

**Cowpea seed coats and their extracts:  
Phenolic composition and use as  
antioxidants in sunflower oil**

**Lethabo B Mokgope**

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Phenolic composition and use as  
antioxidants in sunflower oil**

**By**

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**Submitted in partial fulfilment of the requirements for the degree  
MInstAgrar: Food Production and Processing  
in the  
Department of Food Science  
Faculty of Natural and Agricultural Sciences  
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## DECLARATION

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

Lethabo B Mokgope

June 2006

## **DEDICATION**

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This dissertation is dedicated to papa and mama for their constant support and unconditional love. Thank you for teaching us what parental love is all about. You are the best...I love you very much!!! May the Lord bless you richly.

## ACKNOWLEDGEMENTS

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I wish to express my sincere gratitude and acknowledgements to the following:

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My nephew Seabi and nieces Lesego & Makgabo, thank you for putting a smile on my face...I love you very much guys. Makgabo keep dancing to the tunes...

My friends! You guys are amazing, thank you very much for your support and prayers. Like wine, let our friendship improve as time advances.

Above all to my heavenly Father, my Lord and saviour Jesus Christ whom I love and serve with all of my heart, thank you for giving me the strength and wisdom to complete my studies. I can do all things through Christ who strengthens me.

ABSTRACT

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**Cowpea seed coats and their extracts: Phenolic composition and use as antioxidants in sunflower oil**

**By**

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**Supervisor:** Dr K G Duodu

**Department:** Food Science

**Degree:** MInstAgrar: Food Production and Processing

Seed coats of cream-coloured *Bechuana white* and purple-coloured *Agriblue* cowpea varieties and the freeze-dried forms of their acetone extracts (CPE) were analysed for total phenol content using the Folin-Ciocalteu and Ferric Ammonium Citrate methods. The seed coats and CPE of both cowpea varieties were analysed for antioxidant activity using the Trolox Equivalent Antioxidant Capacity (TEAC) assay. HPLC (High Performance Liquid Chromatography) was done to determine the free phenolic acid composition of the CPE. The effect of the seed coats and CPE on the peroxide value (PV) of sunflower oil was determined during storage at 65°C over a 16-day period. The seed coats and CPE were added to the sunflower oil at concentrations of 1800 ppm and 900 ppm and their effect compared to that of the synthetic antioxidant tertiary butyl hydroquinone (TBHQ) added to the oil at 200 ppm.

Seed coats and CPE from *Bechuana white* cowpeas contained higher levels of total phenols than the corresponding samples of *Agriblue*. This differed from the generally observed trend that cowpeas with darker-coloured seed coat generally contain higher levels of total phenols than light-coloured cowpeas. More cowpea varieties need to be tested to confirm this observation.

The ABTS<sup>•+</sup> radical scavenging activity of the seed coats and CPE of *Bechuana white* cowpeas were higher than those of *Agriblue*. According to the HPLC chromatograms,

both cowpea varieties had similar types of phenolic acids namely, the cinnamic acid derivatives *p*-coumaric, ferulic and sinapic acids and the benzoic acid derivatives *p*-hydroxybenzoic acid, 3,4 dihydroxybenzoic, syringic, gallic and vanillic acid. These were present in higher amounts in *Bechuana white* than *Agriblue*. Phenolic acids derived from cinnamic acid tend to have higher antioxidant activity than benzoic acid derivatives. The higher antioxidant activity of *Bechuana white* seed coat acetone extracts and CPE was attributed to the presence of phenolic acids, especially the cinnamic acid derivatives in higher amounts in *Bechuana white* than *Agriblue*.

CPE and seed coats of both cowpea varieties reduced the formation of hydroperoxides in sunflower oil as shown by their lower peroxide values compared to oil without added antioxidant during storage. This antioxidant effect of the additives was attributed to the presence of phenolic compounds. The ability of the CPE and seed coats of both cowpea varieties to reduce formation of hydroperoxides in sunflower oil was dependent on their concentration in the oil. However, the seed coat and CPE of both cowpea varieties were not as effective as TBHQ in reducing the formation of hydroperoxides in the oil.

*Bechuana white* CPE and seed coats were more effective than *Agriblue* in reducing the formation of hydroperoxides. This was because *Bechuana white* had higher levels of total phenols and higher radical scavenging activity than *Agriblue*.

For both cowpea varieties, the seed coats were less efficient in retarding the formation of hydroperoxides than the CPE. This was because the extracted phenolic compounds in the CPE were in a freer form to diffuse in the oil to exert antioxidant effects compared to the unextracted seed coat.

