PHENOLIC ACID COMPOSITION AND ANTIOXIDANT PROPERTIES OF AQUEOUS EXTRACTS FROM WHEAT AND SORGHUM FLOURS AND COOKIES

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Phenolic acid composition and antioxidant properties of aqueous extracts from wheat and sorghum flours and cookies

BY

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Submitted in partial fulfilment of the requirements for the degree MSc Food Science in the Department of Food Science

Faculty of Natural and Agricultural Sciences

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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.

Carien J. Schoeman

November 2013

DEDICATION

This dissertation is dedicated:

To my mother, Dr Ulrike Schoeman, for her constant support, encouragement and love. Thank you for being with me through thick and thin during this journey of learning.

To my grandfather, Dr Günter Garlipp, who always inspired my hunger for knowledge with his wisdom of “WAS DU GELERNT HAST, KAN KEINER DIR WEGNEHMEN”

To my brother Jurgens Schoeman, for his intellectual support throughout the process.

Finally to my heavenly Father who gave me the gift of thought, wisdom and strength to persevere, thank you for these blessings my Lord. To you Lord all the praise and thanks.

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ABSTRACT

Phenolic acid composition and antioxidant properties of aqueous extracts from wheat and sorghum flours and cookies

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Department: Food Science

Degree: MSc Food Science

Cereal grains are important food staples and sources of antioxidant phenolics. Dietary phenolics have the potential to reduce oxidative stress and help combat associated conditions like cardiovascular disease. The availability of phenolics from sorghum and wheat products (e.g. cookies) for absorption depends on their fate during processing and during digestion in the gastro-intestinal tract.

The effect of simulated GIT pH conditions on total phenolic content (TPC), phenolic acids and antioxidant properties of whole wheat, non-tannin sorghum and condensed tannin sorghum flours and their cookies was determined. The TPC (Folin-Ciocalteu method) of all the samples analysed ranged between 37.0 – 405.3 mg Catechin equivalent / 100 g sample. Extracts from cookies had higher overall TPC than from flours, possibly due to release of bound phenolics or formation of Maillard reaction products (with reducing properties) during baking. Extracts from condensed tannin sorghum samples had higher TPC than the other cereal extracts, possibly due to presence of tannins in the tannin sorghum samples.

Phenolic acids were analyzed using High Performance Liquid Chromatography-Mass Spectrometry. p-Hydroxybenzoic, caffeic, p-coumaric and ferulic acids were identified in extracts from the flour and cookie samples. Ferulic acid was the dominant phenolic acid in both wheat and sorghum samples ranging between 109.8 – 1389.4 mg / 100 g sample.

ABTS radical scavenging capacity of all the extracts analysed ranged between 0.1 – 4.7 mM Trolox equivalent/ 100 g sample. Condensed tannin sorghum sample extracts had the highest ABTS radical scavenging capacity, possibly due to tannins in the extracts. Metal chelation capacity of all the extracts analysed (ferrous ion chelation assay) ranged between 325.6 – 8424.9 µg EDTA equivalent/ g sample. Wheat sample extracts had the highest metal chelating capacity. Inhibition of copper-mediated LDL oxidation (thiobarbituric acid reactive substances assay) of all the samples analysed ranged between 1.0 – 5.4 µM Trolox equivalent / g sample, with wheat extracts having the highest inhibition. Inhibition of LDL oxidation by the extracts (especially from wheat) may be related to their metal chelation ability as shown by high and positive correlation ($r = 0.86$) between inhibition of LDL oxidation and Iron Chelating effect (%).

There were positive overall correlations between TPC and antioxidant properties (radical scavenging or metal chelation). Antioxidant properties of the extracts may be related to their phenolic contents. Phenolic acids identified in the extracts such as ferulic and caffeic acids may exert antioxidant effects by radical scavenging or metal chelation.

The presence of phenolic acids in the GIT pH extracts of the wheat and sorghum cookies shows that they are stable under GIT pH conditions. Overall, this study shows that the cookies have potential as a health promoting ready-to-eat snack to protect against diseases related to oxidative stress such as atherosclerosis.

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CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and problem statement

According to the Heart and Stroke Foundation South Africa, 195 people died of cardiovascular related diseases between 1997 and 2004, 33 people die per day because of a heart attack, and 37 people die per day because of heart failure. Despite the high death rates caused by AIDS in South Africa, projections suggest that the rate of chronic heart disease is going to increase by 15% by 2010 (Fourie, 2007). A major risk factor for atherosclerosis, which leads to cardiovascular disease, is high levels of plasma human-low-density lipoprotein (h-LDL) cholesterol (as reviewed by Jialal & Devaraj, 1996). The body regulates the cholesterol uptake of h-LDL by feedback inhibition (Brown & Goldstein, 1983). If the LDL is modified by oxidation resulting in oxidized-LDL (OX-LDL), the ability to regulate the uptake of cholesterol is lost, resulting in the accumulation of cholesterol in the OX-LDL. This uptake of cholesterol is believed to play a major role in the formation of a fatty streak in the artery resulting in atherosclerosis. It has been shown that antioxidant compounds in food sources inhibit the oxidation of h-LDL (Yu, Zhou & Parry, 2005; Liyana-Pathirana & Shahidi 2005).

Cereals such as wheat and sorghum are sources of phenolic compounds which exhibit antioxidant activity. It has been shown that extracts from wheat whole grain, flour, germ and bran, which contained phenolic compounds, were able to reduce the oxidation of h-LDL (Liyana-Pathirana & Shahidi, 2005). Sorghum, for instance, is regarded among the cereals as having relatively high levels of phenolics and antioxidant activity (Awika & Rooney; 2004). However, there seems to be a lack of information in the literature on the ability of extracts from sorghum and sorghum products to inhibit oxidation of h-LDL.

Sorghum is a very important staple food crop in semi-arid developing countries in Africa and Asia. In South Africa, sorghum is processed into various products for human consumption such as sorghum meal, malt and breakfast cereals. Sorghum meal is used to produce

flatbreads and fermented or unfermented porridge. Whole sorghum is used in boiled products, which are comparable to products prepared from maize grits and rice (FAO, 1995). Sorghum is also used to produce traditional alcoholic beverages. Recent advances in technologies have resulted in the development and commercialization of sorghum lager beer in East and Central Africa.

Wheat is mainly milled into flour and used for the production of bread, pasta, cakes, cookies and breakfast cereals. The use of refined flour, which is flour where the bran has been removed and only the endosperm is milled, reduces the levels of antioxidant compounds such as phenolics in the food products since these phenolics are mostly localized in the outer bran layers of the grain (Chiremba, Taylor & Duodu, 2009). To produce foods with high levels of phenolics for optimum health benefits, such as prevention of h-LDL oxidation, it may be desirable to use whole grain flour.

Whole grain cookies prepared from wheat or sorghum could be a good choice as a source of dietary antioxidant phenolics due to its dual target market. Firstly it could serve as a healthy snack food for the ever increasing health-conscious individuals looking for a healthy, high antioxidant snack. Secondly, cookies are nutrient dense. Therefore they will deliver much needed macro and micro nutrients, and in addition, dietary antioxidant phenolic compounds with potential health benefits (Slavin, 2003).

In cereals, phenolic compounds such as phenolic acids are found mainly bound to protein and cell wall components such as hemicelluloses and lignin (Robbins, 2003). This is due to the ability of phenolics to readily form ester, ether or acetyl links to structural components of the cell. These interactions with other compounds in the food matrix determine the extent and rate at which the phenolics are absorbed into the gut. Bound phenolics need to be hydrolyzed by intestinal enzymes before they can be absorbed (Scalbert & Williamson, 2000). During fermentation in the gut, bound phenolics can be released from the food matrix (Scalbert & Williamson, 2000; Manach, Scalbert, Morand, Rémésy & Jiménez, 2004). The use of acidic or alkaline conditions can also lead to hydrolysis of ester bonds and the release of phenolic compounds (Robbins, 2003). Liyana-Pathirana and Shahidi (2005) showed that by using simulated gastric pH conditions, phenolic acids such as vanillic acid and ferulic acid from wheat grain were released into aqueous extracts. It

is therefore likely that the pH conditions that exist in the gastro-intestinal tract could bring about the release of dietary phenolic compounds for absorption.

1.2 LITERATURE REVIEW

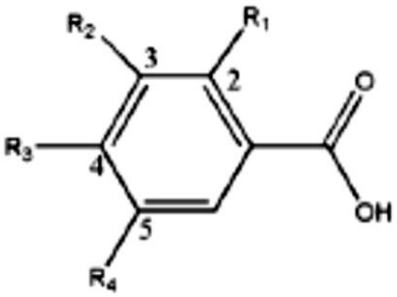
This literature review will discuss the types and levels of phenolic compounds present in wheat and sorghum grains and their cookies and the effect of different extraction solvents on phenolic compounds analysed. As phenolic compounds are closely associated with antioxidant activity, the mechanism of the activity will also be discussed for each type of phenolic group. The principles behind the analytical methods used in the study will also be discussed.

1.2.1 Chemistry of plant phenolic compounds

Phenolic compounds are universally present in the plant kingdom- from brightly coloured flowers to small millet grains. Phenolics are not homogenously distributed throughout a plant (Naczk & Shahidi, 2006). In cereals like sorghum and wheat the majority of the phenolics are present in the pericarp, testa and aleurone layers of the grain (Liyana-Pathirana, Dexter & Shahidi, 2006; Hahn, Rooney & Earp, 1984). A phenolic compound is defined as a substance that possesses a benzene ring bearing one or more hydroxyl substitutes, including their functional derivatives (Waterman & Mole, 1994). Phenolic compounds can be divided into three groups based on their size and structure: phenolic acids, flavonoids and tannins.

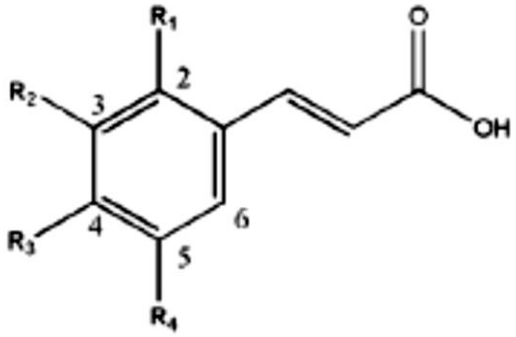
Phenolic acids are defined as the hydroxylated derivatives of benzoic acid and cinnamic acid (Mattila, Pihlava & Hellstorm, 2005). The derivatives are due to the substitution of hydroxyl (-OH) and methoxy (-OCH_3) groups onto the phenol ring at various positions (Caballero, Trugo & Finglas, 2003). Benzoic acid derivatives include gallic, p-hydroxybenzoic, vanillic, syringic and protocatechuic acids. Cinnamic acid derivatives include coumaric, caffeic, ferulic and sinapic acids. The benzoic acid derivatives are more prevalent than the cinnamic acid derivatives in edible plants (Manach et al., Jiménez, 2004; Mattila, Pihlava & Hellstorm, 2005). Figure 1.1 gives examples of both benzoic acid and cinnamic acid derivatives.

Benzoic acid derivatives



Name of benzoic acid derivatives	Functional group			
	R1	**R2**	**R3**	**R4**
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
p-hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Syringic acid	H	OCH_3	OH	OCH_3

Cinnamic acid derivatives



Name of cinnamic acid derivatives	Functional group			
	R1	**R2**	**R3**	**R4**
Caffeic acid	H	OH	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	H	OCH_3
p-Coumaric acid	H	H	OH	H

Figure 1.1: Chemical structures of benzoic acid and cinnamic acid derivatives (from Awika & Rooney, 2004).

Flavonoids are benzopyran derivatives, which consist of a C6-C3-C6 structural skeleton. In Figure 1.2 the generic structure of flavonoids is shown. Rings A and C form the benzopyran nucleus to which an aromatic substitute (ring B) is attached at carbon 2 of the C-ring (Cook & Samman, 1996; as reviewed by Di Carlo, Mascolo, Izzo & Capasso, 1999).

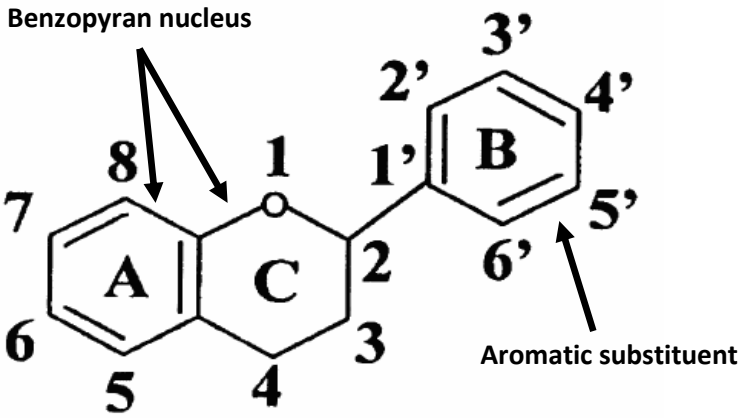


Figure 1.2: The generic structure of flavonoids (from Cook & Samman, 1996).

Flavonoids are classified into six subclasses according to their chemical structure, namely flavonols, flavones, flavanones, flavanols (catechins), anthocyanidins and isoflavones (Cook & Samman, 1996; as reviewed by Hollman & Arts, 2000). Substitutions give rise to multiple derivatives of the subclasses. Substitution can include hydrogenation, methylation and malonylation (Cook & Samman, 1996). Figure 1.3 and Table 1.1 show the chemical structures and characteristic structural features of flavonoid subclasses.

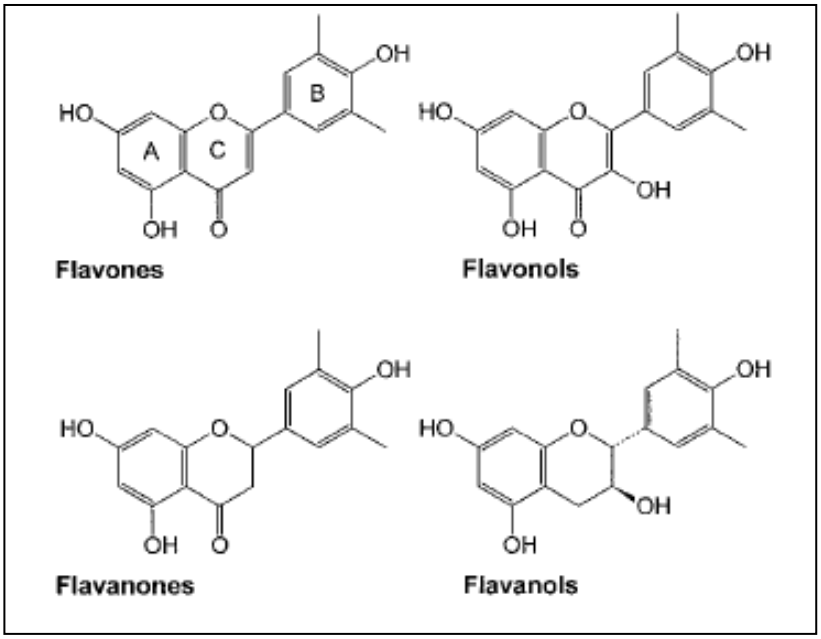


Figure 1.3: Chemical structures of selected subclasses of flavonoids (adapted from Hollman & Arts, 2000)

Table 1.1: Characteristic structural features of flavonoid subclasses (Sugihara, Arakawa, Ohnishi & Furuno, 1999)

Flavonoid subclass	Carbonyl (C-4)	Double bond (C-2 and C-3)	Hydroxyl (C-3)	Example
Flavones	✓	✓	×	Apigenin
Flavonols	✓	✓	✓	Quercetin
Flavanones	✓	×	×	Taxifolin
Flavanols	×	×	✓	Catechin

Flavonoids can be present in the aglycone form (unbound flavonoid) or in the glycoside form, in which the flavonoid is esterified to a sugar molecule such as D-glucose or L-rhamnose (Di Carlo et al., 1999; Cook & Samman, 1996).

Tannins are high molecular weight molecules that are rich in phenolic hydroxyl groups. Tannins can be divided into two classes: hydrolysable tannins and condensed (non-hydrolysable) tannins. Hydrolysis of hydrolysable tannins with acid, alkali or tannase (hydrolytic enzyme) breaks the ester bonds between the phenolic acid (gallic or ellagic acid) and sugar (glucose) molecules. According to Dykes and Rooney (2006) tannic acid/ hydrolysable tannins have not been reported in sorghum. No reports on hydrolysable tannins in wheat could be found. Condensed tannins (proanthocyanidins) are polymers of flavan-3-ols and / or flavan-3,4-diols. These units can be linked in a type A or a type B formation. Type A is characterized by two interflavan linkages between flavan-3-ol units; the first between C4→C8 and the second between C2→C7 (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz & Prior, 2003). Type B interflavan linkages can be between flavan-3-ol and / or flavan-3,4-diol flavonoids at position C4→C8 (Dykes & Rooney, 2006). Tannins found in sorghum are condensed tannins mainly of the B-type with catechin as the terminal units, and (-)-epicatechin as the extension units. This is shown in Figure 1.4 (Gu et al., 2003). Condensed tannin content in sorghum depends on the genotype. Both condensed tannin and condensed tannin-free sorghum types are available.

