition of hydroperoxides to secondary oxidation products in the presence of ferric ions is reflected in the corresponding significantly higher AVs for the control oil compared to the samples containing extracts and ferric ions (Table 2.3.2). The AVs for the control are also higher compared to oil without any additive, whose AVs during the storage period were 2.76, 8.08, 8.33, 7.43, 9.68, 11.70, 12.00 and 12.58 for day 0, 2, 4, 6, 8, 10, 12 and 14, respectively (Fig. 2.3.1B). From day 2 to the end of the storage period in the presence of ferric ions, the PVs of oil samples containing TBHQ were generally lower than that for oil containing CPE (at all the three concentrations) (Table 2.3.1). However, Table 2.3.2 shows that in the presence of ferric ions the AVs for oil samples containing TBHQ were significantly higher than the oil samples containing the CPE and ferric ions. This indicates that for the samples containing TBHQ, the ferric ions accelerated the decomposition of hydroperoxides to secondary oxidation products leading to lower PVs but higher AVs. TBHQ does not chelate metal ions (Shahidi & Wanasundara, 1992); hence the ferric ions would be expected to freely influence the decomposition of the hydroperoxides as in the control samples. For the oil samples containing the CPE, chelation of the ferric ions (presumably by the phenolic compounds in the extract) implies that the ions were not freely available to influence the decomposition of the hydroperoxides to secondary oxidation products. This is reflected by the generally lower AVs for oil samples containing CPE alone compared to the samples containing CPE and ferric ions: Oil samples containing CPE only at 1000 ppm had AVs of 2.71, 5.87, 6.36, 5.28, 6.48, 7.38, 6.26 and 5.34; that containing CPE only at 1500 ppm had AVs of 1.63, 6.04, 5.32, 5.15, 6.10, 6.68, 6.94 and 5.69, and the oil containing CPE alone at 2000 ppm had AVs of 1.83, 4.99, 6.03, 5.78, 7.11, 6.64, 6.75 and 5.53, during day 0, 2, 4, 6, 8, 10, 12 and 14 for each case. Since secondary oxidation products (aldehydes in particular) are the source of the characteristic odour of rancid fats (Coultate, 2002), the CPE may be considered as potential agents in preventing development of rancidity in sunflower oil.

Table 2.3.2. Effect of storage time on anisidine values of sunflower oil samples containing either sorghum crude phenolic extract (at different concentrations) or TBHQ, both in the presence of ferric ions

Treatment	Storage time (days)							
	0	2	4	6	8	10	12	14
Control (+2.2ppm Fe)	3.39[1] a[2]	14.46 c	16.67 c	17.03 c	20.61 c	23.06 b	22.99 b	25.75 cd
Control (+4.4ppm Fe)	3.27 a	14.22 c	15.98 bc	18.59 d	21.63 d	24.31 bc	26.22 c	28.26 d
TBHQ+2.2ppm Fe	2.62 a	11.46 b	14.73 b	18.26 cd	20.58 c	22.99 b	23.14 b	25.25 c
TBHQ+4.4ppm Fe	2.96 a	11.76 b	15.44 bc	18.70 d	22.00 d	26.38 c	26.44 c	27.80 cd
[3]1000E+2.2ppm Fe	2.64 a	5.77 a	5.23 a	5.74 a	7.56 ab	7.75 a	7.40 a	6.29 ab
1000E+4.4ppm Fe	2.37 a	6.53 a	6.64 a	7.66 b	8.01 b	8.67 a	8.54 a	6.50 ab
1500E+2.2ppm Fe	2.20 a	5.63 a	6.09 a	5.46 a	6.89 a	6.79 a	6.57 a	6.34 ab
1500E+4.4ppm Fe	1.92 a	5.05 a	5.95 a	5.74 a	7.05 a	6.68 a	7.08 a	8.43 b
2000E+2.2ppm Fe	1.85 a	5.45 a	5.56 a	6.53 ab	7.14 ab	6.70 a	6.45 a	5.69 a
2000E+4.4ppm Fe	2.05 a	6.70 a	6.10 a	7.13 b	6.84 a	7.70 a	7.77 a	7.33 ab
[4]SEM	0.73	0.67	0.51	0.43	0.32	0.71	0.91	0.87

[1] Results are means of six determinations obtained in three independent experiments

[2] Means within each column followed by a different letter are significantly different ($p < 0.05$)

[3]1000E = 1000 ppm crude phenolic extract from sorghum bran in sunflower oil (similar interpretation for 1500E and 2000E)

[4] Standard error of the mean.

In all the oil samples, the formation of both the primary (hydroperoxides) and secondary (aldehydes) oxidation products, as monitored by PVs (Table 2.3.1) and AVs (Table 2.3.2), respectively, increased with increasing ferric ion concentration. Metals promote the decomposition of lipid peroxides which would both initiate free radical propagation reactions and the formation of volatile breakdown products (Satue-Gracia *et al*., 2000). It would therefore be expected that a higher ferric ion concentration would result in a higher generation of both primary and secondary oxidation products.

Guillen & Cabo (2002) observed that hydroperoxide generation and degradation made it difficult to obtain reproducible PVs, and hence concluded that hydroperoxide generation and degradation rates have more influence on the accuracy of PV determination than the amount of hydroperoxides present in a sample. Nawar (1996) also noted that the usefulness of PV measurement depends on the system and conditions in which lipid oxidation is studied which would have a bearing on the fate of the hydroperoxides generated. It would therefore appear that though PVs are often used as an indicator of initial stages of oxidation (O'Brien, 2004), hydroperoxides decompose rapidly during storage, upon heating or in the presence of ferric ions; hence PV may not necessarily indicate the extent of lipid oxidation. It has been shown in this study therefore that in the presence of ferric ions, PV measurements alone may not give a true representation of the rate of lipid oxidation.

2.3.4.5 Colour development in oil samples

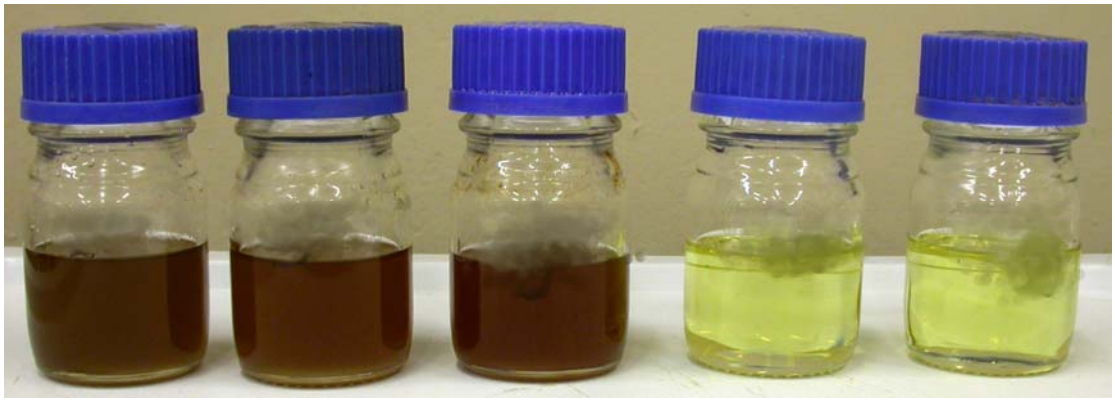
The oil samples containing the CPE developed a brownish colour due to the colour of the extracts, with the colour intensity increasing with increasing concentration of the extracts (Figure 2.3.3). The colour of the CPE, which led to the colour change of the oil samples, could be attributed to presence of phenolics such as anthocyanins, tannins and other flavonoids in the extract (Nip & Burns, 1969; Blessin, van Etten & Dimler, 1963; Hahn & Rooney, 1986).