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## CHAPTER 1

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### 1. INTRODUCTION AND LITERATURE REVIEW

#### 1.1 Introduction and statement of the problem

The cowpea (*Vigna unguiculata* L. Walp) is considered a grain legume or pulse (Uwaegbute, 1991) (legumes that store their energy in the form of starch or those that have small amounts of fat). It is believed to have originated in Africa and Asia (reviewed by Taiwo, 1998), and is widely cultivated in the tropics (Chavan, Kadam & Salunkhe, 1989). Other names commonly used for cowpeas include catjang, black-eyed bean or chinapea (reviewed by Taiwo, 1998). As a legume, cowpeas are rich and low-cost sources of dietary proteins and nutrients (Egounlety & Aworh, 2003) and they form part of a staple diet in most African and Asian countries (Aykroyd & Doughty, 1964).

Cowpeas have been consumed by humans since the earliest practice of agriculture (Phillips & Mcwaters, 1991) and they have various food uses in Africa and Asia. Traditionally, cowpeas are consumed as boiled vegetables using fresh or rehydrated seeds or processed into flour to make other food products. The seeds are decorticated before they are used. The reason for dehulling or decortication of cowpeas is to improve the appearance, texture, aroma and taste (Phillips & Mcwaters, 1991) and to reduce the cooking time (Tharanathan & Mahadevamma, 2003). After the seeds are decorticated, the seed coats (or hulls) are normally thrown away as waste.

A major limiting factor to the utilization of cowpeas as food is the presence of antinutritional factors such as trypsin inhibitors, oligosaccharides and phenolic compounds (Chavan, Kadam & Salunkhe, 1989). Phenolic compounds (tannins in particular) are an important group of such antinutritional factors. They are able to form complexes with food nutrients such as minerals and protein, thus rendering them less soluble or less susceptible to enzymatic degradation and less available for absorption (Towo, Svanberg & Ndossi, 2003). Processes such as dehulling, soaking, heating and fermentation are known to reduce the presence of the antinutritional factors (Vijayakumari, Siddhuraju, Pugalenti & Janardhanan, 1998; Egounlety & Aworh, 2003). However, phenolic compounds have a beneficial role as well. They are

naturally concentrated in the seed coat (Preet & Punia, 2000) where they play a major role in the physical and chemical defense system of the seeds when exposed to environmental factors such as oxidative damage and microbial infections thus contributing to antioxidant and antimicrobial activity (Troszynska, Estrella, Lopez-Amores & Hernandez, 2002).

Oxidation is a major cause of lipid deterioration in the edible oil processing industry. The exposure of lipids to factors such as air (presence of oxygen), light and high temperature causes oxidation reactions and results in the production of rancid odours and undesirable flavours thus reducing the shelf life of lipids and lipid-rich foods. Currently, the edible oil processing industry uses synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ) to retard lipid oxidation and prevent development of rancidity in edible oils (Frankel, 1996). In South Africa, TBHQ, BHA & BHT are currently used as synthetic antioxidants in fats and oils at a concentration of 200 mg/kg (Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972). However, there is concern about the safety and toxicity of synthetic antioxidants in relation to their metabolism and accumulation in body organs and tissues (Ito, Hirose, Fukushima, Tsuda, Shirai & Tatematsu, 1986; Lindenschmidt, Tryka, Goad & Witschi, 1986; Kahl & Kappus, 1993; Whysner, Wang, Zang, Iatropoulos & Williams, 1994; Frankel, 1996; Moure, Cruz, Franco, Dominguez, Sineiro, Dominguez, Nunez & Parajo, 2001; Malecka, 2002). Synthetic antioxidants are known among other things to cause impairment of blood clotting, lung damage and to act as tumor promoters (Kahl & Kappus, 1993). As a result of this, consumers have a preference for natural ingredients and there is a growing interest in the potential use of antioxidants from natural sources (Duh, Du & Yen, 1999). Phenolic extracts from herbs and spices (Abdalla & Roozen, 1999), cereals (Onyeneho & Hettiarachchy, 1992; Sikwese, 2005) and legumes (Onyeneho & Hettiarachchy, 1992) have been reported to effectively retard lipid oxidation in oils and fatty foods.

Cowpeas are processed and consumed extensively in developing countries and the large amounts of seed coats discarded as waste may be considered potential sources of phenolic compounds for application as natural antioxidants in foods. This will be of particular relevance to the edible oil processing industry where there is currently a

drive towards the use of more natural food ingredients. This will also benefit the rural communities where lipid oxidation is a problem.

## **1.2. LITERATURE REVIEW**

### **1.2.1 Cowpea – Production, physical characteristics and proximate composition**

Cowpeas (*Vigna unguiculata* L. Walp) are starchy legumes, which are grown in the tropical and subtropical regions of the world (Kochhar, Walker & Pike, 1988) including Africa and Asia (reviewed by Taiwo, 1998). Globally in the year 2003, about 12.4 million hectares of land is used to cultivate cowpeas, with Central and West Africa contributing about 8 million hectares (FAO Statistical Database, 2004). World cowpea production was 3,721,850 metric tons (Mt) during the year 2003, with Africa and Asia contributing 90% and 7.6% respectively (FAO Statistical Database, 2004).