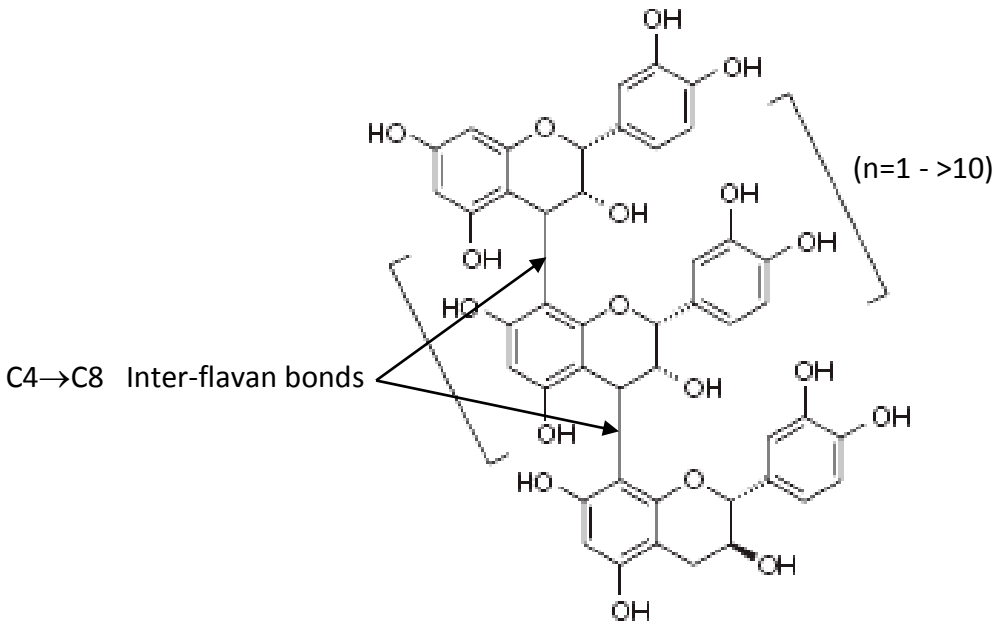


Figure 1.4: Structure of condensed tannin, a polyflavan-3-ol with a B-type linkage found in sorghum (Dykes & Rooney, 2006)

1.2.2 Phenols as antioxidants: Mechanisms and structure-activity relationships

The antioxidant potential of phenolic compounds has been attributed to mainly two mechanisms: (1) scavenging of free radicals, accompanied by stabilization via resonance, and (2) metal chelation (Khokhar & Apenten, 2003). In principle, the antioxidant activity of a phenolic compound is related to its structure (Khokhar & Apenten, 2003; Rice-Evans, Miller and Paganga 1997). For both flavonoids and phenolic acids the free or aglycone form has a higher antioxidant activity compared to the conjugated or glycoside form.

According to Rice-Evans et al., (1997) the activity of an antioxidant is predicted by:

- The antioxidant's reduction potential or how readily it donates electrons
- The ability of the antioxidant to stabilize the resonance structure resulting from donating the electron
- How readily the antioxidant reacts with other antioxidants
- The potential of the antioxidant to inhibit the formation of free radicals by metal catalysts

The structure-activity relationships of these two classes of phenolics (phenolic acids and flavonoids) will be discussed in the next two sections.

1.2.2.1 Antioxidant activity of phenolic acids

Natella, Nardini, Di Felice and Scaccini (1999) confirmed that the antioxidant activity of monophenols is increased with an increase in the number of hydroxy groups (-OH) on the molecule. The presence of one or two methoxy substitutions, especially in the ortho position to the hydroxy group, also increases the antioxidant activity. It has been shown that cinnamic acid derivatives have higher antioxidant potential than their benzoic acid derivative counterparts. This is due to the presence of the propenoid side chain in the cinnamic acid derivatives (Natella et al., 1999). It is hypothesized that the conjugated double

bond in the side chain has a stabilizing effect on the resonance structure and this enhances the antioxidant activity of the aromatic ring (Dueñas, Hernández & Estrella, 2006; Rice-Evans et al., 1997)

1.2.2.2 Antioxidant activity of flavonoids

The antioxidant activity of flavonoids is increased with the presence of 2,3-double bonds present in the C ring. This is due to electron delocalization across the molecule for stabilization of the radical formed during electron donation to the free radical. This is shown in the red area on the quercetin structure in Figure 1.5. The presence of the 4-oxo function on the C-ring (green area) is also important for antioxidant activity. The yellow area in Figure 1.5 shows the *ortho* 3'4'-dihydroxy moiety in the B-ring which increases the antioxidant activity (Rice-Evans et al., 1997). An increase in hydroxyl groups does not increase the antioxidant activity of the flavonoids compared to phenolic acids (Rice-Evans et al., 1997). The difference in potential to scavenge free radicals in flavonoids is more related to the unsaturation of the C-ring than the presence of hydroxyl groups. However, the ability to chelate metal ions like copper and iron may be increased by an increase in hydroxyl groups (blue area) (Williams, Spencer & Rice-Evans, 2004).

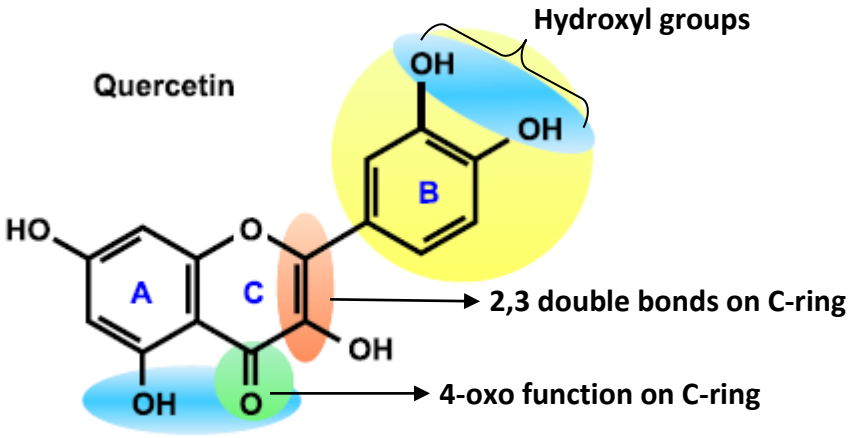


Figure 1.5: Structure of quercetin highlighting important structural features for antioxidant activity (Williams et al., 2004)

1.2.3 Levels of phenolic compounds in wheat and sorghums

In the case of sorghum it has been shown that the condensed tannin sorghum type has higher total phenolic content than the non-tannin sorghum type; see Table 1.2 (Dlamini, Taylor & Rooney, 2007). It has also been shown that decortication (removal of the outer layers of the grain) reduced the total phenolic content by between 18.5 and 62% in non-tannin sorghum and between 65 and 79% in condensed tannin sorghum (Dlamini et al., 2007). The same trend has been shown for wheat bran compared to refined flour (Siwela, Taylor, Duodu, 2010).

Table 1.2: Total phenolic content and antioxidant activity of whole grain wheat and sorghum, and solvents used for their extraction

Sample	Extraction solvent	Total phenolics[a]	Standard	Antioxidant activity (ABTS)[b]	Reference
Hard red wheat mixture	Methanol-acetone-water (7:7:6, v/v/v)	3.456	Ferulic acid	11205	Liyana-Pathirana & Shahidi (2006)
Soft red/white wheat mixture		3.818		12217	
Hard red wheat (commercial)	80% Methanol	3.834	Gallic acid	-	Kim, Tsao, Yang & Cui (2005)
Hard red wheat		3.967		-	
Soft red winter wheat	50% Acetone	0.4 – 0.8	Gallic acid	14.3 – 17.6	Moore, Hao, Hou, Luther, Costa & Yu (2005)
Red wheat mixture	Gastric pH conditions	0.252 – 0.294	Ferulic acid	1.5 – 2.0	Liyana-Pathirana & Shahidi, (2005)
Red wheat mixture (whole grain)	Aqueous extract	0.064 – 0.081		0.5 – 0.6	
Macia (White, Non-tannin) Sorghum	1% HCl in Methanol	2.7	Catechin	22	Dlamini et al., (2007)
NK 283 (Red, Non-tannin) Sorghum		5.3		52	
Red Swazi (Red, Tannin) Sorghum		19.7		359	
NS 5511(Red, Tannin) Sorghum		22.4		384	
Framida (Red, Tannin) Sorghum		24.5		427	
Red grain (non-tannin)	70% Aqueous acetone	5	Gallic acid	53	Awika, Rooney, Wu, Prior & Cisneros-Zevallos (2003)

Notes: [a] Values expressed as mg/g dry weight basis, Folin-Ciocalteu method, [b] Values expressed µMol TE/g dry weight basis, ABTS radical scavenging capacity, - indicates that antioxidant activity was not determined in study.

In a study done by Sosulski, Krygier and Hogge (1982) it was found that in freshly milled whole wheat flour the total bound phenolic acids contributed 84% of the total phenolic compounds. In the same study it was found that ferulic acid represented 89% of the total phenolics present in the wheat flour. Adom and Liu (2002) found that ferulic acid is 98.8% concentrated in the insoluble bound fraction of the wheat analysed. Zhou et al., (2004) analysed the phenolic acid composition of Swiss Red wheat. The following phenolic acids were identified: p-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, and ferulic acid. These phenolic acids were present in the following concentrations in the whole grain: 5.0 µg/g wheat, 4.9 µg/g wheat, 13.7 µg/g wheat, 1.9 µg/g wheat, and 33.7 µg/g wheat, respectively.

In sorghum, as in wheat the majority of the phenolic acids is in the bound form and located in the bran (Hahn, Faubion & Rooney, 1983). The major phenolic acid in sorghum is ferulic acid which contributes between 24% and 47% of the bound phenolic acids. These authors also found that gallic acid is only present in the bound form and cinnamic acid only present in the free form, with the exception of the SC0719 variety where it is also found in the bound form. The following phenolic acids were also identified in four different varieties of sorghum: protocatechuic, p-hydroxybenzoic acid, vanillic acid, caffeic acid and p-coumaric acid.

Literature on the flavonoid composition of wheat is very limited. According to Adom and Liu (2002) there is a large difference between the flavonoids in the free fraction (7%) and the bound fraction (97%) in wheat. Adom, Sorrells and Liu (2003) also showed that the flavonoid content of the free and soluble-esterified fraction together acounted for between 7% to 13%, and the insoluble bound acounted for 87% to 93% of the total flavonoid content. The difference coud be that Adom and Liu (2002) included the soluble-esterified fraction into the bound fraction and not into the free fraction as Adom et al., (2003) did. Adom et al., (2003) found that the average total flavonoid content of 11 wheat varieties were 105.85 - 141.83 µmol of catechin equivalents / 100 g of grain. Feng and Mc Donald (1989) found that the average flavonoid amounts were higher in durum and white wheat varieties than in the hard red spring wheat and hard red winter wheat. According to Abdel-Aal and Hucl (2003)

whole meal from blue wheat has the highest anthocyanins content compared to whole meal from purple and red wheat with 16 mg/100 g, 9 mg/100 g and 0.5 mg/100 g, respectively.

Feng and Mc Donald (1989) found that the flavonoids in wheat are a mixture of 6-C-pentosyl-8-C-hexosylapigenin and 6-C-hoxosyl-8-Cpentosylapigenin which are derivatives of apigenin a flavone, and apigenin.

Luteolinidin and apigeninidin, which confer yellow and red colours, respectively, represent 36% to 50% of total anthocyanin content in black and tannin sorghums and 19% apigeninidin in red sorghums (Awika, Rooney and Waniska, 2004). Other flavonoids reported in red sorghums are flavan-4-ols such as luteoforol and apiforol. Flavones such as apigenin and luteolin have also been isolated and identified in sorghum and are predominant in tan plant sorghums (Awika et al., 2004).

Tannin content of sorghum is based on the presence of genes (B_1 and B_2 genes) that encode for a pigmented testa (Boren & Waniska, 1992), both of which must be present in the dominant genes (Boren & Waniska, 1992). Awika and Rooney (2004) reviewed the literature on sorghum phytochemicals and reported that the condensed tannin content of tannin sorghum is between 10.0 and 68.0 mg catechin equivalents/g (dry wt) for tannin sorghum and between 0.5 and 3.8 mg/g (dry wt) for non-tannin sorghum. In a study done by Dlamini et al., (2007) tannins were not detected in non-tannin sorghum varieties and condensed tannin sorghum varieties had between 33.6 and 49.1 mg Catechin equivalents/g sample. Unlike sorghum it has not been reported that wheat contains any condensed tannins.

1.2.4 Levels of phenolic compounds in wheat and sorghum cookies

Limited work has been done on the phenolic composition of cookies in general. However, there have been a few reports on total phenolic content and antioxidant activity of wheat, sorghum (Chiremba et al., 2009) and wheat-finger millet composite cookies (Siwela et al., Duodu, 2010). Chiremba et al., (2009) showed that the total phenolic content of flour and cookies from condensed tannin sorghum was higher than flour and cookies from non-tannin

sorghum. On a flour mass percentage basis the cookies had higher content of total phenolics than the flour and dough (Chiremba et al., 2009). Mitre-Dieste, Gordan, Awika, Suhendro and Rooney (2000) showed that the addition of sorghum bran to wheat cookies increased the phenolic content. Siwela et al., (2010) did not detect phenolics in wheat dough and cookies made from commercial cake flour which has a low extraction rate due to removal of the bran. However, baking wheat-finger millet composite doughs into cookies decreased total phenolic content by between 30.4% and 61.1% (Siwela et al., 2010). The observed decreases in phenolic content during baking may be due to binding of phenolic compounds to other components such as proteins or carbohydrates (Siddhuraju & Becker, 2007) which renders the phenolics unextractable.

1.2.5 The antioxidant activity in wheat, sorghum and their cookies

Liyana-Pathirana and Shahidi (2007) and Adom and Liu (2002) showed that there is generally a positive correlation between phenolic concentration and antioxidant activity in corn, wheat, oats and rice. The higher the phenolic content of an extract fraction or morphological fraction the higher the antioxidant activity. They found this correlation only for extracts from wheat prepared with methanol as compared to extracts prepared with only water. Zielinski and Kozlowaska (2000) hypothesized that other compounds apart from phenolics in the water extracts may contribute to the antioxidant activity. Iwama, Hattori and Ibuki (1987) reported that the wheat protein gliadin exhibits strong antioxidant activity. This indicates that the presence of wheat protein contributes to the antioxidant activity. Thus in the water extracts not only phenolic compounds were extracted. Liyana-Pathirana and Shahidi (2006) found that the total phenolics content of the insoluble bound fraction contributed 90% of the total phenolics in wheat, and the antioxidant activity contributed by this fraction was more than 80% of the total antioxidant activity of the wheat. They also showed that the insoluble-bound fraction had 8.6 and 7.8 times the antioxidant activity of the free and soluble-esterified fractions, respectively. Table 1.2 shows some values reported in the literature for ABTS radical scavenging properties of wheat, as well as the solvents used during extraction. Zhou et al., (2004) compared the chelating activity of 50% acetone

extracts from red whole grain wheat and the bran. The study showed that extracts from the bran exhibited around 60% higher chelating activity than extracts from the whole grain.

Antioxidant activity of sorghum depends on the variety. Whole grain condensed tannin sorghum has a higher antioxidant activity than non-tannin sorghum. According to Dlamini et al., (2007) the antioxidant activity of acidified methanol extracts from sorghum correlates well with the total phenolic content and tannin content. They found that processing, especially decortication, reduced the antioxidant activity of sorghum by between 73% and 87%. Some reported values of ABTS radical scavenging properties of sorghum are shown in Table 1.2

The majority of the work done on wheat cookies and antioxidant activity is more related to the effect of different formulations and baking conditions on the antioxidant activity and acrylamide content. Morales, Martin, Açar, Arribas-Lorenzo and Gökmen (2008) showed that both cookie formulation and baking conditions have an effect on the antioxidant activity of wheat flour cookies. The effect of the cookie formulation, with respect to type of sugar used, showed that cookies produced with glucose had higher antioxidant activity than cookies made with sucrose. This could be due to glucose being a reducing sugar, whereas sucrose is a non-reducing sugar, resulting in glucose being more reactive in the Maillard browning and caramelisation reactions resulting in products which have been shown to have antioxidant activity (Goya, Delgado-Andrade, Rufián-Henares, Bravo & Morales, 2007; Benjakul, Visessanguan, Phongkanpai & Tanaka, 2005). Morales et al., (2008) showed that the effect of the leavening agent on the antioxidant activity depended on the sugar type used. When ammonium bicarbonate and sucrose were used, the formation of water soluble products, which exhibit higher antioxidant activity, was increased, in contrast to a decrease when glucose was used. Examining the effect of baking conditions showed that increasing either baking time or temperature results in an increase in antioxidant activity (Morales et al., 2008). It was shown that for an increase in baking time and temperature there was a significant increase in the formation of ferric reducing products and products with electron transferring capabilities. Summa, Wenzl, Brohee, De La Calle and Anklam (2006) found that the type and amount of sugar used in the formulation and baking time had a significant effect on the antioxidant activity of wheat cookies. When sucrose was used in the formulation, cookies had up to an 85% increase in antioxidant activity when baking time

increased from 5 min to 20 min. When fructose was used the increase in antioxidant activity could be as high as 98%.

Chiremba et al., (2009) showed that using decorticated sorghum in cookie formulation decreased the antioxidant activity compared to using whole grain sorghum by between 43% and 66%. Cookies from whole grain condensed tannin sorghum flour had higher antioxidant activity than cookies from non-tannin sorghum flour and wheat flour. They also found a decrease in the antioxidant activity from flour to cookies for both condensed tannin sorghum and non-tannin sorghum. The decrease was the greatest in the condensed tannin sorghum with 39% as with the non-tannin sorghum between 17% and 23%.

1.2.6 Effect of extraction solvent on the level of phenols extracted from plant materials

Various solvents and solvent combinations have been used along with various procedures for the extraction of phenolic compounds from cereal grains and products. The utilization of different extraction solvents and procedures has resulted in a large volume of measured phenolic content of cereal grains and products. The main problem with this is that it makes direct comparisons between individual findings difficult and must be done with extreme caution.

The majority of work on phenolic content and antioxidant activity has used organic solvents for extraction. Different organic solvents give differences in the measured phenolic content and antioxidant activity. Zhou, et al., (2004) reported that 50% acetone extracts of wheat bran had 60% and 79% higher total phenolic content and antioxidant activity respectively than extracts prepared with absolute ethanol.

Zhao, Dong, Lu, Chen, Li, Shan, Lin, Fan and Gu (2006) found that 80% acetone was the most effective extraction solvent compared to 80% ethanol, 80% methanol and water in terms of total phenolics extracted and antioxidant activity of barley extracted. It was found in the same study that extraction with water resulted in the highest metal chelating activity, followed by 80% methanol, 80% acetone and 80% ethanol having the lowest.