(a) **(b)** **(c)** **(d)** **(e)**

Figure 2.3.3. Colour changes in sunflower oil containing (a) 1000 ppm CPE; (b) 1500 ppm CPE; (c) 2000 ppm CPE; (d) 200 ppm TBHQ; (e) no additive (control)

In the presence of ferric ions, the oil samples containing CPE developed a dark colour, while the control sample and the oil samples containing TBHQ did not develop the dark colour (Fig. 2.3.4). The dark oil solution formed looked turbid throughout the storage period. The development of the dark colour in the oil samples containing the CPE in the presence of ferric ions could be due to the suggested ferric ion chelation by the CPE. The development of coloured compounds has been reported when phenolic compounds form complexes with metal ions (Miller, Castellucio, Tijburg & Rice-Evans, 1996; Makris & Rossiter, 2000). Condensed tannins are known to form highly coloured complexes with ferric ions, the iron complexation being formed with the *ortho*-dihydroxyl group on the B-ring of the condensed tannin molecule (Slabbert, 1992). The absence of colour development in the oil samples containing TBHQ and ferric ions at both concentrations (Figure 2.3.4A (d) and 2.3.4B (d)) indicates that TBHQ did not complex with the ferric ions.

(A) Ferric ions at 2.2 ppm concentration



(a) **(b)** **(c)** **(d)** **(e)**

(B) Ferric ions at 4.4 ppm concentration

(a) **(b)** **(c)** **(d)** **(e)**

Figure 2.3.4. Colour development in sunflower oil samples containing (a) 1000 ppm CPE; (b) 1500 ppm CPE; (c) 2000 ppm CPE; (d) 200 ppm TBHQ, and (e) no additive (control) in the presence of ferric ions at **(A)** 2.2 ppm and **(B)** 4.4 ppm

The imparting of colour to some foods by plant pigments could be a constraint to their use as food additives (Bonilla, Mayen, Merida & Medina, 1999), and such a characteristic does not satisfy one of the criteria for acceptance of antioxidants for use in foodstuffs (Dugan, 1976; Schuler, 1990; Milic, Djilas, & Canadonovic-Brunet, 1998). The imparting of colour to the oil by the extract could hence be a negative attribute of the tannin sorghum used in this study as a source of natural antioxidants. Removal of the colour pigments from the extract may therefore be considered. However, this should be balanced against the potential of reducing the potency of the extracts as antioxidants if the pigments are removed. Using the CPE in combination with metal chelators such as citric acid may on the other hand alleviate the colour

development due to the suggested metal chelation. Propyl gallate (PG) causes colour development in oil when it chelates iron ions, and the use of citric acid in combination with PG prevents this colour development (Shahidi & Wanasundara, 1992; Eskin & Przybylski, 2001).

2.3.5 Conclusions

CPE from condensed tannin sorghum bran inhibits the formation of both primary and secondary oxidation products in sunflower oil. Ferric ions have an influence on the effectiveness of the sorghum CPE as well as that of TBHQ as antioxidants. The CPE exhibits a lower ability to suppress formation of primary oxidation products compared to TBHQ both in the absence and presence of ferric ions. In the absence of ferric ions (Fig. 2.3.1B), the CPE and TBHQ show a similar ability to inhibit formation of secondary oxidation products but the CPE is more effective in the presence of ferric ions. This may be due to the ability of phenolic compounds in the CPE to chelate ferric ions, which prevents the ferric ions from influencing the decomposition of hydroperoxides to secondary oxidation products. CPE from condensed tannin sorghum bran may therefore be considered as a potential source of natural antioxidants to prevent lipid oxidation and development of rancidity in sunflower oil. A potential drawback to the use of the CPE however is that it imparts colour to the oil, which is an unacceptable characteristic for a food antioxidant. Tannin sorghum bran crude phenolic extracts may therefore require further purification if the extracts are to be used as antioxidants in edible oils, and the colour development due to the suggested metal chelation may be alleviated by combined use of the sorghum extracts with metal chelators such as citric acid.

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CHAPTER 3

3. GENERAL DISCUSSION

The first section of the general discussion will deal with the methodological considerations of the research project. The second part will discuss the potential of sorghum as a source of natural antioxidants with reference to the phenolic content and antioxidant activity of crude extracts from sorghum, their storage stability, and their effectiveness as antioxidants in sunflower oil.

3.1 Discussion of methods used

The initial part of this study involved determining the phenolic contents and antioxidant activity of two Malawian sorghum varieties: a brown-coloured condensed tannin sorghum (*Phatafuli*) and a white-coloured condensed tannin-free sorghum (*Shabalala*). The presence or absence of condensed tannins in the sorghums was determined by using a rapid screening method, the Chlorox bleach test (Waniska *et al*., 1992). The method determines the presence or absence of a black-pigmented testa layer, an indication of the presence or absence of condensed tannins, respectively. Sorghum grains are soaked in chlorox reagent (5% NaOH in commercial bleach, 3.5% sodium hypochlorite). The bleach oxidizes the constituents in the pericarp and testa, causing them to dissolve, revealing the presence or absence of a black-pigmented testa layer (Rooney *et al*., 1980; Earp *et al*., 1981; Waniska *et al*., 1992). The test is simple and inexpensive (Waniska *et al*., 1992). It is however qualitative, hence does not quantify the tannins in the sorghum. The results of the Chlorox test were therefore confirmed by the vanillin-HCl method, which is a quantitative spectrophotometric method for the determination of condensed tannins.

In view of the fact that phenolic compounds in sorghum are known to be concentrated in the outer layers of the grain (bran) (Rooney *et al*., 1980; Hahn *et al*., 1984; Hahn & Rooney, 1986), the sorghum grain was decorticated (approximately 10% bran yield), and the phenolic contents and antioxidant activity of the bran were determined. This was also in view of the fact that the bran could be a potential cheap source of natural antioxidants since it is a waste product during the utilization of sorghum. The determinations were also done for the whole grain to assess the relative proportions of these in the grain and bran. The sorghum was decorticated using a sorghum dehuller,

and a 10% bran yield was obtained, which ensured minimal endosperm contamination of the bran, considering that pericarp forms about 7.9% by weight of the sorghum kernel. A high endosperm contamination of the bran could have reduced the assayable phenolic content and antioxidant activity from the bran, since the endosperm contains insignificant amounts of phenols (Hahn & Rooney, 1986; Waniska, 2000).