The cowpea seeds are made up of cotyledons, germ and a seed coat with testa and hilum (Chavan *et al.*, 1989). The seeds may vary in colour, shape and size. The size and the shape range from 2 to 12 mm long and globular to kidney shaped, respectively (Chavan *et al.*, 1989). The seed coat colour ranges from white, purple to black. The seed composition, predominantly the proteins and starch varies considerably according to cultivar and seed origin (reviewed by Taiwo, 1998).

Cowpeas are a good source of proteins, minerals and energy. However, the nutrient content of cowpeas varies mainly because of genetic background as well as climate, fertilisation, season and agronomic practices (Kochhar *et al.*, 1988). Most of the nutrients are concentrated in the cotyledons as it makes up most of the seed weight. The proximate composition of cowpeas is shown in Table 1.1 (Chavan *et al.*, 1989).

**Table 1.1:** Proximate composition of cowpeas

Constituents	Range (%)
Crude Protein (N*6.25)	18.3-35.0
Crude Fat	0.7-3.5
Crude Fibre	2.7-7.0
Starch	31.5-48.0
Ash	2.5-4.9

### 1.2.2 Cowpeas – Food uses and factors limiting utilisation

Cowpeas are an important part of human diet in developing countries. Generally they are incorporated into a variety of local foods, weaning foods and snack foods in Africa and Asia (Giami, 2005). Traditionally, cowpeas may be cooked alone or processed into flour to make other food products. In Nigeria, whole cowpea seeds are cooked together with spices and palm oil to produce a thick bean soup known as *gbegiri* (Onyenekwe, Njoku & Ameh, 2000), which is eaten either alone or with yams, maize or rice (reviewed by Taiwo, 1998). Decorticated (seed coat removed) cowpea flour may also be mixed with chopped onions and spices, made into cakes and either deep-fried (*akara*) or steamed (*moin-moin*) in Nigeria (reviewed by Taiwo, 1998). Fried *dhals* made with cowpea flour are common in India (Tharanathan, & Mahadevamma, 2003). Generally cowpeas are decorticated to improve their acceptability by consumers (Phillips & Mcwaters, 1991) and to reduce the cooking time (Tharanathan & Mahadevamma, 2003).

Despite the good nutritional content of cowpeas, their consumption is limited due to the presence of antinutritional factors. The antinutritional factors found in cowpeas include inhibitors of enzymes such as trypsin, raffinose group of oligosaccharides and polyphenols (Chavan *et al.*, 1989). Trypsin inhibitor is known to inhibit the action of the enzyme trypsin. It does not necessarily interfere with the ultimate digestion of proteins but it may retard the liberation of the amino acid methionine from the protein (Richardson, 1977). Thus methionine cannot be used effectively for protein synthesis (Aykroyd & Doughty, 1964). Oligosaccharides are not digested by monogastric animals and they are thus fermented by microbes in the colon, which results in the production of flatus and other discomfort (Onyenekwe *et al.*, 2000). Phenolic compounds are able to form complexes with food nutrients such as minerals and



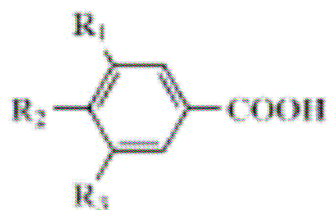
protein, thus rendering them less soluble or less susceptible to enzymatic degradation and less available for absorption (Towo *et al.*, 2003). However, despite the antinutritional activity of phenolic compounds, they also have a beneficial role in the seeds. They are concentrated in the seed coat (Preet & Punia, 2000) and they protect the seeds against oxidative damage and microbial infections because of their ability to act as antioxidants and antimicrobials (Troszynska *et al.*, 2002).

### **1.2.3 Chemistry of plant phenolic compounds**

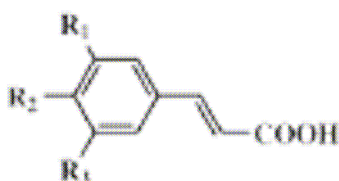
Phenolic compounds are defined as substances possessing a benzene ring bearing one or more hydroxyl substituents, including their functional derivatives (Waterman & Mole, 1994). There are different sources of phenols such as grapes, olive oil, sorghum, beans, spices and herbs (Moure *et al.*, 2001). Phenols have many favourable effects on human health. They decrease the risk of heart diseases by inhibiting the oxidation of low-density lipoproteins (LDL) (Bonilla, Mayen, Merida, & Medina, 1999). A large range of low and high molecular weight phenols exhibiting antioxidant properties have been studied and proposed to be used as antioxidants against lipid oxidation (Moure *et al.*, 2001). This is particularly true for those phenolics with multiple hydroxyl groups that are generally the most efficient for preventing lipid oxidation. Phenolic compounds are also known to possess antibacterial, antiviral, antimutagenic and anticarcinogenic properties (Moure *et al.*, 2001).