The use of organic solvents to extract phenolics from food samples may show the phenolic content and antioxidant activity potential of the food, but this raises questions about the relevance of these results in terms of the human digestive system where enzymes operating in an aqueous environment are at play. It has been shown that extraction with organic solvents renders a higher total phenolic content and antioxidant activity than extraction with water. Zielinski and Kozlowska (2000) studied the antioxidant activity of different morphological fractions of wheat, oats, rye, barley and buckwheat in 80% methanol and water extracts. They reported a positive relationship between the total phenolic content and antioxidant activity for the 80% methanol extracts. This was not the case for the water extracts as no linear relation could be established. The differences in the amount of phenolic and phenolic compounds extracted when using organic solvents compared to using water is based on the solubility of the phenolic compounds and the extraction solvent used.

The only water extract that showed antioxidant activity was that from whole grain buckwheat (Zielinski & Kozlowska, 2000). The extracts from wheat, rye, oats and barley exhibited a prooxidant effect. This is in contrast with the findings of Liyana-Pathirana et al., (2005) who found that extracts prepared with water under simulated gastric pH conditions had about 74% higher total phenolic content and 60% higher antioxidant activity than extracts prepared with only water without simulating gastric pH conditions.

1.2.7 Oxidation of human Low-Density Lipoprotein (h-LDL) and the influence of antioxidants

1.2.7.1 Low-density lipoprotein (LDL)

According to Esterbauer, Geebicki, Puhl & Jurgens (1992) human LDL is defined as the accumulative lipoproteins with a density range of 1.019-1.063 kg/L. The LDL particle consists of two areas, namely the core and the outer layer, and these two are separated by a monolayer of phospholipid molecules and free cholesterol molecules. Half of the fatty acid content of the LDL particle is polyunsaturated fatty acids (PUFAs) with linoleic acid being the most abundant, but variation does occur between LDL particles and this is the major contributing factor for the differences in oxidation behaviour between particles (reviewed by Jialal & Devaraj, 1996). The core is composed of cholesteryl ester molecules and triglycerides. The main component of the outer layer is a very large protein, apolipoprotein (apo) B-100, which is embedded throughout the outer layer. Figure 1.6 shows a low-density lipoprotein particle.

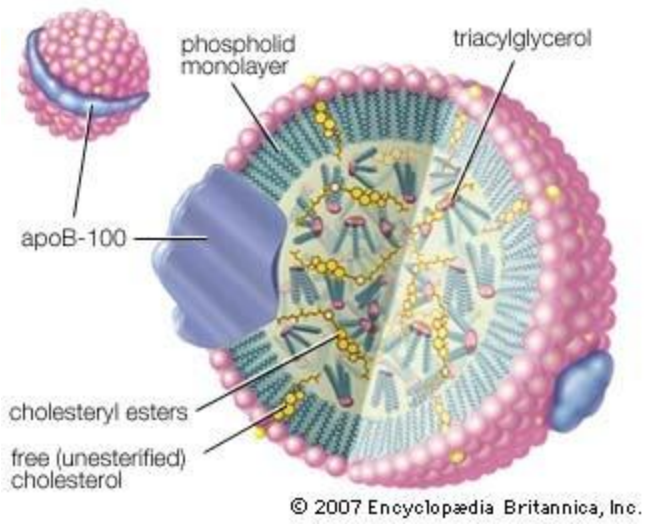


Figure 1.6: Illustration of a low-density lipoprotein particle
(Encyclopædia Britannica Online, 2007)

1.2.7.2 Oxidation of LDL

Oxidation of LDL is in actual fact the oxidation of the individual PUFAs in the LDL particle by means of a radical-mediated process. The oxidation of PUFAs can be divided into three phases (1) initiation phase, (2) propagation phase, (3) degradation or termination phase (reviewed by Jialal & Devaraj, 1996; Logani & Davies, 1980).

The initiation phase is set off by the presence of reactive oxygen species in the initiation reaction. During the initiation reaction the reactive oxygen species react with a double bond in the PUFA by H• abstraction, resulting in a fatty acid free radical. The following equation shows the initiation reaction (• denotes a radical molecule):

X• + PUFA-H → PUFA• + XH

The initiation reaction is followed by molecular rearrangement. The rearrangement of the PUFA results in the formation of conjugated dienes (as reviewed by Jialal & Devaraj, 1996). The presence of antioxidants can cause an oxidation lag phase, by scavenging the free radicals.

During the propagation phase, the PUFA• react with oxygen to generate a reactive peroxy radical PUFA-OO• (Logani & Davies, 1980). The peroxy radicals can react with other PUFAs forming hydroperoxides (PUFA-OOH), and a new free radical (PUFA•). The formation of a new PUFA• causes a chain reaction. The PUFA-OOH can also break down into radicals and have much the same effect as the newly formed PUFA• (Logani & Davies, 1980). The following equation shows the reactions occurring during the propagation phase (• denotes a radical molecule):

PUFA• + O2 → PUFA-OO•

PUFA-OO• + PUFA-H → PUFA-OOH + PUFA•

2PUFA-OOH → PUFA-O• + PUFA-OO• +H2O

The termination phase follows when the concentration of free radicals is such that they start to interact with each other and form stable end products (Logani & Davies, 1980).

Cleavage of double bonds also occurs during the termination phase resulting in formation of aldehydes (as reviewed by Jialal & Devaraj, 1996). The main aldehydes formed during LDL oxidation include malondialdehyde, 4-hydrononenal and hexanal, which can form cross-links with the apo B-100 protein.

1.2.7.3 Oxidative modification and biological effect of oxidized LDL

Apart from the PUFAs, the cholesterol and protein present in the LDL particle are also oxidized. The cholesterol is oxidized and forms oxysterols such as 7-ketocholesterol (as reviewed by Jialal & Devaraj, 1996). The apo B-100 protein undergoes oxidative scission resulting in fragmentation and loss of function (as reviewed by Jialal & Devaraj, 1996; Diaz, Frei, Vita & Keaney, 1997).

There are different extents of oxidation of the LDL particles. Initially, only minor oxidation results in minimally modified LDL (MM-LDL) (Diaz et al., 1997). The MM-LDL can then be modified by macrophages into a more oxidized form, OX-LDL. The OX-LDL particle loses the ability to be recognised by the LDL receptors. Oxidation also results in the LDL losing the ability to regulate the uptake of cholesterol, resulting in the accumulation of cholesterol in the OX-LDL (as reviewed by Jialal & Devaraj, 1996). The OX-LDL is taken up by the scavenger receptors on monocyte-macrophages in the arteries which results in the formation of foam cells (Diaz et al., 1997). The increase in cholesterol of the OX-LDL will decrease the motility of the macrophage thus increasing the retention of macrophages in the arterial wall resulting in a fatty streak in the artery (as reviewed by Jialal & Devaraj, 1996; Diaz et al., 1997). The uptake of cholesterol and thus the formation of fatty streaks in the arteries are believed to play a major role in atherosclerosis.

1.2.7.4 Effect of food antioxidants on the oxidation of LDL

Antioxidants have been shown to decrease the rate of the initiation reaction during LDL oxidation by introducing a lag phase (as reviewed by Jialal & Devaraj, 1996). Figure 1.7 shows the effect of different concentrations of elderberry extract antioxidants on copper-mediated LDL oxidation (Abuja, Murkovic & Pfannhauser, 1998). It can be seen that an increase in the concentration of the extract results in an increase of the lag time in the oxidation of LDL. It is important to note that the maximum rate of oxidation remains constant for all the extract concentrations.

Liyana-Pathirana and Shahidi (2006) showed that whole wheat extracts as well as extracts from the milling fractions, the germ, flour and bran all had inhibitory effect on LDL oxidation. The extracts were prepared to two different concentrations (0.5 mg/kg and 1.0 mg/kg). For both concentrations the inhibitory efficiency was highest in the germ, followed by the bran and whole grain with the flour having the lowest inhibitory effect. Yu et al., (2005) also found that extracts from wheat bran had an inhibitory effect on LDL oxidation.

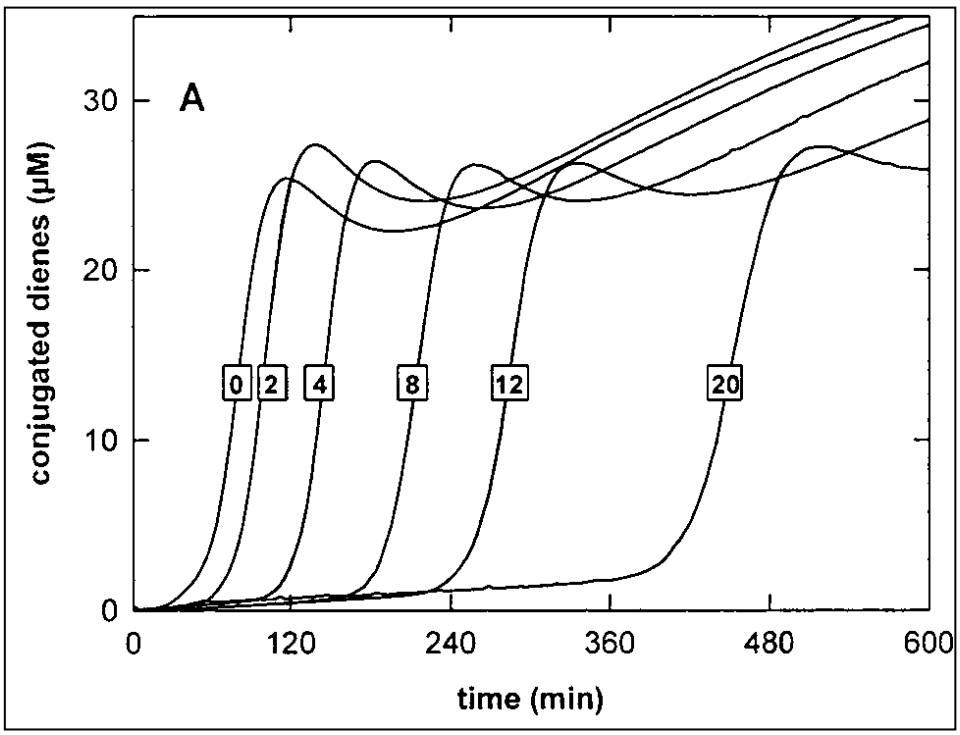


Figure 1.7: Effect of elderberry extract on copper-mediated LDL oxidation (Abuja et al., 1998)

Other food sources whose extracts have shown inhibitory effect on LDL oxidation include oats (Gray, Clarke, Baux, Bunting & Salter, 2002), yellow peas, green peas chick peas yellow and black soybeans, lentils and red kidney beans (Xu, Yuan & Chang, 2007), essential oils from cumin, cloves, thyme, basil, fennel, parsley and tarragon (Teissedre & Waterhouse, 2000) and honey (Hegazi & El-Hady, 2007).

1.2.8 Analytical methods for phenols and antioxidant activity

1.2.8.1 Folin-Ciocalteu assay for total phenolics

The Folin-Ciocalteu assay for total phenolics (Singleton & Rossi 1965) is based on measuring the total reducing phenolic groups present in the extract. The assay is based on an electron transfer redox reaction between the phenolic hydroxyl groups of the extract (reducing agent) and the Folin-Ciocalteu reagent (oxidizing agent) which determines the total reduction capacity or total phenolic hydroxyl groups of the extract. Although the assay is used widely and gives reliable estimates of the levels of reducing phenolic groups present, it is not specific for only phenolic compound such as phenolic acids, flavonoids and tannins. Compounds like sugars, aromatic amines, sulfur dioxide, ascorbic acid and some inorganic compounds can interfere with the assay and result in an over estimation of the phenolic content (Phipps, Sharaf & Butterweck 2007).

It is sometimes difficult to do direct comparison of total phenolic content between studies as different standards can and are used to express the total phenolic content. Standards that have been used include ferulic acid, catechin and gallic acid (Table 1.2). Other factors that also have an influence on the total phenolic content measured in cereal samples is the extent of refining of the flour compared to whole grain, type of grain (for instance wheat compared to condensed tannin sorghum) and extraction procedure where extraction solvent, time, acid or alkaline hydrolysis and temperature plays a role in the amount of phenolics extracted from the sample. According to Phipps et al., (2007) the assay is simple, sensitive and precise, except that the reaction is slow to occur.

1.2.8.2 High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

High performance liquid chromatography combined with mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) is an extremely powerful tool used for molecular mass determination (Lee & Kerns, 1999) and compound identification. HPLC-MS has been used for the identification of phenolic acids in various foods ranging from plums (Gil, Tomás-Barberán, Hess-Pierce & Kader, 2002) to Chinese noodles (Asenstorfer, Wang & Mares, 2005). The analysis can be done in positive and negative mode, with negative mode being the more sensitive of the two modes. The mass spectrum given after analysis of a sample in negative mode shows the parent ions $[M-H]^-$ which can be used for the identification of phenolics. Identification of the phenolic can also be aided with the use of MS/MS analysis. MS/MS analysis gives rise to daughter ions which is the parent ion minus the product ion. Some of the most common product ions observed in the analysis of phenolic acids is: $[M-15]^-$ which is caused by the loss of a methyl group (CH_3) via an alpha-cleavage; $[M-30]^-$ which is caused by the cleavage of a formaldehyde molecule (CH_2O) of the methoxyl substituent of the phenyl ring; $[M-44]^-$ involves the cleavage and rearrangement of the carboxyl group (COOH) (Robbins, 2003).

1.2.8.3 Trolox equivalent antioxidant capacity (TEAC) assay

Various methods have been developed to determine the antioxidant capacity of food extracts based on their ability to scavenge free radicals in vitro. One such method is the ABTS antiradical assay (also known as the Trolox Equivalent antioxidant activity assay, TEAC) (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). It measures the ability of the sample or extract to scavenge the 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical cation chromogen (ABTS•+) (Figure 1.8), as compared to a Trolox (water-soluble vitamin E analogue) standard. The ABTS is oxidized to its radical cation, ABTS•+, which has a deep blue colour and is used to determine the antioxidant activity of the extract. During the assay decolourisation of the deep blue solution is measured spectrophotometrically at a

wavelength of 734 nm. The antioxidant activity is calculated by comparing the absorbance of the solution after reaction with the extract to that of the Trolox standard.

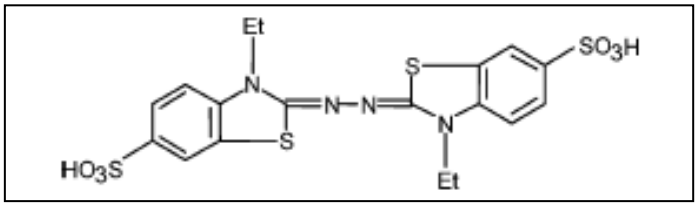


Figure 1.8: Structure of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) (Prior, Wu & Schaich, 2005)

1.2.8.4 Determination of h-LDL oxidation

During the oxidation of LDL, the polyunsaturated fatty acids (PUFAs) are converted to fatty acid hydroperoxides with conjugated double bonds (dienes), which absorb at 234 nm (Puhl, Waeg & Esterbauer, 1994). The basis of the assay is to induce oxidation of the PUFAs in the h-LDL using either Cu^{2+} ions or 2-amidinopropane hydrochloride (AAPH) and then measure the time it takes for conjugated dienes to form. Antioxidants added to the system will scavenge the free radicals formed and thus slow down the oxidation rate and increase the lag phase (Cook & Samman, 1996; Gray et al., 2002).

1.2.8.5 Ferrous Ion Chelating (FIC) Assay

The transition metal ion, Fe^{2+} possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions (reviewed by Jialal & Devaraj, 1996; Logani & Davies, 1980). The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions, which is a mechanism of antioxidative action (Prior et al., 2005). Phenolic extracts have been reported to have the ability to chelate transition metals, like iron, copper and zinc (Karamać, 2009). In this assay, ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulphonic acid) forms complexes with Fe^{2+} in the absence of a metal chelating agent which results in the

formation of stable magenta coloured complexes in solution. If a metal chelating agent is present it reacts with the transition metal instead of the ferrozine which results in no coloured complexes formation. The inhibition of colour formation allows the metal chelation effect to be quantitatively measured spectrophotometrically at 562 nm (Liyana-Pathirana and Shahidi, 2006).

1.2.9 Gaps in knowledge

Despite the enormous amount of research done on phenolic content in cereals such as wheat and sorghum, most of these are done on extracts that were prepared with organic solvents. These organic solvents are not present in the human digestive tract where enzymes operating in an aqueous environment are at play. Apart from the work done on wheat by Liyana-Pathirana and Shahidi (2005), there appears to be a lack of information regarding the effect of simulated gastrointestinal tract pH conditions on phenolic compounds and antioxidant properties of cereals (especially sorghum) and cereal products such as cookies.

The effect of plant phenolic extracts have on the inhibition of h-LDL oxidation is well documented. However, there is lack of information about how simulated gastrointestinal tract pH conditions can influence the ability of extracts from cereal products such as sorghum cookies to inhibit h-LDL oxidation and how this can be related to phenolic composition. Such information will provide some insights about the potential health benefits of a cereal product such as sorghum cookies with regard to prevention of cardiovascular disease by the ability of its antioxidant phenolics to inhibit h-LDL oxidation.

1.3 Hypotheses

1. Aqueous (water and gastrointestinal tract pH) extracts from wheat and sorghum flour will have lower total phenolic content, radical scavenging activity and inhibitory effect on LDL oxidation than extracts from their cookies. High molecular weight compounds containing the furan ring and nitrogen-containing browning compounds like melanoids (Yilmaz, Toledo, 2005) that are produced during Maillard reactions (Goya et al., 2007) and caramelisation (Benjakul et al., 2005) during the baking process, have reducing properties (Morales et al., 2008; Dittrich, El-Massry, Kunz, Rinaldi, Peich, Backmann, Pischetsrieder, 2003) and will contribute to higher total phenolic content, antioxidant activity and inhibition of LDL oxidation in the cookies.