Various extraction solvents have different extraction efficiencies on different types of material. For sorghum, a number of solvents have been used to extract phenols, the most widely used being methanol (Yasumatsu *et al*., 1965; Deshpande *et al*., 1986; Beta *et al*., 2000), acidified methanol (Nip & Burns, 1971; Kaluza, McGrath, Roberts & Schroeder, 1980; Deshpande *et al*., 1986; Awika *et al*., 2004), ethanol (Deshpande *et al*., 1986) and aqueous acetone (Kaluza *et al*., 1980; Deshpande *et al*., 1986; Awika *et al*., 2003; Awika *et al*., 2004). For the determination of total phenols and antioxidant activity, samples were extracted with 75% aqueous acetone (Kaluza *et al*., 1980). The samples were extracted for 2 h (Awika *et al*., 2004) with periodic vortex mixing every 10 minutes, and then the sample residue was rinsed twice with the solvent.

For condensed tannins, a 20-minute extraction using absolute methanol was employed. Acidified methanol, acetone and ethanol and their aqueous solutions and water have previously been used for this purpose, but absolute methanol has been recommended as the choice solvent for tannin extraction, considering that it can be easily removed in cases where purification of tannins from crude extracts is desired (Deshpande *et al*., 1986). The choice of methanol extraction was also made considering that the vanillin-HCl assay for condensed tannins was to be used, in which samples and standards are required to be in absolute methanol (Waterman & Mole, 1994).

A number of methods are used to determine phenolic compounds, and the choice of a method depends on the type of phenolic compound to be determined. The Folin-Ciocalteu method (Singleton & Rossi, 1965) as modified by Waterman & Mole (1994) was used in this study for the determination of total phenols. Determination of total phenols quantifies the total concentration of phenolic hydroxyl groups present in a sample, regardless of the specific molecules in which the hydroxyl groups occur (Waterman & Mole, 1994). The Folin-Ciocalteu method was chosen for this purpose

due to its wide applicability for biological materials (Waterman & Mole, 1994). The method is based on the reducing power of the phenolic hydroxyl groups (Hahn *et al*., 1984), which react with the Folin-Ciocalteu phenol reagent (an oxidizing agent comprised of heteropolyphosphotungstate-molybdate) under basic conditions to form chromogens that can be detected spectrophotometrically at 760 nm. One disadvantage of oxidation-reduction methods such as the Folin-Ciocalteu method is that any substance present in a sample which is capable of being oxidized by the reagents will produce the reduced (coloured) forms of the reagents and would appear as phenolic (Waterman & Mole, 1994). This would therefore overestimate the phenol content. Ascorbic acid has been found to be one of the main interfering compounds in such assays (Deshpande *et al*., 1986; Waterman & Mole, 1994). Sorghum however does not contain ascorbic acid (vitamin C) (USAID, 2005) and is therefore of no concern in total phenol assays. The use of the Folin-Ciocalteu method for the determination of total phenols has however been questioned since the basic mechanism of the method is an oxidation/reduction reaction, which is a basis for the measurement of antioxidant activity (Prior, Wu & Schaich, 2005).

The vanillin-HCl method (Price *et al*., 1978) was used for the determination of condensed tannins due to its specifity in quantifying the condensed tannins (Deshpande *et al*., 1986; Waterman & Mole, 1994), and its wide application in sorghums (reviewed by Awika & Rooney, 2004). It is also reported to be reproducible (Julkunen-Tiitto, 1985; Deshpande *et al*., 1986). The vanillin reagent (4% HCl in methanol and 0.5% vanillin in methanol) reacts with flavanols with a single bond between C-2 and C-3 of the C ring (Earp *et al*, 1981; Deshpande *et al*., 1986) (see Figure 1.2.3 for basic flavonoid structure) in the presence of HCl to give a bright red colour (Hahn *et al*., 1984) that is determined spectrophotometrically. Since condensed tannins are condensation products of flavan-3-ols and flavan-3,4-diols, both with a single bond between C-2 and C-3 of the C ring (Figure 1.2.6), they give a positive reaction with vanillin (Gupta & Haslam, 1980). Other non-tannin flavonoid compounds such as anthocyanidins also develop red colour in mineral acids (Deshpande *et al*., 1986), which could overestimate condensed tannin content in a sample. A blank subtraction was therefore done to attempt the elimination of the non-tannin compounds that give such positive reactions that can overestimate the tannins (Price *et al*., 1978). The sample blanks were done by reacting the samples with 4% HCl in methanol in place of the vanillin reagent. Using this method, results of this

study compared well with other results reported in the literature for tannin sorghums, also using catechin as a standard (Earp *et al*., 1981; Beta *et al*., 1999).

Determination of antioxidant activity of samples is generally based on the inhibition of a particular reaction in the presence of an antioxidant. Common approaches used to test antioxidants in foods and biological systems involve using free-radical-trapping protocols which measure the ability of antioxidants to scavenge free-radicals, or the oxidizing of a lipid or lipoprotein substrate under standard conditions and assessing the antioxidant activity by determining how much oxidation is inhibited (Frankel & Meyer, 2000). For screening purposes however, radical scavenging methods are preferred and widely used due to their high-throughput (Van den Berg *et al*., 1999). The TEAC assay (Miller, Rice-Evans, Davies, Gopinathan & Milner, 1993) is one of the widely used screening methods for antioxidant activity. The original assay by Miller *et al*. (1993) was improved upon by Re *et al*. (1999), and this study used a modification of the assay as described by Awika *et al*. (2003). The assay measures the relative ability of an antioxidant to scavenge the 2,2'-azinobis (3-ethyl-benzothiazoline-6 sulfonic acid) radical ($ABTS^{\bullet+}$) generated in aqueous phase, as compared with Trolox (water soluble vitamin E analogue) standard. The degree of decolorization reflects the extent of the scavenging of the $ABTS^{\bullet+}$ radicals and is determined spectrophotometrically at 734 nm. The assay is applicable for assessing antioxidant capacity of single compounds, food components, and food extracts as well as biological systems (Van den Berg *et al*., 1999), and is rapid, relatively cheap and has good repeatability (reviewed by Awika *et al.*, 2003). It is also a simple and easy method to use (Makkar & Becker, 1994; Pietta *et al*., 2000; Arnao *et al*., 2001; Huang, Ou & Prior, 2005), avoids interference due to endogenous reaction, and antioxidant activity can be studied over a wide range of pH (Arnao *et al*., 2001). One limitation of the TEAC assay is that it may not only reflect the antioxidant capacity of the compound under test alone, but rather the sum of the compound and its reaction products formed by the reaction of the parent compound with $ABTS^{\bullet}$, which may further react with the $ABTS^{\bullet+}$ (Arts, Dallinga, Voss, Haenen & Bast, 2003). Despite this limitation, results of this study using the TEAC method were comparable to other studies (Awika *et al*., 2003).