Phenolic compounds in plants may generally and conveniently be divided into three major classes based on their size. These are phenolic acids, flavonoids and tannins (Scalbert, Morand, Manach, & Remesy, 2002).

Phenolic acids are derivatives of benzoic acid and cinnamic acids with hydroxyl groups (OH) and methoxy (OCH<sub>3</sub>) groups substituted at various points on the aromatic ring (Marinova & Yanishlieva, 2003). Caffeic acid, ferulic acid, vanillic acid and syringic acid are all examples of phenolic acids (Pratt & Hudson, 1990). Structures of some phenolic acids are shown in Figure 1.1.

**Benzoic acid derivatives**

Name of acid	Position of the functional groups		
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Gallic	OH	OH	OH
<i>p</i> -Hydroxybenzoic	H	OH	H
Protocatechuic	OH	OH	H
Vanillic	OCH <sub>3</sub>	OH	H
Syringic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>

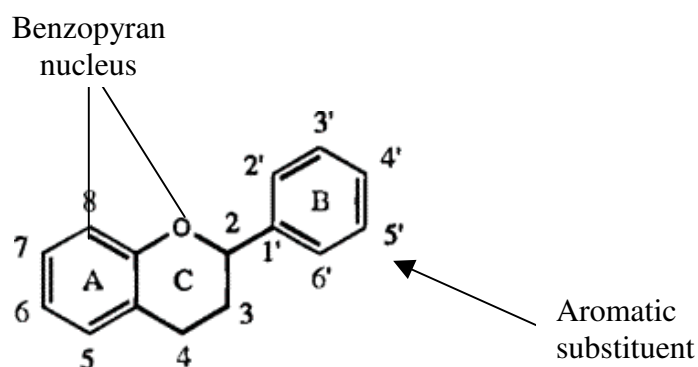
**Cinnamic acid derivatives**

Name of acid	Position of the functional groups		
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
<i>p</i> -Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH <sub>3</sub>	OH	H
Sinapic	OCH <sub>3</sub>	OH	OCH <sub>3</sub>

**Figure 1.1.** Structures of some phenolic acids (Naczka & Shahidi, 2004)

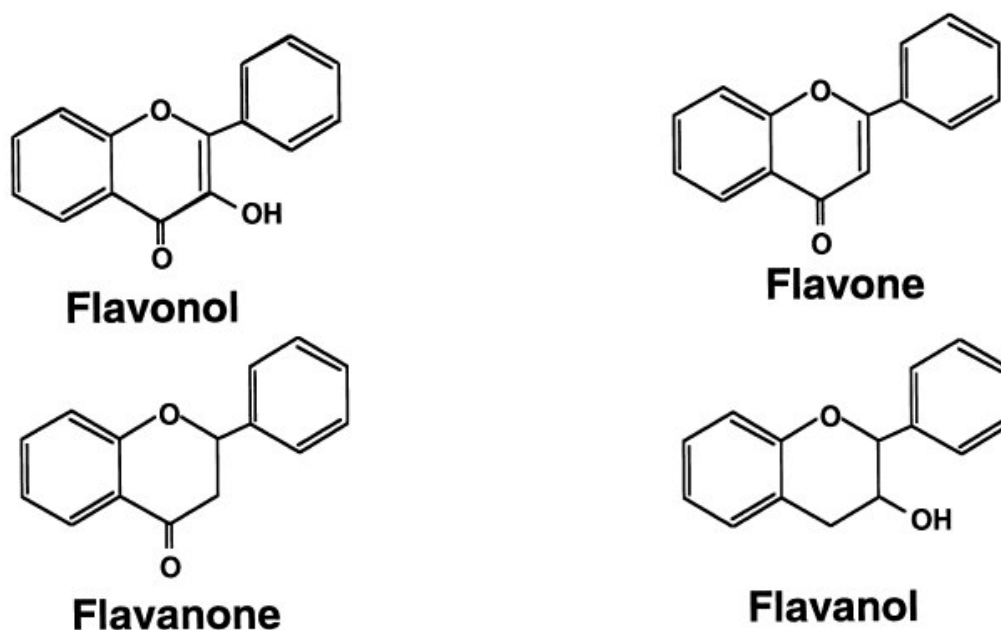
Flavonoids are a variety of phenolic compounds with a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> structural skeleton (Madhavi, Singhai & Kulkarni, 1996) The C<sub>3</sub>-C<sub>6</sub> is from cinnamic acid and the other C<sub>6</sub> fragment is from 3 molecules of malonyl-coenzyme A (Hahn, Rooney & Earp, 1984). The general flavonoid structure may be described as consisting of a

benzopyran nucleus with an aromatic substituent at carbon number 2 of the C ring (Figure 1.2) (Waterman & Mole, 1994).