2. Aqueous (water and gastrointestinal tract pH) extracts from condensed tannin sorghum flour and its cookies will have higher total phenolic content, radical scavenging activity and inhibitory effect on LDL oxidation than extracts from the condensed tannin free sorghum and the wheat flour and their cookies. In an acidic environment tannins are hydrolysed into their basic flavonoid dimers (Schofield, Mbugua & Pell, 2001). Therefore extracts from condensed tannin sorghum flour and its cookies will contain high levels of such flavonoid dimers and therefore higher phenolic content and antioxidant activity.

3. The gastrointestinal tract pH extracts will have a higher content of bound phenolics, such as ferulic acid, than the aqueous extract (extracts with no pH manipulation) which will result in the gastric pH extract having a higher total phenolic content, radical scavenging activity and inhibitory effect on LDL oxidation. Bound phenolics are hydrolysed from the food matrix in acid conditions (Robbins, 2003; Naczk & Shahidi, 2006).

1.4 Objectives

To determine the total phenolic content and antioxidant properties of water and gastrointestinal tract (GIT) pH extracts of wheat, sorghum and their cookies.

To determine the phenolic acid composition of water and gastrointestinal tract (GIT) pH extracts of wheat, sorghum and their cookies.

CHAPTER 2

2.1 CHARACTERIZATION OF PHENOLIC ACIDS IN AQUEOUS EXTRACTS OF WHOLE GRAIN WHEAT, SORGHUM AND THEIR COOKIES

2.1.1 Abstract

The effect of GIT pH conditions on the total extractable phenolic content and phenolic acid composition of whole wheat, non-tannin sorghum and condensed tannin sorghum flour and their cookies was determined. The total phenolic content was determined by using the Folin-Ciocalteu method and ranged between 37.0 – 405.3 mg Catechin equivalent /100 g sample. Extracts from cookies had higher total phenolic content than from flours and sorghum samples had higher total phenolics than wheat samples. Phenolic acids were identified and quantified in all the extracts by means of High Performance Liquid Chromatography-Mass Spectrometry. The phenolic acids identified in the water and GIT pH extracts were p-hydroxybenzoic, caffeic, p-coumaric and ferulic acid in whole grain wheat and sorghum flour and cookie samples. Ferulic acid was the dominant phenolic acid in wheat and sorghum samples ranging between 109.8 – 1389.4 mg / 100 g sample. The presence of phenolic acids in the GIT pH extracts from cookies suggests that they could be potentially available for absorption when the cookies are consumed and may therefore offer health benefits by virtue of their bioactive properties.

Keywords: Wheat, Sorghum, Phenolic content, Phenolic acid, Water extracts, GIT pH conditions extract, Cookies

2.1.2 Introduction

Sorghum and wheat contain phenolic compounds such as phenolic acids (Dykes & Rooney, 2006; Liyana-Pathirana & Shahidi, 2006). The cinnamic acid derivatives such as coumaric, caffeic, ferulic and sinapic acids are more commonly found in edible plants than benzoic acid derivatives (Manach et al., 2004). Ferulic acid is the main phenolic acid present in cereals (Sosulski et al., 1982; Hahn et al., 1983).

Phenolic compounds are known to be powerful antioxidants in vitro (Temple, 2000). It has been shown that phenolic compounds can prevent DNA damage (Zhang, Yang, Zhang, Wang & Zhang, 2011) and inhibit LDL oxidation (Liyana-Pathirana & Shahidi, 2005) in vitro. These properties have led to the hypothesis that phenolic compounds could help in the prevention of cancer and cardiovascular diseases (Kris-Etherton, Hecker, Bonanome, Coval, Binkoski, Hilpert, Griel & Etherton, 2002).

The phenolics in cereals like sorghum (Hahn et al., 1983) and wheat (Liyana-Pathirana & Shahidi, 2006) are mainly found in bound form. The release of these bound phenolics requires alkaline or acid hydrolysis to be made available for absorption (Robbins, 2003). Free phenolics are present in cereals to a lesser extent than bound phenolics, but have a higher chance of being absorbed into the gastrointestinal tract (GIT) (Scalbert & Williamson, 2000).

The GIT with its varying pH conditions ranging from as low as pH of 2 in the stomach to a pH of 6.0 in the small intestine has been shown to release bound phenolics in wheat flour (Liyana-Pathirana & Shahidi, 2006) and wheat-based ready-to-eat breakfast cereals (Baublis, Decker & Clydesdale, 2000).

As wheat and sorghum flours are not consumed in the raw state it is important to look at the phenolic acids in the ready–to-eat products. Furthermore, cookies are shelf-stable food products that are nutrient dense and offer convenience as ready-to-eat functional foods (Chiremba et al., 2009). They can therefore be a good vehicle for delivery of health-promoting phenolic antioxidants.

The objective of this portion of the study was to determine the phenolic acids that are extractable under simulated GIT pH conditions in whole wheat and sorghum flours as well as the ready-to-eat cookies made from these flours.

2.1.3 Materials and methods

2.1.3.1 Wheat and Sorghum samples

Whole wheat (Soft Red Winter Wheat), red type III condensed tannin (ex. Nola '97) and red non-tannin (NK 283) sorghum whole grain samples were used. The whole grain samples were milled into flour using a hammer mill to pass through a 500 µm sieve. Flour samples were stored at 4^{o}C until required.

2.1.3.2 Preparation of cookies

The cookies were made using the AACC sugar snap cookie Method 10-50D (American Association of Cereal Chemists International, 2000) as modified by Chiremba (2008). The formulation of the cookies on an as is basis comprised of 8 g sodium bicarbonate, 225 g sorghum flour or wheat flour and 50 ml water. The components were mixed for 3 min until homogenous dough was formed. The dough was then rolled out to a thickness of 6 mm and circular dough pieces were cut out using a 50 mm diameter cookie cutter. The dough pieces were then baked at 180 °C for 10 min, allowed to cool to room temperature and then stored at 4°C until analysed. The finished cookies are shown in Figure 2.1.1. Before analyses, cookie samples were ground in a commercial Waring blender and passed through a 500 µm sieve.

Figure 2.1.1: Whole wheat cookies (A), non-tannin sorghum cookies (B) and condensed tannin sorghum cookies (C).

2.1.3.3 Preparation of extracts

Water extracts and gastrointestinal tract pH (GIT pH) extracts (prepared simulating gastrointestinal tract pH conditions) of the flour and cookie samples were prepared using a combination of the extraction procedure used by Baublis et al., (2000) and Liyana-Pathirana and Shahidi (2005) (Figure 2.1.2). For GIT pH extracts, the flour and cookie samples (10.0 g) were first incubated in 150 ml double distilled deionised water at a pH of 6.5-7 for 30 min, while being stirred continuously. The pH was then decreased to 2.0 using 6 M HCl, which simulates the transition into the stomach, and allowed to incubate for 30 min at 37 °C. The pH was then increased to 6.0 using 4 M NaOH, which simulates the transition into the small intestinal tract, and allowed to incubate for 30 min at 37 °C. The extract was then centrifuged at 8000 x g at 20 °C for 30 min. The clear supernatant was then collected and freeze dried, and the analyses were done on the solids.

For water extracts, flour and cookie samples (10.0 g) were extracted in 150 ml double distilled deionised water for 90 min, while being stirred continuously. The mixture was then centrifuged at 8000 x g at 20 °C for 30 min. The clear supernatant was collected. NaCl was added to the clear supernatant equal to the amount that was formed during the GIT pH's extraction process. The supernatant was freeze dried and the analyses were done on the solids.

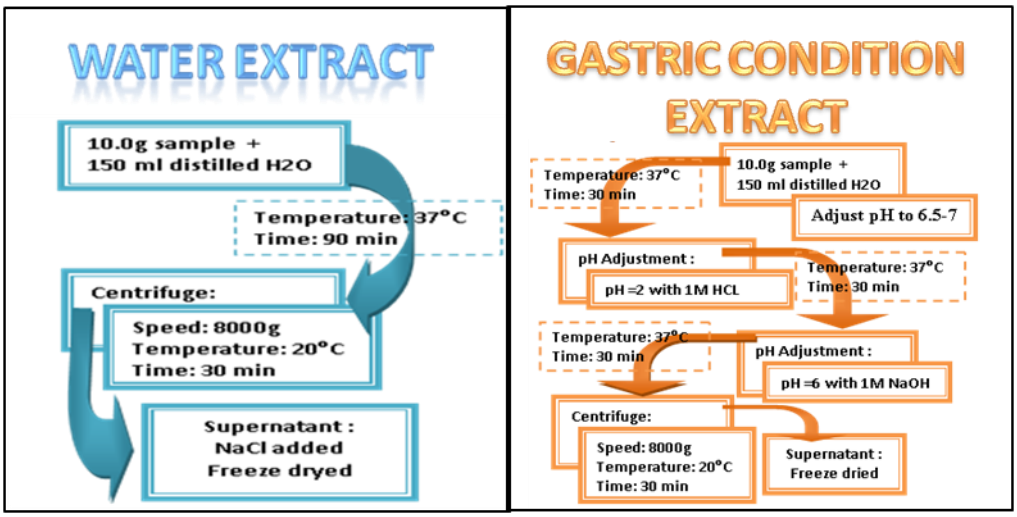


Figure 2.1.2 Illustration of preparation of water and gastro-intestinal (GIT) pH extracts

2.1.3.4 Determination of total phenolic content of water and GIT pH extracts

The total phenolic contents (water soluble TPC) of the freeze-dried water and GIT pH extracts of the flour and cookie samples were determined by the Folin-Ciocalteu assay (Singleton & Rossi, 1965), as described by Waterman and Mole (1994). Extracts were dissolved in distilled water to obtain a concentration of 5 mg/ml. An aliquot (0.25 ml) of the extract was then placed in a 25 ml volumetric flask and 5 ml distilled water was added. Folin-Ciocalteu's phenol reagent (1.25 ml) was added and mixed. After 2 min, 3.75 ml 20% (w/v) sodium carbonate (Na_2CO_3) solution was added. The contents were mixed and distilled water was added to volume and the content was mixed. After the addition of the 20% (w/v) sodium carbonate (Na_2CO_3), timing started for 2 h. After the 2 h the absorption was measured at 760 nm. The standard used was catechin and the results were expressed as mg catechin equivalents per 100 g sample on a dry basis.

2.1.3.5 Characterization and quantification of phenolic acids using High Performance Liquid Chromatography-Mass Spectrometry (LC-MS)

Whole grain flour, water extracts and GIT pH extracts of the flours and cookies were hydrolysed and analysed for the predominant phenolic acids using a modification of the LC-MS method used by Hirawan, Ser, Arntfield and Beta (2010). Thirty ml of 4 M NaOH was added to whole grain flour (2 g) and freeze dried extract (2 g) and the mixture was hydrolysed at ambient temperature for 4 h. The container was flushed with nitrogen for 5 min every hour during hydrolysis. After hydrolysis, the mixture was acidified to pH 1.5 – 2.5 using ice-cold 6 M HCl. The solution was then centrifuged at 5^{o}C for 20 min at 7800 x g. The supernatant was collected and extracted three times using 35 ml ethyl acetate. The organic phase was then dehydrated with sodium sulphate, filtered and then evaporated to dryness with a rotary evaporator. The residue was then redissolved in 2 ml 50% methanol and filtered through a 0.45 µm PTFE syringe filter prior to HPLC analysis. The hydrolysed extracts are shown in Figure 2.1.3.

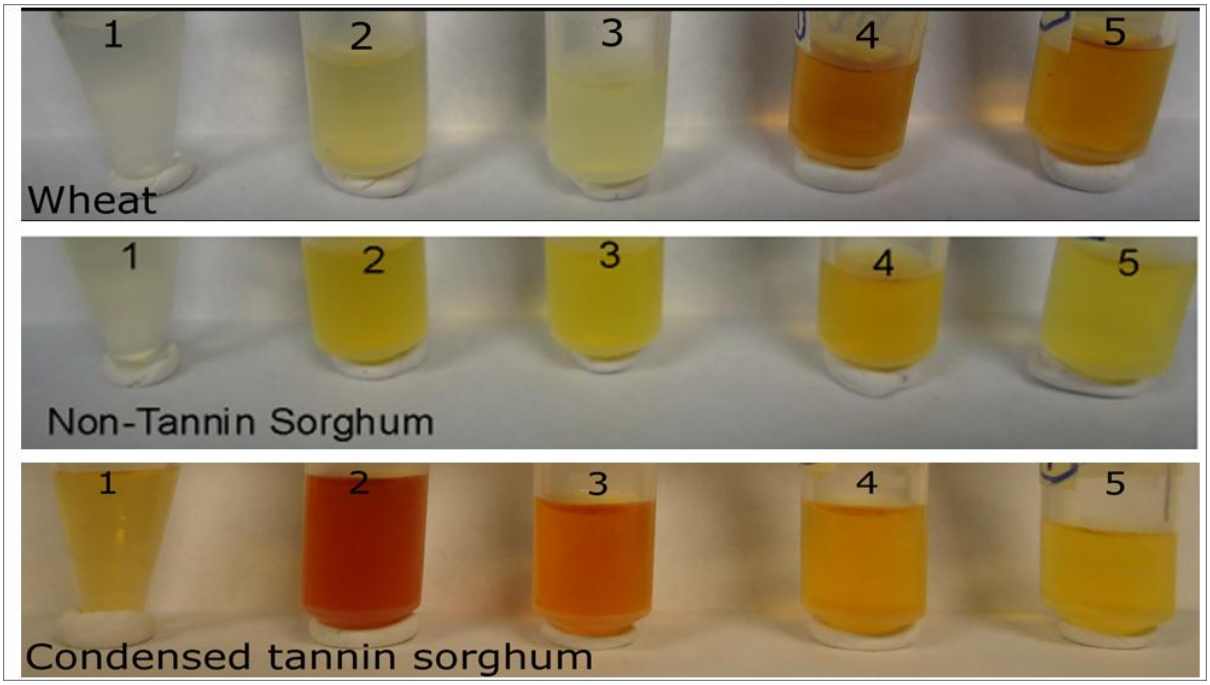


Figure 2.1.3: Appearance of phenolic acid extracts prepared from whole wheat, non-tannin sorghum and condensed tannin sorghum flours, cookies and their freeze-dried aqueous extracts. (1) whole grain, (2) whole grain flour water extract, (3) whole grain flour gastro-intestinal (GIT) pH extract, (4) cookies water extract and (5) cookies GIT pH extract.

The chromatographic separation of the phenolic acid extracts was carried out on a Waters 2695 chromatograph equipped with a photodiode array (PDA) detector (Waters 996) and autosampler (Waters 717 plus) (Waters, Milford, MA). The analytical column was a Gemini 150 mm × 4.6 mm, 5 µm RP 18 column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of A (1% acetic acid in HPLC grade water) and B (1% acetic acid in HPLC methanol) with a flow rate of 0.7 ml/min. A 60 min-linear gradient was programmed as follows: at 0 min 20% B, 4 min 23% B, 8-14 min 25% B, 14–19 min 27% B, 19–35 min 20% B. During analysis, a 10-µL sample solution was injected by autosampler.

Full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 45 V. The flow rate of desolvation gas (N_2) and cone gas (N_2) were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350 and 150 °C, respectively.

2.1.3.6 Statistical analysis

The experiments were repeated in triplicate for all the analyses done. Results were subjected to one-way analysis of variance (ANOVA). Fisher's least significance difference (LSD) test at the 95% significance level (STATISTICA version 7.1 StatSoft, Tulsa, OK) was used to determine the effects of the independent variables (grain samples, flours, cookies and the extracts) on the dependent variables (total phenolic content and phenolic acid content).

2.1.4 Results and Discussion

The effect of extraction under GIT pH conditions on the water soluble total phenolic content is shown in Table 2.1.1. Across all samples and extracts the tannin sorghum had the highest water soluble TPC, with up to 87% higher water soluble total phenolic content than wheat and 71% higher than non-tannin sorghum. This could be due to the tannins present in the water soluble portion of the condensed tannin sample. It has been shown that tannins can be extractable with water as a solvent (Shelembe, Cromarty, Bester, Minnaar & Duodu, 2012). Compared to other simple phenolics, tannins have more phenolic hydroxyl groups per mole which can react with the Folin-Ciocalteu reagent (Chiremba et al., 2009). The higher total phenolic content of the tannin sorghum samples could also be due to the higher anthocyanin content in condensed tannin sorghums in comparison with non-tannin sorghums (Awika, Rooney & Waniska, 2004) and wheat (Abdel-Aal, Young & Rabalski, 2006). Anthocyanins are water soluble (Harborne, 1984) and thus would be readily extracted into the water and GIT pH extracts. The major anthocyanins in sorghum are the 3-deoxyanthocyanins (Awika et al., 2004) which have been shown to exhibit more stability at pH greater than 3.5 than the 3-hydroxylated anthocyanins. The pH of the extracts (water extracts: 7.0 and gastric pH extracts: 2 to 6.0) falls within the pH range for stability of the 3-deoxyanthocyanins (pH > 3.5).

Table 2.1.1: Effect of extraction under gastro-intestinal (GIT) pH conditions on the water soluble total phenolic content (mg Catechin equivalent/ 100 g sample on dry basis) of whole wheat and sorghum flour and their cookies

Extracts	Wheat	Non-tannin sorghum	Tannin sorghum	Overall extraction effect	Overall cookie making effect
Flours					
Water	39.9 ab[1] (6.2)[2]	87.4 a (3.7)	305.7 c (7.7)	145.3 c	124.1 a
GIT pH	37.0 a (2.9)	88.1 a (2.0)	183.6 b (4.5)	102.9 b	
Cookies					
Water	55.8 c (1.1)	157.6 b (8.4)	405.3 d (15.4)	206.2 d	147.7 b
GIT pH	43.2 b (1.7)	81.8 a (7.2)	142.7 a (10.2)	89.2 a	

[1]Mean values in the same column with different letters are significantly different ($p<0.05$)

[2]Standard deviations given in parentheses

The overall extraction effect shows that for both flour and cookies, extraction with water resulted in 29% – 57% higher water soluble TPC than extraction under GIT pH conditions. These findings disagree with that reported by Liyana-Pathirana and Shahidi (2005), who reported higher TPC of gastric pH extracts than water extracts from wheat flour and bran. This could be due to differences in extraction procedure. Liyana-Pathirana and Shahidi (2005) prepared water extracts by extracting for 15 min while in this study, water and GIT pH extracts were prepared by extracting for 90 min. It may be hypothesised that the extended period of extraction increases the chances of oxidation of phenolic compounds, which could lead to lower TPC of the GIT pH extracts compared to the water extracts. For example, phenolic compounds such as tannins can be oxidatively degraded in low pH conditions (Price et al., 1978, as reviewed by Appel, 1993), such as used during preparation of the GIT pH extracts. This could reduce the TPC of the GIT pH extracts, especially in the condensed tannin sorghum samples in comparison to the water extracts.