Methods used to determine the extent of oxidation of lipid foods such as sunflower oil are based on the measurement of the concentration of primary or secondary oxidation products. Effectiveness of an antioxidant to retard lipid oxidation can therefore be assessed by the extent to which it prevents the formation of primary or secondary oxidation products. Sensory evaluation methods are recognized to be more reliable to evaluate oxidative state of lipid foods but are rarely applied in routine analysis because they are time-consuming and expensive (Rossell, 1986; O'Brien, 2004). Oil stability tests are therefore usually determined by objective methods and under accelerated conditions since use of ambient temperatures could require long test periods (Abou-Gharbia *et al*., 2000). The tests involve subjecting the oil to accelerated oxidation conditions such as high temperature, airflow, presence or absence of light or other catalyzers such as metal ions (Guillen & Cabo, 2002), and measuring the extent of oxidation by monitoring the levels of the primary or secondary oxidation products. In this study, the oil samples were subjected to a temperature of 65°C in the dark. The effect of the sorghum extracts on the oxidative stability of sunflower oil was assessed in the presence or absence of ferric ions. Presence of metals at concentrations as low as 0.1 ppm (0.1 mg/kg) increases the rate of oxidation (Nawar, 1996).

Common indicators to measure lipid oxidation products include the measurement of peroxide value (Guillen & Cabo, 2002) and conjugated diene hydroperoxides (Moure *et al*., 2001; Guillen & Cabo, 2002) for primary oxidation products; anisidine value (Guillen & Cabo, 2002), volatile compounds (Moure *et al*., 2001; Guillen & Cabo, 2002) and carbonyl value (Guillen & Cabo, 2002) for secondary oxidation products. These various compounds measured by different methods imply that each method is capable of providing information on the concentration of a particular product during the oxidation process; therefore one test method may not provide a whole picture of the progression of oxidation of oil or the extent of effectiveness of an antioxidant in retarding lipid oxidation (Nawar, 1996). A combination of tests may therefore be required to monitor the various stages of the oxidation process. Monitoring the extent of lipid oxidation in this study was therefore done by determining both the primary and secondary oxidation products by the measurement of peroxide values (PVs) and anisidine values (AVs), respectively.

The use of both PVs and AVs in this study provided information on the extent of the effectiveness of the tannin sorghum bran crude phenolic extracts (CPE) in the inhibition of both the primary and secondary oxidation products. Using PVs, the results showed that the CPE was effective in inhibiting formation of hydroperoxides but their effectiveness was lower than that for TBHQ. On the other hand AVs showed that the CPE was equally effective as TBHQ in inhibiting the generation of secondary oxidation products. This suggests that the tannin sorghum bran CPE was more effective in inhibiting the generation of secondary oxidation products than primary oxidation products. The use of the two tests also enabled the monitoring of the influence of ferric ions on the lipid oxidation process. It was observed that the presence of ferric ions in oil samples led to accelerated decomposition of hydroperoxides to secondary oxidation products as shown by low PVs and corresponding higher AVs. The use of only one of these tests could hence have not provided such information. The use of both PVs and AVs therefore provides a clearer picture of how the oil is being oxidized as compared to using only one of them.

3.2 Discussion of study results

As reported (section 2.1), *Phatafuli* sorghum contained significantly higher total phenols and antioxidant activity in both the bran and whole grain compared to *Shabalala* sorghum. The higher total phenol content in both the bran (56 times more) and grain (156 times more) of *Phatafuli* sorghum compared to that of *Shabalala* sorghum bran and grain is due to the presence of condensed tannins in *Phatafuli* sorghum, which was not detected in *Shabalala* sorghum. Condensed tannin molecules posses multiple hydroxyl groups that constitute a large proportion of phenols present in the sorghum grain of high tannin varieties (Butler, 1981; Hahn & Rooney, 1986), thus largely contributing to the high total phenol content of *Phatafuli* sorghum. Since phenolic compounds are largely responsible for antioxidant activity of plant extracts (Awika *et al*., 2003; Guo *et al*., 1997; Velioglu *et al*., 1998), it followed that *Phatafuli* sorghum bran and grain had very high antioxidant activity compared to *Shabalala* sorghum bran and grain. The antioxidant activity of *Phatafuli* bran was 53 times higher than *Shabalala* bran while that of *Phatafuli* whole grain was 102 times higher than *Shabalala* whole grain. High molecular weight phenolic compounds such as condensed tannins have higher antioxidant activities, up to a certain extent, than lower molecular weight phenolics (Hagerman *et al*., 1998; Plumb *et al*., 1998), probably because they contain more significant areas of charge delocalization that enable the

scavenging of free radicals and stabilization of antioxidant radicals. The reported high antioxidant activity of condensed tannins (Hagerman *et al*., 1998) may therefore account for the high antioxidant activity of *Phatafuli* sorghum bran and grain than that of *Shabalala* sorghum bran and grain.

Both *Shabalala* grain and bran yielded significantly lower phenolic content and antioxidant activity than *Phatafuli* grain and bran (section 2.1.4.2, Table 2.1.2); it may therefore not be cost-effective to use condensed tannin-free sorghum as a source of natural antioxidants, due to their low antioxidant activity. Further studies on the stability of the phenolic extracts during storage and their effectiveness in preventing oxidative stability of sunflower oil were therefore done using crude extracts from the bran of the condensed tannin sorghum (*Phatafuli*). As shown in section 2.1.4.2 (Table 2.1.2), both the phenol concentration and antioxidant activity in the bran of *Phatafuli* was three times higher than that in the whole grain. Freeze-drying of the crude phenolic extract from the sorghum bran produced a yield of 10% soluble solids and further concentrated the phenol content and antioxidant activity. The 10% yield of soluble solids may be considered high enough to render it cost-effective to use condensed tannin sorghum bran as a source of natural antioxidant compared to tannin-free sorghum (where the yield was less than 0.05%). The 10% yield is even comparable to 9.7% reported for durum wheat bran (Onyeneho & Hettiarachchy, 1992), which was also considered as a potential alternative source of natural antioxidant. Comparatively, black and brown sorghum bran has even been shown to have better antioxidant properties than fruits such as grapes, strawberries and blueberries (Awika & Rooney, 2004). In addition to the level of yield of soluble solids and antioxidant activity, the freeze-dried form would also be a more appropriate form for storage and for incorporation in edible oils in that the freeze-dried form would be more stable than a liquid extract during storage, and that it could easily be reconstituted in an appropriate solvent for better dispersion in the oil.