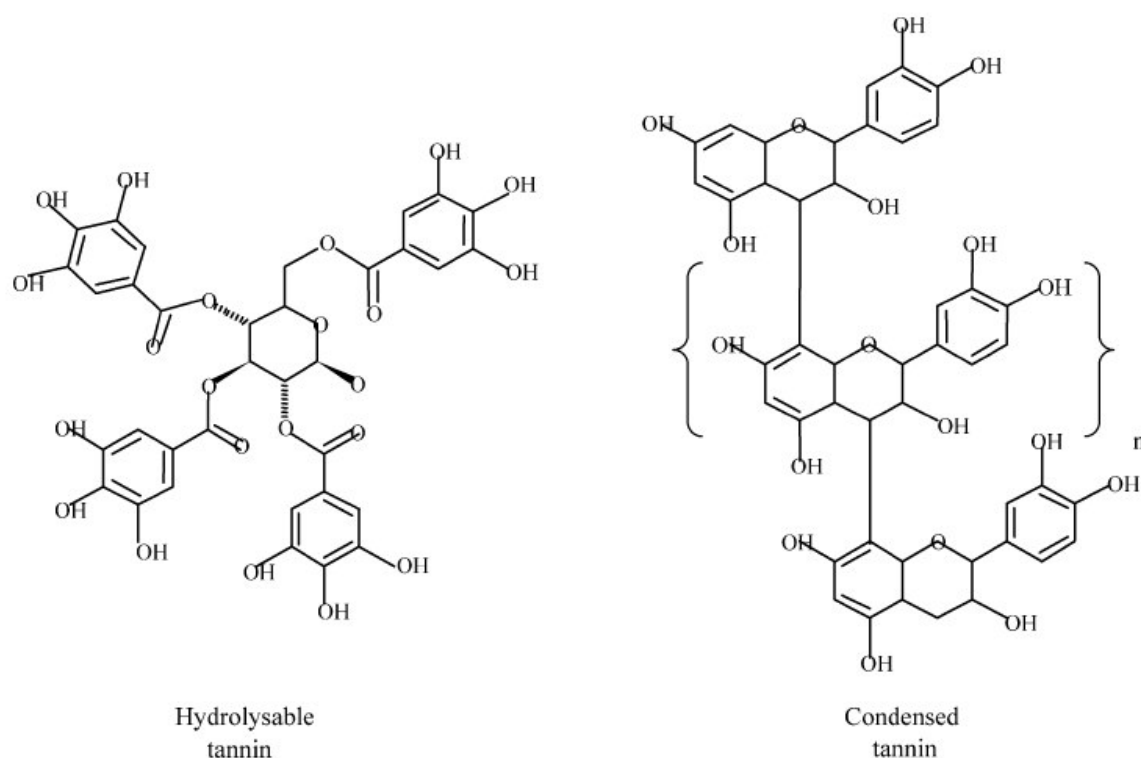
**Figure 1.2.** Basic structure of flavonoids (Sugihara, Arakawa, Ohnishi & Furuno, 1999)

Flavonoids are commonly found in edible fruits, leaves and other parts of plant foods as either glycosides (esterified to a sugar molecule) or aglycones (not esterified to a sugar molecule). The subgroups are classified based on the substitution pattern of the C ring and the position of the B ring. The major subgroups include flavonols, flavanones, flavanols (or flavans) and flavones (Figure 1.3). Flavonols (e.g. quercetin and kaempferol) have a carbonyl at C-4, double bond between C-2 and C-3, and hydroxyl at C-3; flavanones (e.g. taxifolin) have a carbonyl at C-4, no double bond between C-2 and C-3 and no hydroxyl at C-3; flavanols (e.g. catechin) have no carbonyl at C-4, no double bond between C-2 and C-3 and a hydroxyl at C-3 and flavones (e.g. apigenin and luteolin) have a carbonyl at C-4, a double bond between C-2 and C-3 and no hydroxyl at C-3 (Sugihara, *et al.*, 1999). Flavonones give rise to other family members such as anthocyanins by undergo a series of transformation that affects the heterocyclic ring (Cao, Sofic & Prior, 1996), which are responsible for the colour of fruits, legumes and vegetables (Mazza & Miniatti, 1993). Anthocyanins such as Delphinidin-3-O-glucoside, Peonidin-3-O-glucoside, Malvidin-3-O-glucoside and cyaniding-3-O-glycoside were previously reported in cowpeas (Chang & Wong, 2004).