The major portion of the phenolics in cereals are found in the bound form and located in the pericarp (Hahn et al., 1983). The use of acidic or alkaline conditions can lead to hydrolysis of ester bonds and the release of phenolic compounds from the food matrix (Robbins, 2003).

Organic solvents (Liyana-Pathirana & Shahidi, 2006; Zhao et al. 2006), sometimes with mild acidification (Kim et al., 2005), have been used to extract free phenolics from food. For the extraction of the bound phenolics, Kim et al., (2005) used alkali and a more severe acid hydrolysis. It could be reasoned that the extraction method used in this study may have allowed extraction of predominantly water soluble free phenolics from the wheat and sorghum samples and perhaps some bound phenolics as well. Although the low pH could release bound phenolics, the application of low pH for an extended period of time could also cause oxidative degradation as discussed previously.

Refining or removal of the pericarp layer in cereals reduces the phenolic content (Liyana-Pathirana et al., 2006). In Table 2.1.1 the TPC of water (39.9 mg Catechin equivalent/ 100 g) and GIT pH extracts (37.0 mg Catechin equivalent/ 100 g) of whole grain wheat flour were lower than TPC of acidified methanol extracts of refined wheat flour (150 mg Catechin equivalent/ 100 g), as reported by Chiremba et al., (2009). Chiremba et al., (2009) also reported higher TPC of acidified methanol extracts from condensed tannin and tannin free sorghum flours and cookies compared to the aqueous extracts of the flours and cookies used in this study. This shows that organic solvents are more effective compared to aqueous solvents such as water in the extraction of phenolic compounds.

The overall cookie making effect indicates that the cookie extracts had 16 % higher TPC than the flour extracts. A similar result has been reported by Morales et al., (2008), where phenolic content increased as baking temperature and time increased during production of wheat-based biscuits. Although Chiremba et al., (2009) reported higher TPC of whole grain sorghum and wheat flours than their cookies, when the flour component in the cookies was considered, the TPC of the cookies were slightly higher than that of the flour. This higher TPC of cookies compared to flours may be attributed firstly to products of the Maillard reaction and caramelisation that are formed during the baking process. Maillard reaction products (MRPs) have reducing properties (Dittrich et al., 2003) and therefore can act as reducing agents in the Folin-Ciocalteu assay. The Folin-Ciocalteu assay is not specific for phenolic compounds but actually measures the total reducing properties (Goya et al., 2007; Benjakul et al., 2005).

Secondly, the presence of polyphenol oxidase in whole wheat (Taneja & Sachar, 1974) and sorghum (Funderburk & Davis, 1963) flours could bring about enzymatic oxidation of phenolic compounds (as reviewed by Anderson, 1968), thus reducing the TPC. In the cookies the polyphenol oxidase would not be present due to inactivation during the baking process.

Thirdly, denaturation of proteins and breakdown of starch during the baking process could facilitate the release and solubilization of any phenolics that are bound to these macromolecules (Abdel-Aal & Rabalski, 2013). Lastly, during the production of the cookies the mixture of flour, sodium bicarbonate and water would have resulted in dough with an alkaline pH. Alkaline conditions can bring about hydrolysis and release of bound phenolic compounds (Barberousse, Roiseux, Robert, Paquot, Deroanne & Blecker, 2008) during preparation of the dough. These released phenolics could contribute to higher TPC of the cookies if they are stable during the baking process.

In contrast to the results obtained in this study, Friedman (2004) reported a decrease in the concentrations of phenolic compounds during baking. Friedman (2004) hypothesised that this is possibly due to the reaction of the free phenolics with the food matrix thereby making phenolic compounds less available.

Table 2.1.2: MS spectral data of phenolic acids identified in water and gastro-intestinal (GIT) pH extracts from whole wheat and sorghum flour and their cookies.

Peak	Assignment	tR (min)	[M−H]− (m/z)	Daughter ions (m/z) and their relative intensity (%)
1	*p*-hydroxybenzoic acid	8.20	137	92 (10); 108 (25); 136 (35)
2	Caffeic acid	10.68	179	107 (5); 135 (100)
3	*p*-coumaric acid	17.72	163	93 (5); 119 (100)
4	Ferulic acid	21.47	193	134 (100); 178 (70)

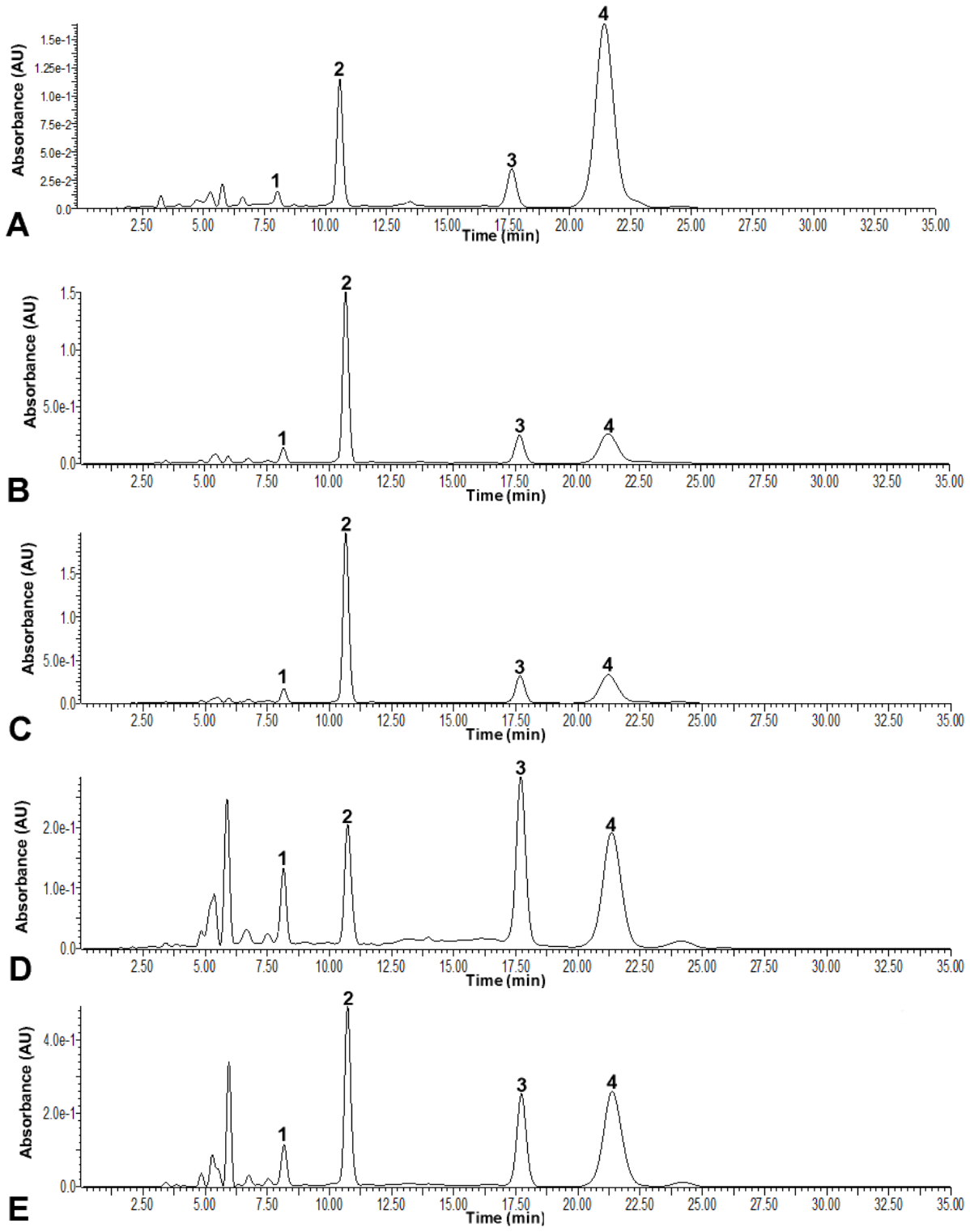


Figure 2.1.4: HPLC chromatograms of samples from condensed tannin sorghum. (A) extract from hydrolysis of whole grain flour with NaOH, (B) whole grain flour water extract, (C) whole grain flour gastro-intestinal (GIT) pH extract, (D) whole grain cookies water extract and (E) whole grain cookies GIT pH extract. Peaks 1 to 4 are identified as (1) *p*-hydroxybenzoic acid, (2) caffeic acid, (3) *p*-coumaric acid and (4) ferulic acid.

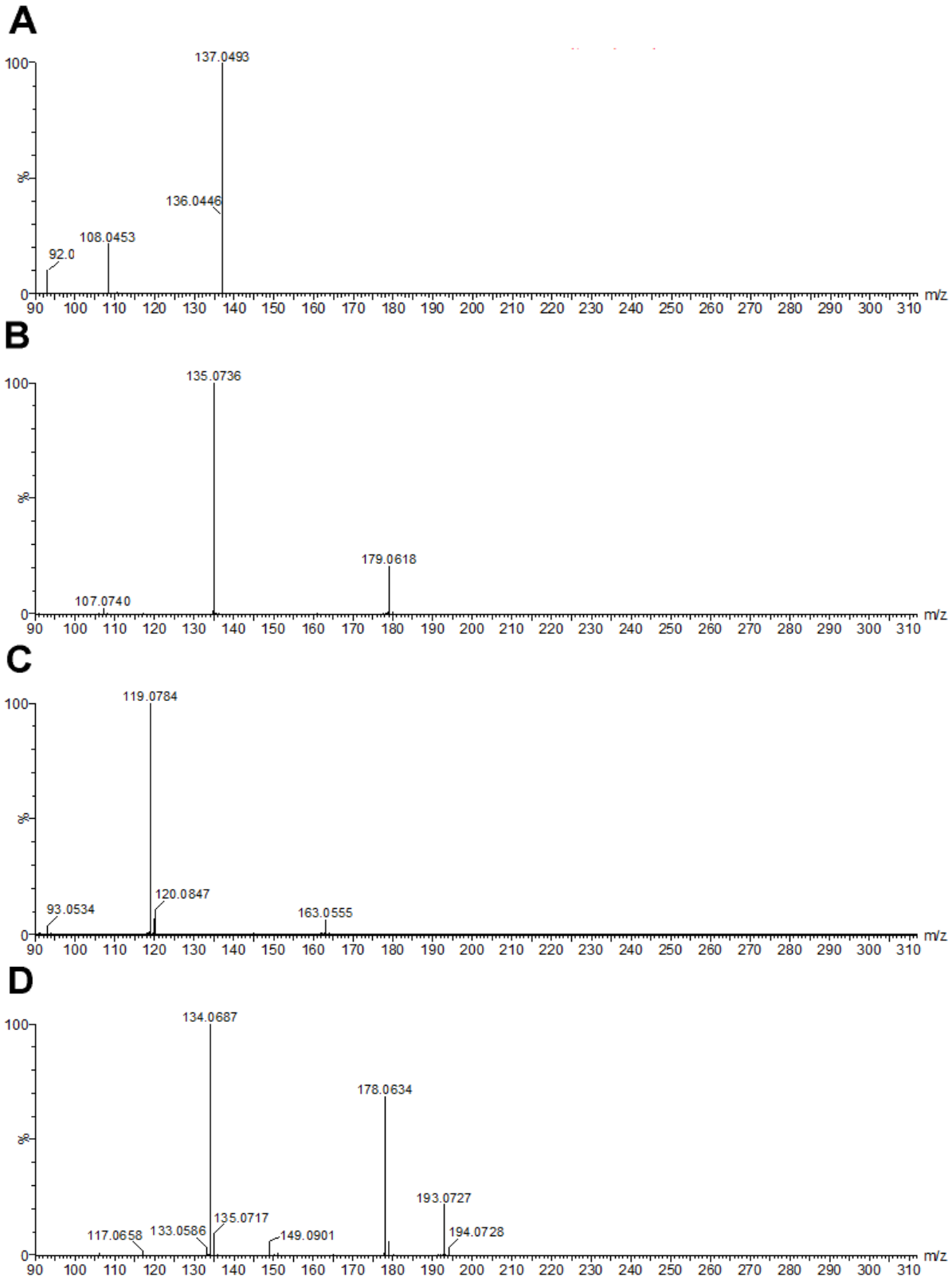


Figure 2.1.5: MS spectra of identified phenolic acids from condensed tannin sorghum samples. (A) peak 1: p-hydroxybenzoic acid, (B) peak 2: caffeic acid, (C) peak 3: *p*-coumaric acid, (D) peak 4: ferulic acid.

Table 2.1.3: Effect of extraction under gastro-intestinal (GIT) pH conditions on phenolic acid concentration (mg / 100 g sample on dry basis) of whole wheat and sorghum flour.

Whole grain Flour		*p*-Hydroxybenzoic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Total extracted phenolic acid content*
Wheat	**NaOH flour extract****	ND[1]	9.9 c (2.7)	42.3 b (1.7)	1844.3 f (9.9)	1896.5
	Water extract	3.1 a[2] (0.8)[3]	0.3 a (0.2)	6.9 a (0.2)	242.2 e (4.2)	252.5
	GIT pH extract	6.1 b (0.8)	4.0 b (1.0)	7.1 a (0.1)	205.7 d (4.9)	222.9
Condensed tannin sorghum	**NaOH extract**	55.1 d (4.6)	218.1 g (1.6)	214.0 e (1.3)	2379.3 g (16.3)	2866.5
	Water extract	47.5 c (1.0)	98.4 d (1.6)	42.7 b (0.7)	109.8 a (1.4)	298.4
	GIT pH extract	74.5 f (1.0)	160.8 f (0.8)	66.9 c (0.8)	171.5 c (2.6)	473.4
Non-tannin sorghum	**NaOH extract**	10.3 b (4.3)	256.7 h (8.1)	156.1 d (5.2)	2118.5 h (38.4)	2541.6
	Water extract	71.9 ef (0.4)	157.7 f (3.2)	62.1 c (2.6)	168.3 c (2.4)	460.0
	GIT pH extract	68.7 e (0.4)	140.3 e (2.4)	45.2 b (0.9)	125.1 b (1.8)	379.3

*Sum of *p*-hydroxybenzoic, caffeic, *p*-coumaric and ferulic acids for each sample; [1]ND indicates compound was not detected; [2]Mean values in the same column with different letters are significantly different ($p<0.05$); [3]Standard deviations in parentheses

**This is the extract obtained after direct hydrolysis of the flour with NaOH.

Table 2.1.4: Effect of extraction under gastro-intestinal (GIT) pH conditions on phenolic acid concentration (mg / 100 g sample on dry basis) of whole wheat and sorghum cookies.

Whole grain Cookies		*p*-Hydroxybenzoic acid	Caffeic acid	*p*-Coumaric acid	Ferulic acid	Total extracted phenolic acid content*
Wheat	**Water extract**	47.3 b[2] (3.8)[3]	41.8 c (4.1)	33.1 a (0.6)	1332.7 e (12.8)	1454.9
	GIT pH extract	142.2 d (3.0)	50.3 d (1.3)	32.0 a (0.4)	1389.4 f (14.0)	1613.9
Condensed tannin sorghum	**Water extract**	230.1 f (1.0)	29.0 b (5.6)	86.6 d (1.1)	138.0 a (2.0)	483.7
	GIT pH extract	227.3 f (0.4)	55.8 de (4.7)	72.7 c (0.9)	173.5 b (2.2)	529.3
Non-tannin sorghum	**Water extract**	166.3 e (0.6)	51.9 d (1.6)	127.8 f (1.2)	322.6 d (4.7)	668.6
	GIT pH extract	123.9 c (0.6)	61.5 e (1.8)	91.7 e (0.6)	257.7 c (1.4)	534.8

*Sum of *p*-hydroxybenzoic, caffeic, *p*-coumaric and ferulic acids for each sample; [1]ND indicates compound was not detected; [2]Mean values in the same column with different letters are significantly different ($p<0.05$); [3]Standard deviations in parentheses

Figure 2.1.4 shows the chromatograms for the extracts from the condensed tannin sorghum flour and cookies. These chromatograms from the condensed tannin sorghum extracts are shown as a sample of the chromatograms obtained in this study. The peak identities are shown in Table 2.1.2, with the parent ions from MS spectra shown in Figure 2.1.5. The phenolic compound profiles from the HPLC chromatograms of the extracts from flour and cookies were similar, with identification of four phenolic acids (p-hydroxybenzoic acid, caffeic acid, p-coumaric acid and ferulic acid). These phenolic acids were positively identified by comparing their retention times and mass spectra to those of known standards and they were quantified using these standards in calibration curves. Three of the water soluble phenolic acids identified were cinnamic acid derivatives, namely; caffeic acid, p-coumaric acid and ferulic acid. Table 2.1.3 and Table 2.1.4 show the phenolic acid content for the flour and cookie extracts, respectively. Ferulic acid was the dominant phenolic acid in the flour and cookie extracts for both wheat and sorghum. This is in accordance with literature for both wheat (Sosulski et al., 1982) and sorghum (Hahn et al., 1983).