As reported (section 2.2.4.2), oxidizing conditions (bubbling of oxygen into the extract) did not have significant effects on the content of total phenols, condensed tannins and antioxidant activity. Oxygen however brings about oxidation of phenolic compounds (Haslam *et al*., 1992; Waterman & Mole, 1994), which may affect their chemical and functional properties such as antioxidant properties. The oxidizing conditions used in this study may therefore not have been to the extent that would

bring about significant changes in the phenols and their chemical and functional properties. The time of bubbling with oxygen (10 min) may have not been enough to cause any such significant changes. Results of the study therefore suggest that exposure of the liquid extract to oxygen under the conditions used in the study may not have any significant negative effect on the phenolic content and antioxidant activity, which may imply that oxidative conditions may not be a significant factor to affect the antioxidant activity of sorghum extracts.

Among the storage conditions studied (presence or absence of air due to packaging, temperature and time), storage time had the most significant effect on the stability of the CPE during storage in relation to its content of total phenols, condensed tannins and antioxidant activity (section 2.2.4.3 – 2.2.4.5). Storage may promote a progressive polymerization of phenolic compounds (Manzocco *et al*., 1998), which can either result in an increase or decrease in radical scavenging properties (Nicoli *et al*., 2000; Pinelo *et al*., 2004). The observed trend of an initial increase in antioxidant activity of the tannin sorghum CPE (section 2.2.4.5) could have therefore been due to the formation of new compounds with higher antioxidant activity as a result of polymerization (Nicoli *et al*., 2000; Pinelo *et al*., 2004). The increase in antioxidant activity is possibly due to the increased ability of the aromatic structure of the polymerized compounds to support unpaired electrons and their delocalization around the aromatic ring (Nicoli *et al*., 2000). Further polymerization of the phenolics could have however resulted in a decrease in antioxidant activity due to steric hindrance, which results in the reduction in the availability of hydroxyl groups to scavenge radicals (Pinelo *et al*., 2004). Such an initial increase and subsequent decrease in antioxidant activity has been observed in phenolic solutions of catechin (Nicoli *et al*., 2000; Pinelo *et al*., 2004), resveratrol and quercetin (Pinelo *et al*., 2004). The sorghum CPE used in this study contained condensed tannins, which are polymers of catechin and epicatechin (Gupta & Haslam, 1980; Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozi & Prior, 2002). When isolated, condensed tannins, as well as the structural units composing them (catechin and epicatechin) get altered with time (Okuda *et al*., 1989), probably due to oxidation and polymerization, which may result in changes in their chemical reactivity, such as the antioxidant properties. The significant reduction in antioxidant activity of the sorghum CPE with time therefore suggests that the extracts may need to be used shortly after extraction, since storing it for longer periods could result in a subsequent decline in antioxidant activity.

Storing the freeze-dried sorghum bran extract in the presence or absence of air (vacuum packaging) did not alter their levels of total phenols, condensed tannins and antioxidant activity significantly. This could probably have been due to better stability of the extract in the dry state, in which it could be difficult for major changes to occur in phenol content and antioxidant activity compared to liquid state. Storage temperature however had some significant effect on the stability of the CPE during some days of storage. Increasing temperature accelerates chemical reactions, hence higher temperatures influence the oxidation of phenolic compounds (Palma *et al*., 2001), and as the number of hydroxyl groups increase, phenolic compounds become increasingly susceptible to further oxidation (Thomson, 1964). Polymeric phenolics such as condensed tannins contain more hydroxyl groups; therefore they could be more susceptible to oxidation at higher temperatures.

For both *Phatafuli* and *Shabalala* sorghum grain and bran, antioxidant activities of the extracts were significantly correlated with the levels of both total phenols, and for *Phatafuli*, the antioxidant activity also significantly correlated with condensed tannin content (section 2.1.4.3). During storage of the CPE from *Phatafuli* sorghum however, there were low correlations between the antioxidant activity and the phenolic content (section 2.2.4.4). This showed that while phenolic compounds were largely responsible for the antioxidant activity of the sorghum grain and bran extracts, new compounds may have been formed due to oxidation and polymerization (Okuda *et al*., 1989; Nicoli *et al*., 2000; Pinelo *et al*., 2004) during storage of the CPE, which resulted in the low correlations, probably because the compounds formed during the storage period may not have had the ability to support free radical scavenging due to change in chemical structure.