**Figure 1.3.** Major flavonoid sub-groups (Sugihara *et al.*, 1999)

Tannins refer to substances of vegetable origin capable of transforming fresh hide into leather (Hahn *et al.*, 1984). Tannins are rich in phenolic hydroxyl groups. They are divided into two classes, namely: Hydrolysable tannins and Condensed (Non-hydrolysable) tannins (Waterman & Mole, 1994). Hydrolysable tannins are phenolic carboxylic acids esterified to sugars such as glucose. They are called hydrolysable tannins because they break down into sugars and a phenolic acid (gallic or ellagic acid) upon hydrolysis with acid, alkali or hydrolytic enzymes (tannase) (Hahn *et al.*, 1984). Condensed (Non-hydrolysable) tannins are polymers of flavan-3-ol units and are also known as proanthocyanins (or proanthocyanidins) (Butler, Riedl, Lebryk & Blytt, 1984) because they yield anthocyanins upon heating in acidic media (Santos-Buelga & Scalbert, 2000). The structures of condensed tannins and hydrolysable tannins are shown in Figure 1.4.



**Figure 1.4.** Structures of condensed tannins (proanthocyanidins), and hydrolysable tannins (Krause, Smith, Brooker & McSweeney, 2005).

#### 1.2.4 Levels of phenolic compounds in cowpeas

Most research into the phenolics of cowpeas also includes an estimation of total phenol content. Values reported for the total phenol content of cowpeas are highly variable. Factors such as the assay method and conditions (e.g. type of extraction solvent), type of standard used and type of cowpea sample used (e.g. variety, colour and maturity) all influence the levels of total phenols obtained (Chang, Collins, Bailey & Coffey, 1994; Nwokolo & Ilechukwu, 1996). Dark coloured seeds generally contain larger amounts of phenols than white or cream-coloured seeds (Nwokolo & Ilechukwu, 1996). Table 1.2 shows levels of total phenols obtained in whole grain of cowpeas reported by different authors.

**Table 1.2:** Levels of total phenols reported in cowpea whole grain

Cowpea sample	Assay	Amount (%)	Reference
Brown	Folin-Denis	0.78-0.93	Preet & Punia, 2000
Louisiana purple hull	Folin-Ciocalteu	0.35	Cai <i>et al.</i> , 2003
Dark red (acc 1222)	Protein precipitation	0.793	Laurena, Van Den & Mendoza, 1984
Black (acc 87)	Protein precipitation	1.03	Laurena, Van Den & Mendoza, 1984
IT81D-699 white	Vanillin-H <sub>2</sub> SO <sub>4</sub>	0.103	Giami & Okwechime, 1993
<i>Vigna aconitifolia</i>	Folin-Ciocalteu	1.36	Vijayakumari, Siddhuraju, Pugalenthi & Janardhanan, 1998

Cowpeas contain phenolic compounds in the three main groups mentioned above namely, flavonoids (quercetin, myricetin and kaempferol) (Lattanzio, Cardinali, Linsalata, Perrino, & Ng, 1997; Chang & Wong, 2004; Duenas, Fernandez, Hernandez, Estrella & Munoz, 2005), phenolic acids (coumaric, ferulic, caffeic, hydroxybenzoic, syringic, sinapic and protocatechuic acids) (Cai, Hettiarachchy & Jalaluddin, 2003; Sosulski & Dabrowski, 1984) and tannins (Morrison, Asiedu, Stuchbury & Powell, 1995; Lattanzio, Cardinali, Linsalata, Perrino, & Ng, 1997; Egounlety & Aworh, 2003). These phenolic compounds are mainly concentrated in the seed coat (Preet & Punia, 2000). Cai *et al.* (2003) analysed 17 cowpea varieties and observed that protocatechuic acid was the major bound phenolic acid. Analyses of tannins in cowpeas have been done using specific methods for condensed tannins such as the vanillin-HCl method (Chang *et al.*, 1994; Morrison *et al.*, 1995; Oigiangbe & Onigbinde, 1996; Oluwatosin, 1999; Egounlety & Aworh, 2003). There does not seem to be any report in the literature on the presence of hydrolysable tannins in cowpeas. It may be that as is the case with sorghum (reviewed by Awika & Rooney, 2004), the tannins present in cowpeas may be of the condensed type. Table 1.3 below shows levels of specific phenolic compounds reported in cowpeas.

**Table 1.3:** Levels of specific phenolic compounds reported in cowpeas

Sample	Phenolic compound	Amount	Reference
	<b>Phenolic acids</b>		
Whole grain	Protocatechuic acid	0.4-3.6 mg/100 g	Cai <i>et al.</i> , 2003
Whole grain	Caffeic acid	0.1-1.0 mg/100 g	Cai <i>et al.</i> , 2003
Whole grain	Ferulic acid	0.6-6.2 mg/100 g	Cai <i>et al.</i> , 2003
Seed coat	Protocatechuic acid	0.8 mg/100 g	Sosulski & Dabrowski, 1984
Seed coat	Syringic acid	1.3 mg/100 g	Sosulski & Dabrowski, 1984
	<b>Flavonoids</b>		
Seed coat	Quercetin	4 mg/100g	Chang & Wong, 2004
Whole grain	Kaempferol 3-O-glycoside	19 mg/100g	Chang & Wong, 2004
Seed coat	Myricetin 3-O-glucoside	24 mg/100g	Chang & Wong, 2004
Whole grain	Quercetin 3-O-glycoside	1.145 mg/100g	Duenas <i>et al.</i> , 2005
	<b>Tannins</b>		
Seed coat	Condensed tannin	413mg/100g catechin equivalent	Egounlety <i>et al.</i> , 2003
Cotyledon	Condensed tannin	1.9 mg/100 g tannic acid equivalents	Oigiangbe & Onigbinde, 1995
Whole grain	Condensed tannin	177.4 mg/100 g catechin equivalent	Chang <i>et al.</i> , 1994