The ferulic acid content of the wheat flour in this study was between 242.2 and 205.7 mg/100 g for the water and GIT pH extracts, respectively. This was between 50 and 100 times higher than that found by Liyana-Pathirana and Shahidi (2005), who reported from only traces to 6.7 mg/100 g. In the Liyana-Pathirana and Shahidi (2005) study the crude extracts were analysed for ferulic acid whereas in the present study the crude extracts underwent a process of alkaline and acid hydrolysis before analysis which could have released phenolics present in the crude extract that were bound to proteins or carbohydrates.

The extract obtained after direct hydrolysis of the flour with NaOH (NaOH flour extract) had between 5.5 and 9.6 times the level of phenolic acids than the water and GIT pH extracts from the wheat and sorghum samples. This could be due to the alkaline hydrolysis that the NaOH flour extract was exposed to during the extraction procedure. It has been shown that alkaline hydrolysis has a greater effect on the release of bound phenolic compounds than acidic hydrolysis (Barberousse et al., 2008). In the NaOH flour extract the bound phenolic

acids present in the food structure could have been released, which would not have occurred under the water or GIT pH extraction conditions.

Water and GIT pH extracts from cookies had higher total quantifiable phenolic acid content than the corresponding extracts from flour for the three grain samples (Tables 2.1.3 and 2.1.4). This is essentially due to the significant increase in levels of ferulic acid in the cookie samples. The most prominent increase was in the wheat cookies which had 5.5 times and 6.7 times higher ferulic acid content than the wheat flour for the water and GIT pH extracts, respectively. This could be due to the phenolic acids being more extractable after baking as a result of the changes in the food matrix structure thus releasing the ferulic acid more readily from the bound form. Mega, Fares, Troccoli, Cattivelli and Baiano (2010) found a 2.7 times increase in the free ferulic acid content during the baking of bread from wheat flour.

2.1.5 Conclusion

Condensed tannin sorghum flour and cookie extracts have higher water soluble TPC than the non-tannin sorghum. Overall the cookies have higher phenolic content than the flour for both the water and GIT pH extracts showing that the consumption of cookies has added health benefits when made from whole grain flour. The phenolic acids identified in the water and GIT pH extracts are p-hydroxybenzoic, caffeic, p-coumaric and ferulic acid in both whole grain wheat and sorghum samples, with ferulic acid being the dominant phenolic acid in both wheat and sorghum samples.

2.1.6 References

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2.2 INHIBITION OF LDL OXIDATION AND ANTIOXIDANT ACTIVITY OF WATER AND GASTROINTESTINAL TRACT pH EXTRACTS FROM WHOLE GRAIN WHEAT, SORGHUM AND THEIR COOKIES

2.2.1 Abstract

The effect of GIT pH conditions on the antioxidant properties of extracts from whole wheat, non-tannin sorghum and condensed tannin sorghum flour and their cookies was determined. The antioxidant properties analysed were ABTS radical scavenging ability, iron chelating capacity (ferrous ion chelation assay) and inhibition of LDL oxidation (measurement of thiobarbituric acid reactive substances after copper-mediated LDL oxidation). The water extracts had higher overall antioxidant activity properties than the GIT pH extracts. For the radical scavenging ability a range of results between 0.1 – 4.7 mM Trolox equivalent/ 100 g sample were found. The condensed tannin sorghum flour and cookies extracts for both water and GIT pH extracts had the highest radical scavenging properties. Metal chelation capability was between 325.6 – 8424.9 µg EDTA equivalent/ g sample. The wheat samples had the highest metal chelating capability. Inhibition of LDL oxidation was between 1.0 – 5.4 µM Trolox equivalent/g sample, and the water extracts from the whole wheat flour sample had the highest h-LDL oxidation inhibition. The observed antioxidant properties of the cookie extracts suggests that these cookies have the potential to contribute to reduction in oxidative stress and associated conditions such as cardiovascular disease

Keywords:

Wheat, Sorghum, Antioxidant activity, Metal chelation, LDL oxidation, GIT pH extract, Water extract.

2.2.2 Introduction

According to Jones (2008), reactive oxygen species (ROS) are molecules and ions derived from molecular oxygen that have an unpaired electron (free radicals), thus rendering them extremely reactive. ROS are produced in the body during various metabolic processes (as reviewed by Turrens, 2003). Cellular structures such as DNA and LDL are susceptible to attack by ROS, contributing to cancer and heart disease respectively (as reviewed by Jones, 2008).

The increasing mortality rate in the world due to cardiovascular disease is a major cause of concern (Ross, 1993). When plasma human-low-density lipoprotein (LDL) is modified by oxidation from ROS, resulting in oxidized-LDL (OX-LDL), the ability to regulate the uptake of cholesterol is lost, resulting in the accumulation of cholesterol in the OX-LDL. Uptake of cholesterol is believed to play a major role in the formation of a fatty streak in the artery resulting in atherosclerosis (reviewed by Jialal & Devaraj, 1996).

Phenolic compounds such as phenolic acids, flavonoids and tannins have been shown to have various degrees of antioxidant activity and thus have the ability to neutralize ROS and prevent oxidative damage. It has been shown that antioxidant compounds (especially phenolics) in food sources like red wine (Nigdikar, Williams, Griffin & Howard, 1998), legumes (Xu et al., 2007) and wheat (Liyana-Pathirana & Shahidi, 2005) inhibit the oxidation of h-LDLs (Yu et al., 2005). The inhibition of ROS by dietary phenolics could contribute to the prevention of chronic and degenerative diseases such as cancer and cardiovascular disease, as these are associated with oxidative damage.

Cereals contain phenolic compounds and sorghum is reported to have higher levels of phenolics than wheat (Liyana-Pathirana & Shahidi, 2006; Kim et al.,2005). Most of the research conducted into cereal phenolics has been done using organic solvents as extractants. Chiremba et al. (2009) used acidified methanol as an extractant and showed that after baking, cookies from sorghum and wheat flour still maintained appreciable levels of total phenolics and antioxidant activity. In order to better understand the potential health benefits of dietary phenolics, it is necessary to utilize a more physiologically relevant system

such as simulated in vitro digestion. The aim of this work was to determine the effect of gastrointestinal pH treatment on bioactive properties of sorghum and wheat flours and their cookies in terms of antioxidant activity and inhibition of LDL oxidation, respectively.

2.2.3 Materials and methods

2.2.3.1 Samples and extract preparation

The grain samples used, method of flour and cookie preparation and how the extracts were prepared have been described in section 2.1.2 (pages 30-32).

2.2.3.2 Methods

2.2.3.2.1 Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity of the extracts was determined using the ABTS antiradical assay (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003). The ABTS•+ (mother solution) was prepared by mixing equal volumes of 8 mM ABTS and 3 mM potassium persulphate ($K_2S_2O_8$) with distilled water in a volumetric flask. The mixture was wrapped in foil and allowed to react for a minimum of 12 h in a dark place. The working solution was prepared by mixing 145 ml phosphate buffer solution (pH 7.4) with 5 ml of mother solution. The phosphate buffer was prepared by mixing 405 ml 0.2 M sodium phosphate dibasic (Na_2HPO_4), 95 ml 0.2 M sodium phosphate monobasic (NaH_2PO_4) and 8.77 g NaCl, and then made up to 1000 ml with distilled water. The working solution (2.9 ml) was added to the freeze-dried extracts, which were previously dissolved in distilled water (5 mg/ml) (0.1 ml). The working solution (2.9 ml) was also added to the Trolox standards (0.1 ml) for obtaining a standard curve. The mixtures were made up in a test tube and mixed with a vortex. The test tubes were allowed to stand for exactly 30 min. The absorbance of the standards and samples was measured at 734 nm. The results were calculated and expressed as µM Trolox equivalents/100 mg sample, on dry weight basis.

2.2.3.2.2 Determination of the formation of thiobarbituric acid reactive substances during LDL oxidation

The effect of the extracts on copper-catalyzed human low density lipoprotein (LDL) oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) (Rüfer & Kulling, 2006; Xu et al., 2007). The freeze-dried extracts were dissolved in distilled water to obtain a concentration of 0.5, 1.0, 1.5, and 2.0 mg/ml for the wheat and non-tannin sorghum extracts and 0.05, 0.1, 0.15, 0.25, 0.5 mg/ml for the condensed tannin sorghum. Trolox was used as the standard. For the assay, 2 µL of LDL solution (5.5 mg/mL), 168 µL 0.02 M phosphate buffer saline (PBS) solution and 10 µL sample extract or standard solutions were placed in eppendorf tubes. Oxidation was initiated by the addition of 20 µl of 100 mM copper sulphate solution to each tube. A positive control was prepared by adding 2 µL LDL solution, 178 µl PBS and 20 µL copper sulphate solution. A negative control was prepared by combining the LDL solution (2 µL) and (198 µL) PBS solution (198 µL). Tubes were incubated in a shaking water bath at 37 °C for 3 h. The following were then added: 200 µL of 10 mM EDTA, 200 µL of 20 % (w/v) trichloroacetic acid solution and 200 µL of 0.67 % (w/v) thiobarbituric acid solution in 0.2 M NaOH. Tubes were then heated (80 °C for 30 min) in a water bath, cooled, centrifuged (1500 g for 15 min) and the supernatant was transferred into a 1 mL cuvette. The absorbance was then measured at 532 nm using a T80+ UV-VIS spectrophometer (PG Instruments, Leicestershire, UK).

2.2.3.2.3 Ferrous Ion Chelating (FIC) Assay

The Fe (II) chelating effect and the Fe (II) chelating activity of the extracts were determined using the method followed by Liyana-Pathirana and Shahidi (2006), as described by Carter (1971). Freeze dried extracts were dissolved in distilled water to obtain a concentration of 5 mg/ml (2.0 ml) and mixed with 0.2 ml 2 mM $FeCl_2$ and 4 ml 5 mM ferrozine. The mixture

was allowed to stand for 10 min at room temperature. The absorbance was measured at 562 nm.

The Fe (II) chelating effect of the extracts was calculated as follows:

$$\text{Fe (II) chelating effect (\%)} = [1-(A_S/A_B)] \times 100$$

Where A_S is the absorbance of sample at 562 nm and A_B is the absorbance of the control at 562 nm (control contains $FeCl_2$ and ferrozine). The Fe (II) chelating activity was then calculated and expressed as µg EDTA equivalents per 100 g sample on a dry basis.

2.2.3.3 Statistical analysis

The experiments were repeated in triplicate for all the analyses done. Results were subjected to one-way analysis of variance (ANOVA) and significant differences between the sample means were determined using Fisher's least significance difference (LSD) test at the 95% significance level (STATISTICA version 7.1 StatSoft, Tulsa, OK, USA).

2.2.4 Results and Discussion

The effect of extraction under gastro-intestinal (GIT) pH conditions on the ABTS radical scavenging capacity is shown in Table 2.2.1. Overall, the water extracts from the condensed tannin sorghum flour and cookies had the highest radical scavenging capacity. This could be due to the fact that the tannin sorghum contains condensed tannins whereas the other samples do not. On a molar or unit mass basis, condensed tannins have more phenolic hydroxyl groups for radical scavenging and contribution to antioxidant activity than other phenolics (Hagerman, Rield, Jones, Sovik, Ritchard, Hartfeld & Reichel, 1998).

Table 2.2.1: Effect of extraction under gastro-intestinal (GIT) pH conditions on the ABTS radical scavenging capacity (mM Trolox equivalent/100 g sample on dry basis) of whole wheat and sorghum flour and their cookies

Extracts	Wheat	Non-tannin sorghum	Tannin sorghum	Overall extraction effect	Overall cookie making effect
Flours					
Water	0.1 a[1] (0.0)[2]	3.0 c (0.7)	4.5 b (1.2)	2.5 b	2.2 a
GIT pH	0.1 a (0.0)	2.5 bc (1.3)	2.8 a (0.6)	1.8 a	
Cookies					
Water	0.1 a (0.0)	1.9 b (0.8)	4.7 b (1.2)	2.2 b	1.7 a
GIT pH	0.1 a (0.0)	0.9 a (0.4)	2.5 a (0.6)	1.2 a	

[1]Mean values in the same column with different letters are significantly different ($p<0.05$)

[2] Standard deviations given in parentheses

Overall extraction effect showed that the water extracts from the flour and cookies had higher radical scavenging capacity than GIT pH extracts. This was more evident with the condensed tannin sorghum samples, where the water extracts from the flour and cookies had up to 2.1 times the radical scavenging capacity of the GIT pH extracts. This is in

agreement with the results in Table 2.1.1 (Chapter 2.1), which showed that water extracts from the wheat and sorghum flours and their cookies had higher overall total phenolic content than GIT pH extracts. This shows that the two extraction conditions solubilise antioxidant compounds differently. The water extraction appears to solubilise more antioxidant compounds than the GIT pH extraction. As mentioned earlier in Chapter 2.1, low pH conditions such as used during preparation of the GIT pH extracts can bring about oxidative degradation of phenolic compounds (Price et al., 1978, as reviewed by Appel, 1993).

Although baking the non-tannin sorghum flour into cookies reduced radical scavenging capacity of water and GIT pH extracts, there was no such reduction for the wheat and tannin sorghum flours. Overall, there was no effect of cookie making on radical scavenging capacity of the extracts.

Table 2.2.2: Effect of extraction under gastro-intestinal (GIT) pH condition on the Fe (II) chelating effect[1] (%) of extracts (5 mg/ml) of whole wheat and sorghum flour and their cookies

Extracts	Wheat	Non-tannin sorghum	Tannin sorghum	Overall extraction effect	Overall cookie making effect
Flours					
Water	98.6 a[2] (1.2)[3]	98.6 b (0.5)	99.8 c (0.1)	99.0 c	96.9 a
GIT pH	96.3 a (2.4)	87.9 a (1.1)	98.4 bc (3.4)	94.7 a	
Cookies					
Water	99.7 a (1.7)	102.2 c (2.8)	86.6 a (2.5)	96.1 b	95.4 a
GIT pH	97.3 a (0.5)	90.5 ab (18.6)	96.4 b (0.9)	94.7 a	

[1]Percentage in comparison with the EDTA standard used, [2]Mean values in the same column with different letters are significantly different ($p<0.05$), [3] Standard deviations given in parentheses

Fe (II) chelating capacity (µg EDTA equivalent/ g sample on dry basis) and Fe (II) chelating effect (%) is measured by the FIC assay. The Fe (II) chelating effect (%) is a measure of the ability of the extract at a given concentration, in this study 0.5 mg/ml, to chelate Fe (II). The Fe (II) chelating capacity is a representation of the chelating ability of the samples as a whole and not just the extracts being analysed. The Fe (II) chelating capacity takes into account the concentration of the extract in relation to the sample. This can increase differences between samples as the amount (g) of extract differs between different grains and treatments. Fe (II) chelating effect (Table 2.2.2) and Fe (II) chelating capacity (Table 2.2.3) will be discussed simultaneously.

Table 2.2.3: Effect of extraction under gastro-intestinal (GIT) pH conditions on the Fe (II) chelating capacity (µg EDTA equivalent/g sample on dry basis) of whole wheat and sorghum flour and their cookies

Extracts	Wheat	Non-tannin sorghum	Tannin sorghum	Overall extraction effect	Overall cookie making effect
Flours					
Water	680.8 a[1] (17.4)[2]	376.7 a (8.5)	387.7 a (22.1)	481.7 b	458.8 a
GIT pH	655.5 a (41.9)	326.2 a (9.7)	325.6 a (17.5)	435.8 a	
Cookies					
Water	8424.9 b (307.3)	4664.6 b (236.5)	3105.8 c (102.2)	5398.4 d	3376.0 b
GIT pH	719.1 a (45.4)	448.3 a (32.1)	2893.1 b (37.3)	1353.5 c	

[1]Mean values in the same column with different letters are significantly different ($p<0.05$)

[2] Standard deviations given in parentheses

In vivo it has been shown that metals like copper and iron can cause oxidation to LDL (as reviewed by Lynch & Frei, 1993), DNA (as reviewed by Aust & Eveleigh, 1999), and other cellular organelles (as reviewed by Zhu, Su, Wang, Smith & Perry, 2007). It has been argued that there is little correlation between iron chelating and the radical quenching process,

which is the method utilized by most antioxidants. However, the ability to mediate the oxidation of radicals to ions can have a large impact on the retarding or stopping of the radical chain reactions (Prior, Wu & Schaich, 2005).

Based on extraction with water, wheat flour and cookies had higher Fe (II) chelating capacity than flour and cookies from the two sorghum types (Table 2.2.3). This could be due to the natural metal chelators that are found in cereals (Alabaster, Tang & Shivapurkar, 1996).

Looking at the overall extraction effect, Table 2.2.3 shows that flour and cookies had higher Fe (II) chelating capacity on the basis of extraction with water than under GIT pH conditions. Water extracts also had a higher Fe (II) chelating effect than GIT pH extracts (Table 2.2.2). This is in agreement with the ABTS radical scavenging capacity results shown above (Table 2.2.1), as well as the TPC in Section 2.1.3. The overall extraction effect showed that water extracts from the flours and cookies had 9.5 % and 74.9 % higher Fe (II) chelating capacity than the GIT pH extracts, respectively. Oxidation of phenolics under the GIT pH extraction conditions could reduce the levels of assayable phenolics that would be available for Fe (II) chelation and thus would reduce Fe (II) chelating capacity of the samples (Liyana-Pathirana & Shahidi, 2006). In contrast, Liyana-Pathirana and Shahidi (2005) found that the Fe (II) chelating capacity of gastric pH extracts were higher than water extracts from wheat flour and bran. This could be due to differences in extraction procedure as discussed in Section 2.1.4.