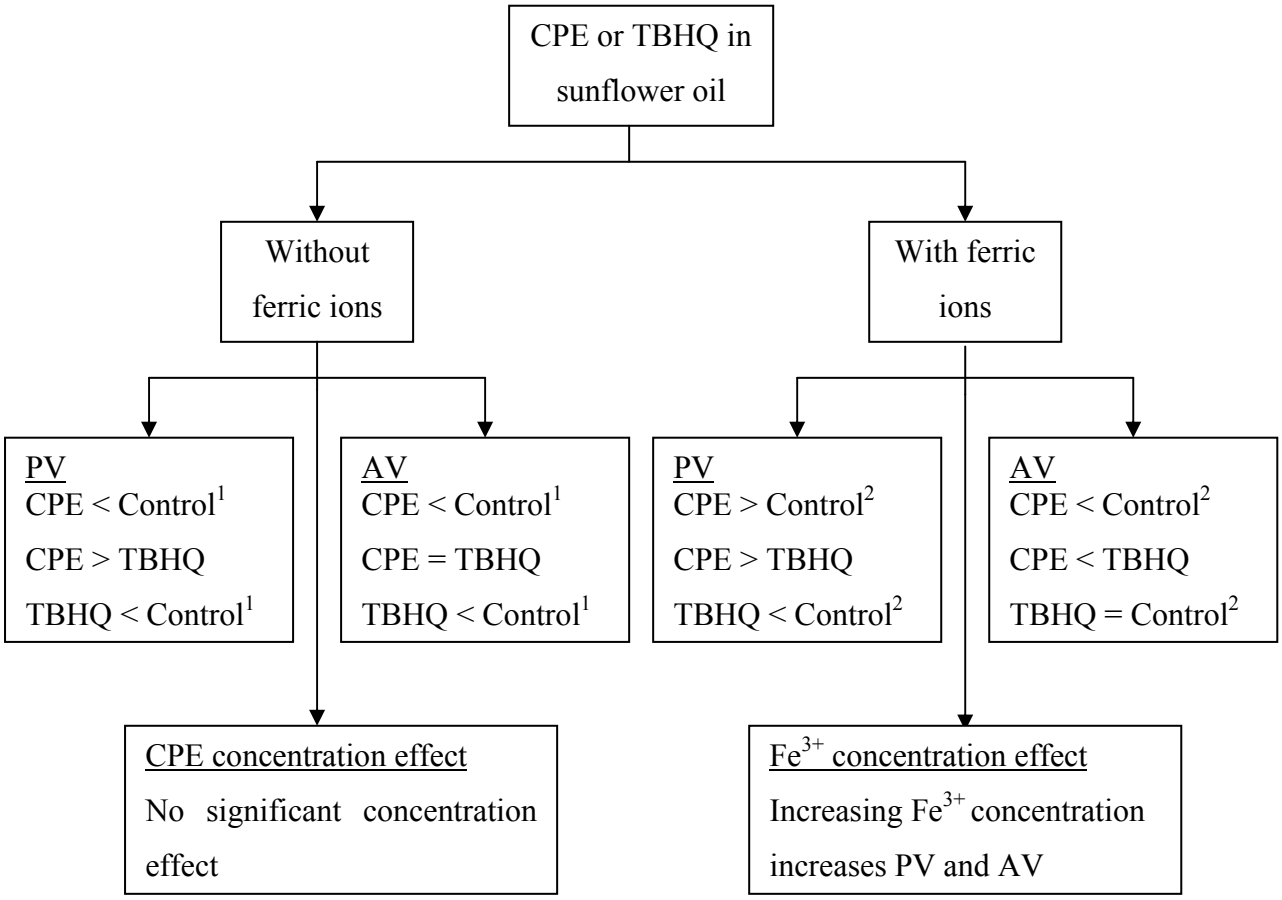

Figure 3.1. Summary of the effect of freeze-dried crude phenolic extract (CPE) from condensed tannin sorghum bran in sunflower oil in the presence or absence of ferric ions. ([1]Oil without Fe^{3+}, CPE or TBHQ; [2]Oil with Fe^{3+}, no CPE or TBHQ).

The antioxidant activity (by free radical scavenging) exhibited by the CPE using the TEAC assay was confirmed by the effectiveness of the CPE to inhibit lipid oxidation in sunflower oil, and this is summarised in Figure 3.1. In the absence of ferric ions, the CPE inhibited oxidation of the sunflower oil as shown by lower PVs and AVs compared to the oil without the CPE (control), but it (CPE) was less effective in controlling PVs compared to TBHQ. The CPE was however equally effective as TBHQ in the prevention of the generation of secondary oxidation products as shown by their similar AVs (section 2.3.4.2). TBHQ is known for its effectiveness in preventing formation of secondary oxidation products (O'Brien, 2004). The similar ability of the CPE to that of TBHQ in preventing secondary oxidation products therefore shows the potential antioxidant potency of the CPE. This could be due to the ability of phenolic hydroxyl groups of the phenolic compounds in the extracts to react with free radicals (probably alkoxyl radicals, $RO^{\bullet}$), therefore interrupting the autoxidation chain reaction, and preventing further decomposition of the radicals to

secondary oxidation products (Eskin & Przybylski, 2001). The phenolic hydroxyl groups of the phenolic compounds (AH) in the extracts may react with the radicals ($RO^{\bullet}$) as shown below, resulting in the formation of stable antioxidant free radicals ($A^{\bullet}$).

$$RO^{\bullet} + AH \longrightarrow ROH + A^{\bullet}$$

The phenolic compound (AH) donates a hydrogen atom to the free radical ($RO^{\bullet}$), and the antioxidant free radical ($A^{\bullet}$) formed is stable because it is not reactive enough to continue the chain propagation (Frankel, 1996; Eskin & Przybylski, 2001) due to stabilization by resonance delocalization of the unpaired electron around the aromatic ring of the phenolic compound (Gordon, 1990; Nawar, 1996; Coultate, 2002) (Figure 3.2).

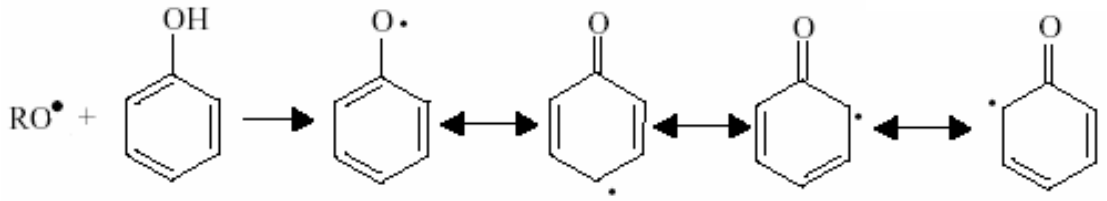

Figure 3.2. Stabilization of phenolic antioxidant free radical by resonance (Adapted from Coultate, 2002)

In the presence of ferric ions, oil samples containing CPE had on average, higher PVs and lower AVs than the control samples (oil with ferric ions only) and samples containing TBHQ (section 2.3.4.4). The lower PVs in the control samples and samples containing TBHQ and ferric ions may however not have been due to lower oxidation rates, but that the increased ability of ferric ions to decompose hydroperoxides prevented the accumulation of the peroxides in the oil, resulting in the low PVs (Mancuso *et al*., 1999) and high AVs. The ferric ions in these two samples were freely available to influence the breakdown of the hydroperoxides to secondary oxidation products, which led to lower PVs and higher AVs. Ferric ions breakdown hydroperoxides to form peroxyl radicals ($ROO^{\bullet}$) (as shown in equation below), which may promote further free radical reactions (Nawar, 1996; Hamilton *et al*., 1997).

$$Fe^{3+} + ROOH \longrightarrow Fe^{2+} + H^{+} + ROO^{\bullet}$$

The peroxyl radicals ($ROO^{\bullet}$) formed may also be broken down to release carbonyl compounds such as aldehydes (Dugan, 1996; Nawar, 1996; McWilliams, 1997). This

may explain the higher AVs in the control samples and samples containing TBHQ and ferric ions.

The ferric ions however did not have the same influence on the oil samples containing the CPE probably due to chelation of the ferric ions by phenolic compounds in the CPE, which made the ferric ions unavailable to participate in the decomposition of hydroperoxides. Though polyphenols may reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Sugihara *et al*., 1999; Paiva-Martins & Gordon, 2005) and that Fe^{2+} has an even greater ability than Fe^{3+} at catalyzing the decomposition of hydroperoxides to free radicals (Dunford, 1987), the ferric ions chelated by the polyphenols may be rendered catalytically inactive to further decompose hydroperoxides and generate free radicals (Shahidi & Wanasundara, 1992; Sugihara *et al*., 1999; Fernandez, Mira, Florencio & Jennings, 2002).