### 1.2.5 Phenols as antioxidants: Mechanisms and structure-activity relationships

Generally the efficacy of phenolic compounds as antioxidants depends on a number of factors such as the number of hydroxyl groups bonded to the aromatic ring, the site of bonding, mutual position of hydroxyls in the aromatic ring (Sroka & Cisowski, 2003) and their ability to act as hydrogen or electron donating agents and free radical scavengers. All polyphenols are capable of scavenging singlet oxygen and alkyl radical through electron donating properties, thus generating a relatively stable phenoxyl radical (Santos-Buelga & Scalbert, 2000).

A relationship exists between the efficacy of phenolic compounds as antioxidants and their chemical structure. The configuration and total number of hydroxyl groups substantially influence the mechanism of antioxidant activity (Heim, Tagliaferro & Bobilya). The phenolic ring with hydroxyl groups are the main structural features required for antioxidant activity. In order for phenolic compounds to act as antioxidants, their hydroxyl groups have to be in free form. This is because the attachment of an external group to the hydroxyl groups reduces the antioxidant power of the phenolic compounds as they lack hydrogen atom for donation (Farag, El-Baroty, & Basuny, 2003).

Flavonoids are known to stabilise radicals by donating hydrogen and electrons from the hydroxyl groups in the B-ring to hydroxyl, peroxy and peroxy nitrite radicals, thus giving rise to relatively stable flavonoid radicals (Cao, Sofic & Prior, 1997.) Flavonoids therefore generally function as primary antioxidants and superoxide radical anion scavengers. The aglycones are more effective than glycosides. The position and the degree of hydroxylation of the B ring determine the antioxidant activity of flavonoids (Madhavi *et al.*, 1996). Flavonoids are also known to have the ability to sequest (or chelate) and thus reduce the activity of oxidative inducing metals such as copper and iron (Soleas, Diamandis & Goldberg, 1997).

Tannins inhibit lipid oxidation by scavenging the initial free radicals or the lipid peroxy radicals. They are also excellent chelators of metals ions such as copper and iron (Soleas *et al.*, 1997).

The antioxidant activity of phenolic acids depends on the degree of hydroxylation. The derivatives of cinnamic acids are generally more effective than the derivatives of



benzoic acid (Marinova & Yanishlieva, 2003). The presence of the CH=CH-COOH group in cinnamic acid derivatives ensures greater efficiency than the COOH group in benzoic acids (Madhavi *et al*, 1996). The double bond has been reported to participate in stabilising the phenoxyl radical by resonance (Cuvelier, Richard & Berst, 1992 according to Marinova & Yanishlieva, 2003). Phenolic acids are known to be scavengers of oxygen species. The position of the hydroxyl groups in the aromatic ring is important in the efficiency of phenolic acids as antioxidants (Sroka & Cisowski, 2003). For phenolic acids for instance, the presence of OH group in the *para* position is important for high antioxidant activity (Pannala, Razaq, Halliwell, Singh, & Rice-Evans, 1998; Pannala, Chan, O'Brien, & Rice-Evans, 2001). For flavonoids, structural features such as the attachment of the 3-OH group to the 2,3 double bond and adjacent to the 4-carbonyl in the C ring (Rice-evans, Miller, & Paganga, 1997), a 3,4 dihydroxy arrangement in the B ring and the meta 5,7 dihydroxy arrangements in the A ring (Rice-Evans Miller, & Paganga, 1996) are important for high antioxidant activity.

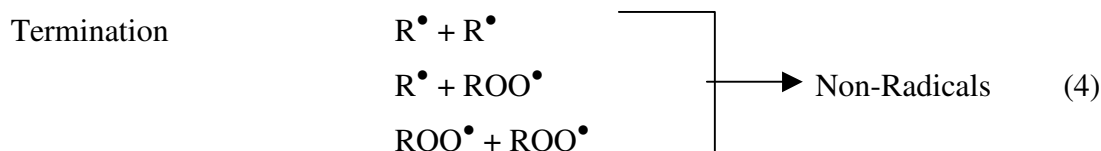
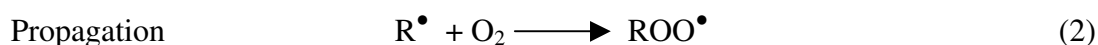
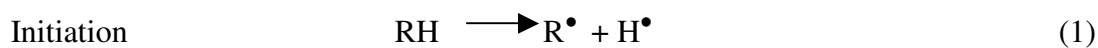
#### **1.2.6. Oxidation of lipids**

Lipid oxidation is a major cause of food quality deterioration during storage of oils, fats and other fat-containing foods (Yagi, 1990). Oxidation of lipids results in changes that may affect the nutritional quality, wholesomeness, colour, flavour and texture of food (Shahidi & Wanasundara, 1992). Moreover, the products of lipid oxidation may be potentially toxic and may lead to adverse effects such as the production of carcinogens, mutagenesis and aging (Yagi, 1990).