On the basis of both water and GIT pH extraction, cookies of the three grain samples had higher Fe (II) chelating capacity than their flours. This result suggests that the Fe (II) chelating agents (phenolics and possibly other compounds) are better solubilised in the cookies than in the flours. Morales et al., (2008) found that during the production of wheat-based biscuits the water-soluble phenolic content increased as baking temperature and time increased. Glucose-lysine Maillard reaction products (MRPs) that are formed during the baking process are known metal chelators (Delgado-Andrade, Seiquer & Navarro, 2004; Wijewickreme, Kitts & Durance, 1997). It has been proposed that the metal binding affinity of MRPs is due to the fact that they can function as electron donors which is also the mechanism by which they function as antioxidants (Jing & Kitts, 2004). According to Yoshimura, Lijima, Watanabe and Nakazawa (1997), the hydroxyl group of MRPs is

important in the reducing activity. MRPs have shown a higher ability to chelate iron than copper (Kim & Lee, 2009).

Table 2.2.4: Effect of extraction under gastro-intestinal (GIT) pH conditions on copper-mediated LDL oxidation based on LDL-TBARS assay (µM Trolox equivalent/g sample on dry basis) of whole wheat and sorghum flour and their cookies

Extracts	Wheat	Non-tannin sorghum	Tannin sorghum	Overall extraction effect	Overall cookie making effect
Flours					
Water	5.4 c[1] (1.6)[2]	3.8 b (1.6)	2.2 b (0.3)	3.8 c	3.2 a
GIT pH	2.3 b (0.7)	4.3 b (1.1)	1.1 a (0.3)	2.6 b	
Cookies					
Water	5.3 c (0.7)	3.5 b (0.8)	3.8 c (0.9)	4.2 d	2.7 a
GIT pH	1.4 a (0.5)	1.1 a (0.4)	1.0 a (0.3)	1.2 a	

[1]Mean values in the same column with different letters are significantly different ($p<0.05$)

[2]Standard deviations in parentheses

Water extracts of wheat flour and cookies had higher LDL oxidation inhibitory capacity than water extracts from the non-tannin and tannin sorghum flours and cookies. This may be related to the higher Fe (II) chelating capacity of the wheat flour and cookies based on water extraction that was observed in Table 2.2.3. The wheat sample may exert high Cu (II) chelating ability and thus render the copper inactive and unable to proceed with the oxidation of PUFAs in the LDL.

The observed higher LDL oxidation inhibitory capacity of water extracts from wheat flour and cookies, compared to those from the sorghum samples, contrasts with the ABTS radical scavenging results reported in Table 2.2.1 above. Water extracts from sorghum (non-tanin and tannin) samples had higher ABTS radical scavenging activity than water extracts from wheat samples. With regard to phenolic composition, a major difference between the sorghums and wheat would be the presence of tannins in the tannin sorghum samples and relatively higher levels of phenolics such as flavonoids in the non-tannin sorghum compared to the wheat. Tannins (in tannin sorghum) and relatively higher levels of flavonoids (in non-tannin sorghum) would offer more phenolic hydroxyl groups with reducing properties and so would be expected to have high radical (e.g. ABTS) scavenging capacity. However, tannins are large molecules and thus may not be able to interact with LDL molecules optimally to prevent their oxidation due to steric hindrance. Secondly, it could also be as a result of the mechanism by which the water extracts of the sorghum samples inhibit LDL oxidation, as ABTS assay is based on the scavenging ability of antioxidants to the radical anion $ABTS^{\bullet+}$ (Prior et al., 2005). This mechanism would stop the oxidation of the PUFA, but it does not remove the oxidation source as such, thus allowing the copper to oxidize other PUFAs within the LDL. The reasoning for the difference in mechanism between the extractable portions from the wheat compared to that of the sorghum samples could be due to differences in the composition of the extractable portions from the two cereals.

Overall, water extracts had 32% and 71% higher LDL oxidation inhibitory capacity than GIT pH extracts for flour and cookies, respectively. As mentioned earlier, oxidation of phenolics under GIT pH extraction conditions could reduce the levels of solubilizable antioxidants, leading to the observed lower LDL oxidation inhibiting capacity of the GIT pH extract. LDL oxidation is accelerated in an acidic environment (Leake, 1997). Thus the relatively more acidic nature of the GIT pH extracts could also contribute to the lowering of their ability to inhibit LDL oxidation. As observed with the ABTS radical scavenging assay, baking into cookies had no significant overall effect on LDL oxidation inhibitory capacity of the samples.

2.2.5 Conclusion

Both the water and GIT pH extracts showed the ability to inhibit the oxidation of h-LDL from the wheat and sorghum flour and cookies. The in vitro antioxidant activities measured in this study have been linked to medical benefits in terms of the possible prevention of in vivo oxidation. The GIT pH extractions simulated the changes in pH in the human digestive tract. The results suggest that the products of GIT digestion may contain antioxidant compounds such as phenolics that may offer potential health benefits associated with radical scavenging and metal chelating properties.

2.2.6 References

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CHAPTER 3

3. GENERAL DISCUSSION

This chapter is divided into two sections: The first section will critically discuss the methodologies used in this research. The second section will attempt to explain the observed trends in terms of the mechanisms of antioxidant activity in the water and GIT pH extracts from the wheat and sorghum flours and their cookies.

3.1 Critical discussion of experimental design and methodologies

Sorghum is an important drought-tolerant cereal which is consumed extensively, especially among rural communities in mainly semi-arid regions of Africa. Wheat is the cereal most used for manufacture of cookies. Due to the importance of sorghum as a food cereal, its utilization could be enhanced if used in cookie manufacture, instead of using wheat. The use of sorghum as a flour base in cookies, instead of wheat, also presents other advantages. Sorghum is gluten-free and can therefore be consumed by people with gluten allergies. Sorghum also generally contains higher levels of phenolic antioxidants than wheat (Liyana-Pathirana & Shahidi, 2005; Awika et al., 2003), and therefore could provide more health benefits. Whole grain flour was selected over refined flour due to the fact that the bran fraction, which is excluded in refined flour, contains more antioxidants (Siwela et al., 2010). Therefore it would be expected to be the more health-conscious and nutritious choice.

In normal cookies the basic ingredients are flour, a sugar source like sugar or golden syrup, a fat source like butter or margarine, and a leavening agent like sodium bicarbonate or baking powder (American Association of Cereal Chemists International, 2000). For the model cookies used in this study the sugar and fat sources were removed and water was used as a binding agent. The rationale behind the above-mentioned is that exclusion of these two ingredients will eliminate potential sources of interference with antioxidant capacity.

The main aim of this study was to determine antioxidant properties of the phenolic compounds extracted from the flours and cookies. It will therefore be desirable to eliminate other non-phenolic compounds which have antioxidant properties as much as possible. This reduces interference with the results and prevents possible overestimation in the effects observed. In the case of sugar it has been shown that during baking, significant levels of Maillard reaction products (MRPs) are formed due to the reaction between the reducing sugars in the sugar and the amino acids in the flour. These MRPs have antioxidant activity (Jing and Kitts, 2004). The fat source (e.g. margarine) could contain vitamin E, which is normally added during the production of margarine. Vitamin E is known for its antioxidant activity (Burton, Le Page, Gabe & Ingold, 1980). Thus adding sugar and fat to the cookies could result in the addition of other non-phenolic sources of antioxidants. This, in turn, could result in an overestimation of the antioxidant properties of the extracts.

Mimicking the pH conditions of the gastro intestinal tract during extraction has been shown to release phenolic compounds from wheat flour (Liyana-Pathirana & Shahidi, 2006) and wheat-based ready-to-eat breakfast cereals (Baublis, Decker & Clydesdale, 2000). A modified form of the extraction procedure used by Liyana-Pathirana and Shahidi (2006) was used in this study. The water extracts had the same extraction time as the GIT pH extracts, unlike the method employed by Liyana-Pathirana and Shahidi (2006), where the time used for extraction with water was only a third of the time for GIT pH extracts. The modification to the method was made to ensure that the change in pH was the only variable between the water extract and the GIT pH extracts.

Due to the differences in the chemical nature of phenolics present in the food matrix there is no ideal extraction method to recover 100% of the phenolics present in the food matrix (Naczk & Shahidi, 2006). Extraction with water, or extraction under gastric conditions would not only extract phenolic compounds, but also some water-soluble substances such as sugars, small peptides and amino acids in the whole grain flours could be extracted. Some of these non-phenolic, water-soluble substances have reducing properties and could also contribute to the results obtained for total phenolic content and radical scavenging properties. It has been shown, for instance, that the Folin-Ciocalteu phenol reagent reacts with the amino acids tyrosine and tryptophan (Lowry, Rosebrough, Farr & Randall, 1951).

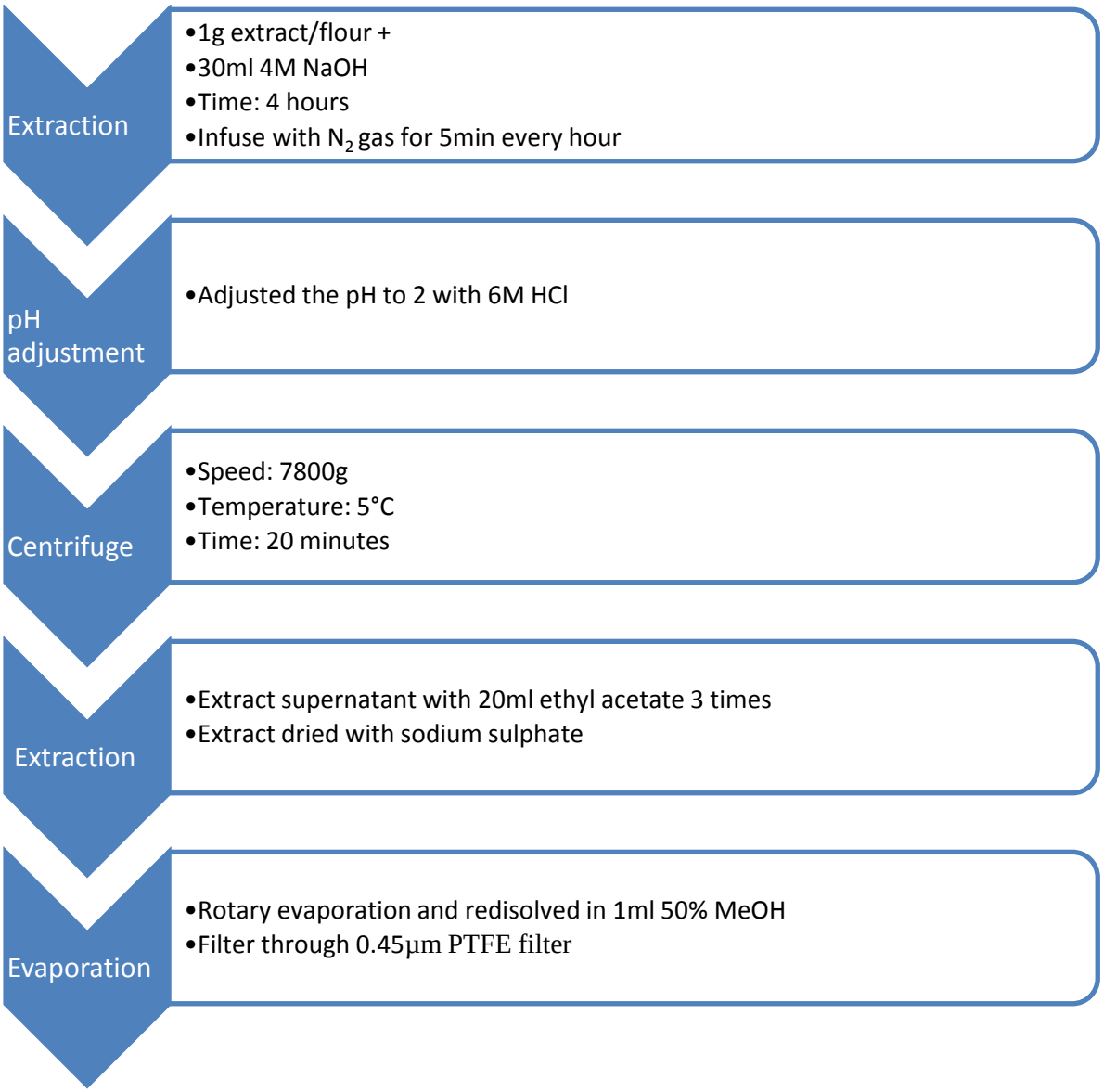

Figure 3.1.1 Illustration of the preparation of water and gastro-intestinal pH conditions phenolic acid extracts from wheat and sorghum flour and cookies

For analysis of phenolic acids, prior to HPLC/MS analysis, the extraction and purification procedure used by Hirawan et al., (2010) was used in this study. This was done to ensure that the presence of small peptides, amino acids, sugars and various other small molecular weight compounds in the crude extract was removed. The procedure involved isolation of the phenolic acids from the extracts by solvent extraction using ethyl acetate. This resulted in a purified form of the extract consisting predominantly of phenolic acids, with

interference from the non-phenolic compounds brought to an absolute minimum. This extraction procedure was employed for extracts obtained after sequential hydrolysis under both alkaline and acidic conditions, which served to release any bound phenolic acids in the extract.

Quantification of phenolic compounds can be achieved by the use of several methods, depending on the type of phenolic compounds to be assayed. The methods range from those that measure total phenolic content to methods that identify the phenolic composition. The method used for determining total phenolic content in this study was the Folin-Ciocalteu method (Singleton & Rossi, 1965). High performance liquid chromatography and mass spectrometry were used to identify and quantify the individual phenolic acids.

The Folin-Ciocalteu assay is a widely used colorimetric assay for total phenolics in cereals (Awika et al., 2005; Liyana-Pathirana & Shahidi, 2006). An advantage of the assay is that it gives reliable estimates of the levels of reducing phenolic groups present. The disadvantage is that it is not specific and is subject to interference from any non-phenolic reducing substances (McGrath, Kaluza, Daiber, Van der Riet & Glennie, 1982). Such substances include sugars, aromatic amines, sulphur dioxide, ascorbic acid and some inorganic compounds (Phipps et al., 2007).

The separation and identification of phenolic acids can be done by utilizing a number of analytical methods like thin-layer chromatography, gas-liquid chromatography and high performance liquid chromatography (HPLC) (as reviewed by Robbins, 2003). The choice of method used depends on the solubility and volatility properties of the compounds to be analysed (Harborne, 1984). In this study the use of HPLC had two functions: The first was to separate the compounds in the extracts, which were then passed through the Mass spectrometer (MS) to be identified. Secondly, it was used to determine the quantity of each phenolic acid present in the extract. The use of HPLC coupled to an MS resulted in the identification of the phenolic acids not just based on comparing their retention times to those of standards, but also based on their molecular masses. This resulted in a more accurate identification of the phenolic acids present in the extracts.

To determine the antioxidant activity in terms of radical scavenging capacity of the water and gastric extracts from wheat and sorghum flour and their cookies, the ABTS radical scavenging assay was used. The assay measures the relative ability of antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation compared to that of the water soluble vitamin E analogue, Trolox. The main advantages of the assay are that it is quick and can be used over a wide range of pH values (Arnao, Cano & Acosta, 1999) in both aqueous and organic solvent systems. The major disadvantage is the free radical generation period of 12 hours and the instability of the radical solution after 16 hours. This step makes the procedure long and analyses cannot be repeated in a single day. Other antioxidant capacity assays that are used include the ORAC and DPPH assays, although they also have their unique merits and demerits. The ORAC assay, although highly automated and standardized, uses expensive equipment and is sensitive to pH (Awika et al., 2003). DPPH radicals react slowly with phenolic compounds. The reaction takes up to 6 hours or longer, while in the case of $ABTS^{\bullet+}$ radical the reaction takes 20 minutes (Awika et al., 2003). DPPH is also prone to interference by colourants in samples that contain pigments such as anthocyanins, which absorb at the same wavelength (515 nm), in turn leading to an underestimation of antioxidant activity (Arnao, 2000). $ABTS^{\bullet+}$ radical scavenging activity was measured at 734 nm, which is outside the absorption range of the sorghum anthocyanin plant pigments, resulting in less interference (Awika et al., 2003). Therefore, the ABTS radical scavenging assay is the favored method compared to the DPPH and ORAC methods as it gives the most reliable results and can be used to measure total antioxidant activity (Awika et al., 2003).

To determine the antioxidant activity in terms of metal chelation capability of water and gastric extracts from wheat and sorghum flour and their cookies, the ferrous ion chelation (FIC) assay was used. Metal chelation, like radical scavenging, has been shown to be a mechanism of antioxidant activity (Prior et al., 2005). A difference between the ability of phenolic compounds to chelate different metal ions has been shown (Karamać, 2009). The advantages of the metal chelation method are that it is simple, speedy, and inexpensive. It

also has the advantage that the results could assist in explaining the observations from the LDL oxidation assay since the LDL assay proceeds by initiation of oxidation with Cu (II).

The LDL-TBARS assay was used to determine the antioxidant activity of the extracts in terms of inhibition of h-LDL oxidation. The assay measures the amount of thiobarbituric acid reactive substances (TBARS) formed after oxidation of the LDL, which is initiated with Cu (II). The LDL-TBARS assay can provide information about the kinetics of the inhibition process if the measurements are conducted during the oxidation process. Otherwise it gives an indication of the ability of the extract to inhibit LDL oxidation within a specified time frame. In actual fact, this assay gives an indication of the ability of the extracts to influence the lag phase during the oxidation of lipids within the LDL molecule (Figure 3.1.2). The presence of antioxidants (e.g. the extracts) increases the lag phase of the oxidation reaction. The longer the lag phase, the lower the amount of late stage products and therefore the higher the inhibition of LDL oxidation.