The development of colour in oil samples containing CPE and ferric ions, but not in samples containing ferric ions alone or TBHQ and ferric ions is also suggestive of ferric ion chelation by the sorghum CPE. The colour formation between phenolic compounds and ferric ions is due to spectral properties, as a result of differences between the ground state and excited state of molecules. Phenols have characteristic absorption bands that are normally in the ultraviolet region of the spectrum, but oxidizing cations such as Fe^{3+} have phenol-to-metal charge transfer bands that are low enough in energy to encroach the visible region of the spectrum (Paiva-Martins & Gordon, 2005). Formation of complexes between phenolic compounds and metal ions has been reported (Miller *et al*., 1996; Makris & Rossiter, 2000), and condensed tannins in particular form strong complexes with ferric ions (Porter, 1992, Slabbert, 1992) that are highly coloured (Slabbert 1992). One possible position for metal chelation by flavonoids is the *ortho*-dihydroxyl group in the B ring (Rice-Evans, *et al*., 1997; Sarma *et al*., 1997; Luzia *et al*., 1998; Sugihara *et al*., 1999; Khokhar & Apenten, 2004). The CPE used in the study contained condensed tannins (section 2.2.4.1), which could be one of the many phenolic compounds present in the extract. One possible position for metal chelation by a condensed tannin molecule could therefore be the 3',4'-*ortho*-dihydroxyl group on the B ring of the molecule. Figure 3.3 shows the suggested possible position for ferric ion chelation by condensed tannin (Sugihara *et al*., 1999).

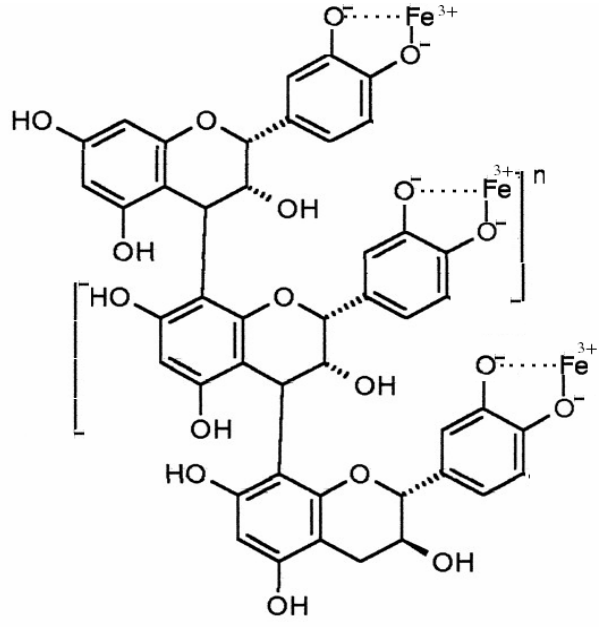

Figure 3.3. Possible position for ferric ion chelation by a condensed tannin molecule.

On the other hand, there was no colour development in samples containing TBHQ and ferric ions because TBHQ does not complex with iron hence it does not discolour the treated oil samples (Shahidi & Wanasundara, 1992). The inability of TBHQ to chelate metal ions may be due to lack of suitable sites for metal chelation such as the *ortho*-dihydroxyl group on the aromatic structure (section 1.2.5.6, Figure 1.2.8).

Effectiveness of the sorghum CPE could ultimately be assessed by the ability of the extract to prevent the development of rancid odours in the sunflower oil. Oxidative rancidity of oils occurs when a certain level of PV (Warner *et al*., 1989; Van der Merwe, 2003) or AV (Rossell, 1986) is reached or exceeded. Rossell (1986) indicates that an AV of less than 10 would be an indication of good oil. It however appears that temperature of storage affects the perception of rancidity or off-flavour in relation to PV; at lower storage temperatures oils are perceived to be rancid at higher levels of PV, while at higher storage temperature rancidity is detected at lower PVs. Semwal & Arya (1992), as referenced by Van der Merwe (2003), did not detect any off-flavours in sunflower oil stored for two years at room temperature even when the PV increased from 8.5 – 22.6 meq/kg oil, and Yousuf Ali Khan, Lakshminarayana, Azeemoddin, Atchyuta Ramayya & Thirumala (1979) only noted development of detectable off-odours in sunflower oil stored at ambient temperature when PV reached 25 meq/kg oil. On the other hand, Warner *et al*. (1989) showed that sunflower oil stored at 60ºC for 8 days developed rancidity when the PV was 13.6 meq/kg. Oxidative stability test

in this study was done at a temperature of 65ºC, and the results could be fairly compared to that of Warner *et al*. (1989). In this study, PVs for samples containing TBHQ reached the highest level of 10.84 meq/kg oil on day 14 of storage, compared to 34.91, 42.04 and 38.68 meq/kg oil for the samples containing the CPE at 1000, 1500 and 2000 ppm, respectively, and 60.20 meq/kg for the control oil without CPE or TBHQ (section 2.3). Assuming a PV of 13.6 at which the sunflower oil used in this study could develop rancid odours (Warner *et al*., 1989), it may therefore be suggested that by the end of the storage period, the control oil and all the oil samples containing the CPE may have developed rancidity, while oil containing TBHQ had not. It could therefore be estimated that the control oil and oil samples containing the CPE may have developed rancidity earlier since the samples exceeded the PV of 13.6 on day 6 (control oil), day 10 (CPE at 1000 ppm) and day 8 (CPE at 1500 and 2000 ppm). Furthermore, the control oil reached the PV of 13.6 earlier compared to oil samples containing CPE, showing the ability of the CPE to retard the development of rancidity in sunflower oil. Since rate of oxidation increases with increasing temperature (Nawar, 1996), it would be expected that the oil stored at 25ºC would reach a PV of 13.6 after a longer storage time.

Using AVs as indicators of rancidity development in the sunflower oil samples however shows a contrasting picture to that shown by PVs for the samples containing the CPE. Using an AV of less than 10 as an indication of good quality oil (Rossell, 1986), the control oil had exceeded this limit by day 10 of storage (AV of 11.7), compared to 5.20 for TBHQ, and 5.34, 5.69 and 4.53 for the samples containing extracts at 1000, 1500 and 2000 ppm, respectively, at the end of the storage period (day 14). It may therefore be suggested that by day 10 of storage, the control oil alone may have developed rancidity. The AV for oil samples containing the CPE and TBHQ however did not reach the level that could lead to the development of rancidity, even up to the last day of storage (day 14), which confirms the suggested effectiveness of the CPE to inhibit the generation of secondary oxidation products.

In the presence of ferric ions however, oil samples containing the ferric ions and those containing TBHQ and ferric ions had already reached AVs greater than 10 by day 2 of storage, with very high AVs of 25-28 by the end of the storage period (section 2.3.4.4). On the other hand the AVs of the samples containing CPE and ferric ions did not exceed 8.5, showing the extent of prevention of the formation of secondary

oxidation products by the CPE in the presence of ferric ions. Using the AVs as the guide for oil quality, oil samples containing ferric ions or TBHQ and ferric ions could be assumed to have developed rancidity by day 2 of storage, while samples containing the CPE and ferric ions were still within the range of good quality oil, which was comparable to the oil samples containing TBHQ alone.

The study also demonstrated that the rate of production of hydroperoxides as well as the decomposition of hydroperoxides to secondary oxidation products was proportional to the concentration of the ferric ions in the oil samples as shown by higher PVs and AVs in oil samples containing 4.4 ppm ferric ions than the oil containing 2.2 ppm ferric ions. Since metal ions promote the decomposition of lipid peroxides, which may both initiate free radical propagation and the formation of volatile breakdown products (Satue-Gracia *et al*., 2000; Fomuso *et al*., 2002), it may therefore be expected that a higher ferric ion concentration would result in a higher generation of both primary and secondary oxidation products.

The CPE from tannin sorghum imparted colour to the oil, probably due to the presence of anthocyanin, tannin and other flavonoids in the extracts (Nip & Burns, 1969; Blessin *et al*., 1963; Hahn & Rooney, 1986), and also due to metal chelation by the CPE. One criterion for the acceptance of a substance as a food additive is that it should not impart an uncharacteristic colour to the food product (Dugan, 1976; Schuler, 1990; Milic *et al*., 1998). The colour development in the oil may therefore be a negative attribute of the tannin sorghum CPE as a source of natural antioxidants for use in sunflower oil if consumers negatively perceive the colour of the oil resulting from the use of the CPE. Removal of the colour pigments from the extract may therefore be considered if this could be balanced against the potential of reducing the antioxidant potency of the extracts. The colour development due to the metal chelation by the CPE on the other hand may be alleviated by the combined use of the CPE with other metal chelators such as citric acid. Propyl gallate (PG) causes colour development in oil when it chelates iron ions, and the use of citric acid in combination with PG prevents this (Shahidi & Wanasundara, 1992; Eskin & Przybylski, 2001). One possible way in which the combined use of citric acid and the CPE would alleviate the colour development due to chelation could be that the combined use of the citric acid with the CPE would result in the use of a reduced amount of the CPE,

which may also result in less intensity in colour development, since the colour development was found to increase with the concentration of the CPE (section 2.3.4.5). Lipid oxidation is a multi-step process (Huang *et al*., 1994). Different antioxidants might have differing abilities in interfering with various steps of the oxidation process. The results of this study suggest that the tannin sorghum CPE was more effective in inhibiting the formation of secondary oxidation products than the primary oxidation products, which was achieved in two ways. In the first place, the CPE was able to react with the radicals (probably alkoxyl radicals) produced from the decomposition of hydroperoxides, which prevented further decomposition of the radicals to secondary oxidation products. Secondly, the extracts were able to chelate ferric ions, which prevented the metal ions from influencing the decomposition of hydroperoxides to secondary oxidation products.

Ferric ions promote the breakdown of hydroperoxides to secondary oxidation products as shown here and in other previous studies (Mancuso *et al*., 1999; Van der Merwe, 2003). Though peroxide values are often used as indicators of initial stages of oxidation (O'Brien, 2004), they may not necessarily indicate the extent of lipid oxidation when ferric ions are present in the substrate, since decomposition of hydroperoxides could be accelerated by the ferric ions, therefore preventing their accumulation in the oil samples. Low peroxide values arising out of such lack of accumulation of hydroperoxides may then give a wrong impression of the state of oil since a lower peroxide value may not necessarily be an indication of good quality oil.

CHAPTER 4

4. CONCLUSIONS AND RECOMMENDATIONS

Phatafuli sorghum bran and grain contains more total phenols and higher antioxidant activity than *Shabalala* sorghum bran and grain due to the presence of condensed tannins in *Phatafuli* that have more phenolic hydroxyl groups, and presumably more sites for scavenging and stabilizing radicals. Bran samples for both sorghums show higher levels of phenolic contents than grain samples, which is in agreement with previous findings that phenolic compounds are highly concentrated in outer layers of sorghum grain. Antioxidant activities of both *Phatafuli* and *Shabalala* sorghums show significant correlations with their phenolic contents, suggesting that phenolic compounds are largely responsible for antioxidant activity of sorghums.

Storage time is a major factor influencing the stability of tannin sorghum bran CPE in relation to its content of total phenols, condensed tannin and antioxidant activity. This suggests that tannin sorghum bran CPE would need to be used shortly after extraction, since storing it for longer times would result in significant reduction in antioxidant activity. Temperature also appears to affect the content of total phenols, condensed tannin and antioxidant activity of the CPE during some days of storage. Bubbling of oxygen in a liquid CPE of the tannin sorghum bran, and storage of the CPE in the presence or absence of air however does not seem to have significant effects on the phenolic content and antioxidant activity of the CPE. The reduced potency of the crude phenolic extracts from condensed tannin sorghum due to storage time may therefore pose practical challenges in their utilization as a source of natural antioxidants. Further studies could however be undertaken by manipulating the storage conditions to investigate the possibility of obtaining a stable product. It is further suggested that future studies on the stability of freeze-dried extracts during storage should consider identifying (using high performance liquid chromatography, HPLC) individual compounds in the extracts that largely contribute to the antioxidant activity, and further investigating how these individual compounds are affected during storage as it relates to their antioxidant activity.

CPE from sorghum bran inhibits the formation of both primary and secondary oxidation products in sunflower oil. The CPE however exhibits a low ability to

suppress formation of primary oxidation products compared to TBHQ both in the absence and presence of ferric ions. In the absence of ferric ions, the CPE and TBHQ show similar ability to inhibit formation of secondary oxidation products, but the CPE is more effective in the presence of ferric ions, probably due to the ability of the CPE to chelate ferric ions, which prevents the ferric ions from influencing the decomposition of hydroperoxides to secondary oxidation products. Since secondary oxidation products are responsible for the rancid odour and taste of oxidized oils, it may be suggested that tannin sorghum CPE is similar to TBHQ in preventing the development of rancidity in sunflower oil in the absence of ferric ions, while the CPE is better than TBHQ in preventing rancidity in the presence of ferric ions. Future work may however need to consider doing a sensory evaluation study to verify the effectiveness of the CPE in controlling rancidity of the sunflower oil. The observed antioxidant activity of the CPE in sunflower oil suggests that the sorghum extract may act as both scavengers of lipid radicals due to the ability of the extracts to interfere with propagation reactions of lipid oxidation by donating hydrogen atoms to free radicals, or as metal chelators. Further study to confirm the metal chelating property of the tannin sorghum bran CPE is suggested, which could be carried out with the inclusion of a metal chelator in the treatments.

Though peroxide values are often used as indicators of initial stages of oxidation, they may not necessarily indicate the extent of lipid oxidation of sunflower oil in the presence of ferric ions, since decomposition of hydroperoxides could be accelerated by the ferric ions, therefore preventing their accumulation in the oil samples. Such low peroxide values arising out of the lack of accumulation of hydroperoxides could be misinterpreted as an indication of good quality oil.

Tannin sorghum CPE imparts colour to sunflower oil, which may be a negative characteristic for a food antioxidant depending on consumers' perceptions. Removal of pigments contributing to the colour may therefore be considered if the tannin sorghum extract could be considered for exploitation as a source of natural antioxidant. Combined use of other metal chelators with the CPE could also be considered to prevent the colour development due to metal chelation by the CPE. Further research would however need to be done using the CPE in combination with other metal chelators such as citric acid to confirm if this would alleviate the colour development due to metal chelation by the CPE.

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