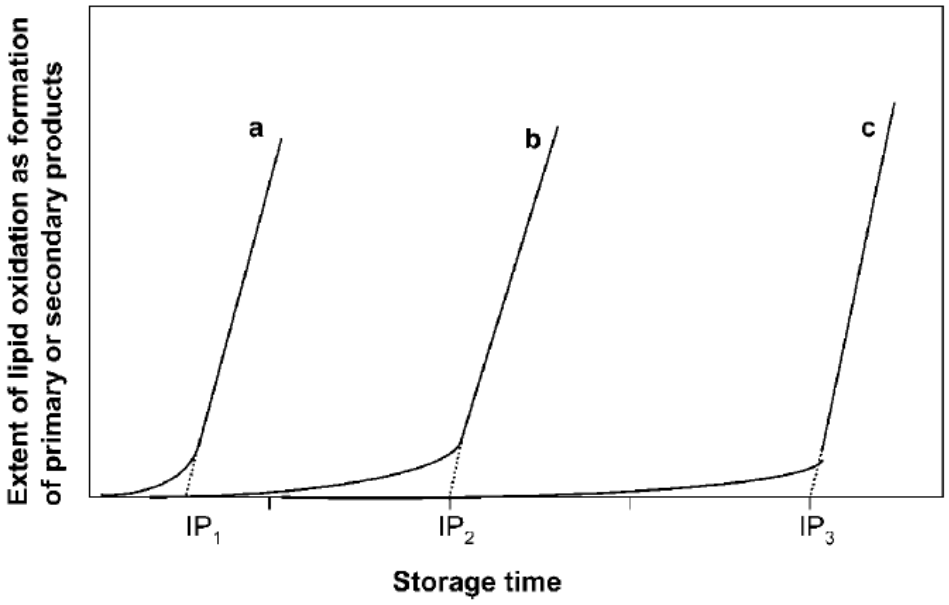

Figure 3.1.2 Typical curves for oxidation of lipids; (a) no antioxidant added, (b) and (c) increased lag phase, depending on the amount of antioxidants added. IP1, IP2 and IP3 indicate the ending of the lag phase in terms of storage time
(Wanasundara & Shahidi; 2005)

3.2 Critical discussion of results

Table 3.2.1 shows the correlation coefficients (r) between the dependent variables for all the treatments. All the correlation coefficients were positive. The correlation between ABTS radical scavenging properties and total phenolic content (TPC) was the highest (r =0.92). Chiremba et al., (2009) showed that there is a positive correlation between ABTS and TPC when analysing cookies made from sorghum and wheat. There was also a high and positive correlation (r = 0.86) between inhibition of LDL oxidation and Iron Chelating effect (%). This suggests that inhibition of LDL by the extracts can be related to their ability to chelate metal ions.

The lowest overall correlation occurred between ABTS radical scavenging and inhibition of LDL oxidation (r =0.57), which was slightly lower than that between total phenolic content and inhibition of LDL oxidation (r =0.63), and in turn much lower than the correlation between Iron chelating effect and LDL oxidation (r = 0.86). This suggests that the LDL oxidation inhibitory capacity of the extracts may be due more to their ability to chelate metal ions than scavenge free radicals. It is likely that both mechanisms may occur at the same time but to different extents.

More detailed correlation analyses within the different sub-groups, namely cereal type (wheat, tannin sorghum and non-tannin sorghum), extraction method and sample type (flour and cookies) resulted in high and positive correlations ($r \geq 0.7$).

Table 3.2.1: Correlation coefficients (r) between the dependent variables for all treatments of the wheat, tannin- and non-tannin sorghum samples

		Wheat						Tannin sorghum						Non-tannin sorghum					
		Water extract			GIT pH extract			Water extract			GIT pH extract			Water extract			GIT pH extract		
	Overall	Flour and cookies	Flour	Cookies	Flour and cookies	Flour	Cookies	Flour and cookies	Flour	Cookies	Flour and cookies	Flour	Cookies	Flour and cookies	Flour	Cookies	Flour and cookies	Flour	Cookies
ABTS vs. TPC	0.92	0.99	0.99	1.00	0.79	1.00	1.00	0.98	0.99	0.99	0.99	1.00	0.98	0.89	1.00	0.99	0.91	0.99	0.91
TPC vs. Fe%	0.79	0.98	0.99	1.00	0.85	1.00	1.00	0.98	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	0.96	1.00	0.98
ABTS vs. LDL	0.57	0.98	0.97	0.99	0.95	0.97	0.95	0.96	0.97	0.99	0.94	0.95	0.92	0.85	0.85	0.97	0.97	1.00	0.79
LDL vs. Fe%	0.86	0.98	0.96	0.99	0.93	0.97	0.94	0.93	0.99	0.97	0.98	0.98	0.98	0.97	0.97	0.98	0.85	0.98	0.91
LDL vs. TPC	0.63	0.95	0.99	0.94	0.70	0.97	0.94	0.99	1.00	0.98	0.98	0.98	0.97	0.91	0.96	0.97	0.97	0.99	0.94
ABTS vs. Fe%	0.73	1.00	1.00	1.00	0.99	1.00	1.00	0.98	0.99	0.98	0.98	1.00	0.97	0.98	0.99	0.99	0.99	0.99	0.87

The positive correlations discussed above between TPC vs. ABTS, TPC vs. %Fe and TPC vs. LDL shows that the radical scavenging capacity, metal chelation ability and inhibition of h-LDL oxidation of the extracts can be related to their content of phenolics.

Cereal grains are consumed in processed form and therefore, what is of importance is the presence of bioactive compounds of interest in the processed foods and their potential health-promoting properties. This section will therefore discuss the relationship between the phenolic acids and the antioxidant properties of the cookies with focus on the GIT pH extracts from the whole wheat, condensed tannin sorghum and the non-tannin sorghum. Specifically, the possible ways by which phenolic acids in the extracts may exert antioxidant effects and the significance of this in terms of potential health benefits will be explored.

Table 2.1.4 shows that ferulic acid (structure shown in Figure 3.2.1) was most prominent in the GIT pH extracts particularly from wheat cookies (86% of total phenolic acids) and non-tannin sorghum cookies (48% of total phenolic acids). It can therefore be hypothesized that ferulic acid could play a significant role in contributing to the observed antioxidant properties of the GIT pH extracts from the cookies reported in this research. Ferulic acid will therefore be used as an example to demonstrate the mechanisms by which it may exert antioxidant effects.

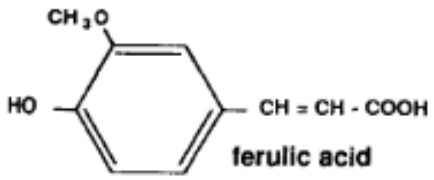

Figure 3.2.1 Structure of ferulic acid

(Nardini et al., 1995)

Antioxidant activity of phenolic acids is related to their structure, especially the substitutions on the aromatic ring. The antioxidant activity of phenolic acids is increased with an increase in the number of hydroxyl groups (-OH) on the molecule. For hydroxycinnamic acids such as ferulic acid, it is hypothesized that the conjugated double

bond in the side chain has a stabilizing effect on the resonance structure and this enhances the antioxidant activity of the aromatic ring (Dueñas et al., 2006; Rice-Evans et al., 1997).

Ferulic acid would be expected to exert antioxidant activity by free radical scavenging through an electron transfer mechanism. Figure 3.2.2 shows how ferulic acid could react with a free radical using the ABTS radical as an example.

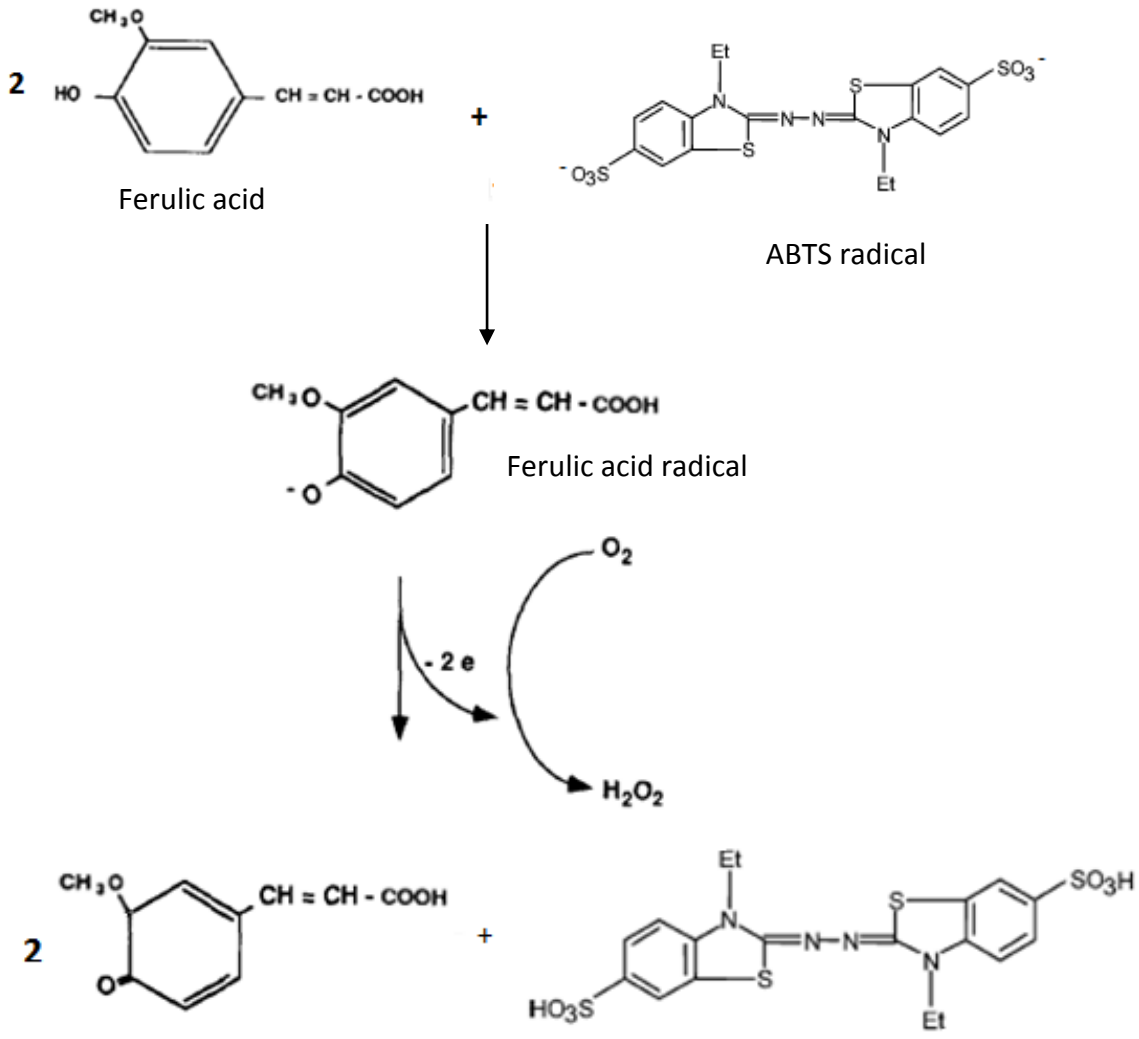

Figure 3.2.2 Interaction between ferulic acid and ABTS radical

(Adapted from Nardini et al., 1995; Prior et al., 2005)

In this reaction sequence, there is transfer of an electron from the ferulic acid to the ABTS radical and a ferulic acid radical is formed. The ferulic acid radical is stabilized by resonance because the unpaired electron can be delocalized around the aromatic ring and this is aided by the presence of conjugated double bond in the side chain (Figure 3.2.3).

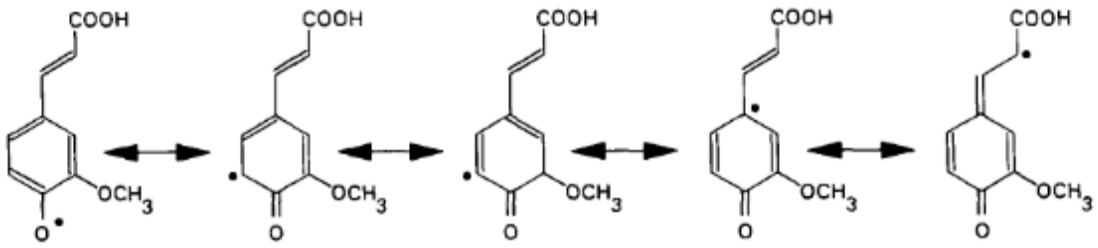

Figure 3.2.3 Resonance stabilization of ferulic acid radical (Graf, 1992)

Metal chelation is another mechanism by which phenolics exert antioxidant activity. According to Graf (1992) it is doubtful that ferulic acid forms iron chelates. Rather, its antioxidant potential arises from its ability to scavenge free radicals, and by absorbing the harmful effects of UV radiation, when present (Graf, 1992).

It has been shown that caffeic acid has much higher metal chelating properties than ferulic acid (Borges, Lima, Pinto, Reis & Siquet, 2003). This is due to the two hydroxyl groups (-OH) adjacent to each other on the aromatic ring in caffeic acid compared to only one phenolic hydroxyl group in ferulic acid. In relation to metal chelation, the significance of having two hydroxyl groups adjacent to each other on the aromatic ring is that they can act as co-ordination points for the metal ion to be chelated. An illustration of this is shown in the copper-caffeic acid complex in Figure 3.2.4 below.

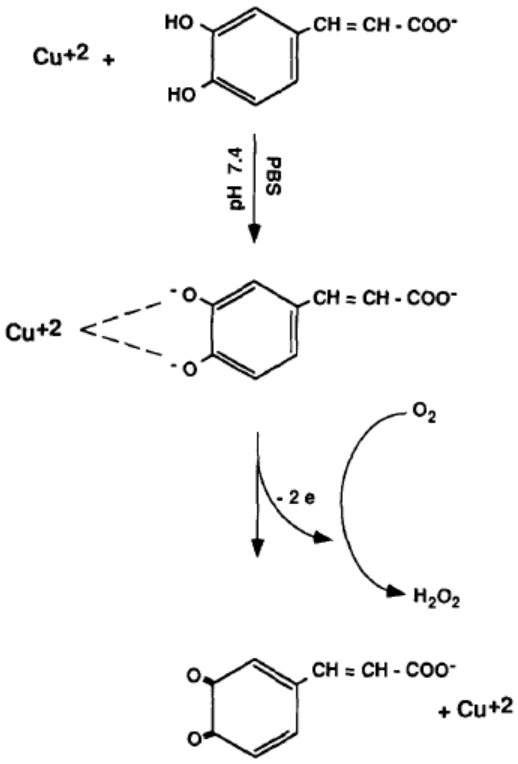

Figure 3.2.4 Schematic for copper-caffeic acid complex formation (Nardini et al., 1995)

From the results presented in this work, it may be proposed that the extracts studied could inhibit h-LDL oxidation through the two mechanisms discussed above – free radical scavenging and metal (i.e. Cu II metal ions) chelation and this by virtue of the presence of phenolic acids (such as ferulic acid and caffeic acid) in these extracts. According to Andreasen, Landbo, Christensen, Hansen and Meyer (2001) ferulic acid is a poor antioxidant in the inhibition of LDL even at levels twice that of caffeic acid. However, they also showed that high levels of ferulic acid retard the rate at which conjugated dienes are formed during h-LDL oxidation. Although the GIT pH extract from wheat cookies had the lowest caffeic acid content of the GIT pH cookie extracts, it had 5 and 8 times the ferulic acid content of non-tannin sorghum and condensed tannin sorghum, respectively. Taking the overall phenolic acid composition of the extracts into consideration, it may be suggested that caffeic acid, ferulic acid and other minor phenolic acids may act together to produce the observed antioxidant effects. Possible synergistic effects may not be ruled out.

This research has shown that aqueous extracts from sorghum and wheat cookies prepared under simulated gastro-intestinal pH conditions contain phenolic compounds (particularly phenolic acids) which may be responsible for antioxidant effects. This observation has

important implications for potential bioacessibility of dietary phenolics and their potential health promoting properties. These findings suggest that bioactive phenolic acids such as ferulic acid and caffeic acid could be potentially available for absorption in the gut after consumption of whole grain wheat or sorghum cookies. Upon absorption, these phenolic acids could act as free radical scavengers or metal chelators thus reducing the occurrence of oxidative stress and possibly any associated diseases. The inhibitory capacity against h-LDL oxidation of the extracts is of particular significance as it suggests that the bioactive phenolics in the cookies may contribute towards prevention or reduction in the occurrence of cardiovascular disease which is related to h-LDL oxidation.

CHAPTER 4

4. CONCLUSION AND RECOMMENDATIONS

Water extracts from whole wheat, condensed tannin and non-tannin sorghum flour and their cookies have higher total extractable phenolic content than corresponding GIT pH extracts. This suggests that the levels of extractable phenolics are closely related to the extraction methods and extraction medium used. As there is limited research available on aqueous extracts from cereals, more investigation is needed to examine trends in behaviour of aqueous extractable phenolic compounds. There is also a need for a standardized method of analysis for aqueous based extraction.

Water extracts from whole wheat, condensed tannin and non-tannin sorghum flour and their cookies have higher antioxidant activity (determined as free radical scavenging ability, metal chelation and inhibition of copper-mediated LDL oxidation ability) than the corresponding GIT pH extracts. Water and GIT pH extracts from the grain samples and cookies have similar phenolic acid profiles, containing the cinnamic acid derivatives caffeic acid, p-coumaric acid, ferulic acid with p-hydroxybenzoic acid as the only predominant benzoic acid derivative.

Water and GIT pH extracts from condensed tannin sorghum flour and cookies have higher total phenolic content and radical scavenging ability than corresponding extracts from wheat and tannin-free sorghum. The phenolic acid content of the condensed tannin sorghum samples are similar to the non-tannin sorghum samples. The wheat samples have the highest phenolic acid content, essentially due to the very high amount of ferulic acid in the wheat cookie samples. The antioxidant capacity, in terms of metal chelation and inhibition of copper mediated LDL oxidation ability, is the highest in the wheat sample. This may be due to the high levels of ferulic acid observed in the wheat samples.

Water and GIT pH extracts from the cookie samples have higher total phenolic content, phenolic acid content and metal chelation capability than the water and GIT pH extract of the flour samples. This is in accordance with generally observed trends in baked products due to Maillard browning and caramelisation.

The observed presence of phenolic acids in the GIT pH extracts of the cookies and their observed antioxidant properties is an indication of the potential health promoting properties of the cookie sample due to their phenolic content. Subjection of the cookies to simulated in vitro gastro-intestinal digestion processes using enzymes could enhance understanding of potential bioaccessibility and bioavailability of phenolics in the cookies and their health promoting properties.

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