Autoxidation occurs when molecular oxygen reacts with unsaturated lipids. The process involves a free radical chain reaction that is most frequently initiated by exposure of unsaturated lipids to light, heat, ionizing radiation, metal ions or metallo-protein catalysts (Shahidi & Wanasundara, 1992). Free radicals are defined as any chemical species having one or more unpaired electrons (Hamilton, Kalu, Prisk, Padley & Pierce, 1997).

### 1.2.6.1 Mechanism of lipid oxidation

Lipid oxidation can be divided into three stages, namely: Initiation (production of lipid free radicals), Propagation, and Termination (production of non-radical products).



**Figure 1.5.** Stages of lipid oxidation

#### 1.2.6.1.1 Initiation

The initiation reactions result in the formation of free radicals that are small numbers of highly reactive molecules with unpaired electrons. These are shown in Figure 1.5.  $\text{R}^\bullet$  and  $\text{H}^\bullet$  represent an alkyl free radical and a hydrogen free radical respectively (Coultae, 1996).

The production of the first few radicals during the initiation stage is necessary to start the propagation stage. The first few radicals normally occur by some catalytic means (Nawar, 1996). The reactions that give rise to the first few radical species involve the short-lived but highly reactive, high -energy form of oxygen known as singlet oxygen ( $^1\text{O}_2$ ). Singlet oxygen molecules arise from low energy, ground state oxygen (triplet oxygen,  $^3\text{O}_2$ ). The triplet oxygen is converted to singlet oxygen by photosensitization of natural pigments such as chlorophyll and riboflavin present in foods (Nawar, 1996; Coultae, 1996). Metal ions may also be involved in activating molecular oxygen to produce singlet oxygen (Shahidi & Wanasundara, 1992). Lipid hydroperoxy radicals can be formed by the reaction of the lipid with molecular oxygen in its excited singlet state ( $^1\text{O}_2$ ), or by metal catalysts or exposure to light (Shahidi & Wanasundara, 1992).

Different initial radicals are produced by different initiation processes, e.g. photolysis, singlet O<sub>2</sub>, etc (Wheatley, 2000).

#### 1.2.6.1.2 Propagation

During the propagation stage, free radicals formed during initiation are converted into other radicals. The radicals are highly reactive and they undergo propagation reactions to produce lipid peroxide radicals by reacting with atmospheric triplet oxygen or by abstracting a proton from the  $\alpha$  position adjacent to a double bond in a lipid molecule. The radical-oxygen reaction occurs rapidly because it requires almost zero activation energy. The reaction results in the formation of hydroperoxy radical (ROO<sup>•</sup>), whose concentration increases and becomes higher than that of the alkyl radical (R<sup>•</sup>) in oxygen-containing food systems (Shahidi & Wanasundara, 1992; Madhavi, Deshpande & Salunkhe, 1996). The resulting lipid peroxide radicals propagate the chain by abstracting more hydrogen from lipid molecules (Madhavi *et al.*, 1996; Wheatley, 2000).

The lipid hydroperoxy radicals (ROO<sup>•</sup>) enter into chain reactions with other molecules and produce lipid hydroperoxides (ROOH) and other lipid free radicals. The lipid hydroperoxides are the primary products of autoxidation and have no taste and odour (Nawar, 1996). The repetition of this reaction produces an accumulation of hydroperoxides. The reaction will continue as long as there are unsaturated lipids or fatty acid molecules still available. Lipid hydroperoxides can also be formed by the reaction of unsaturated fatty acids with oxygen in the singlet-excited state or by the action of lipoxygenase (Wheatley, 2000).

The reaction pathway is usually accompanied by a shift in the position of double bonds because of the resonance stabilization of R<sup>•</sup> species, thus resulting in the formation of isomeric hydroperoxides that often contain conjugated diene groups (Shahidi & Wanasundara, 1992).

#### 1.2.6.1.3 Termination

Free radicals are chemically unstable because they are considered to be bonding deficient. They restore to normal bonding by trying to react whenever it is possible. As a result they are highly reactive (Jadhav, Nimbalkar, Kulkarni & Madhavi, 1996).

A decrease in the amount of unsaturated lipids or fatty acids causes the radicals to bond with one another, thus forming stable non-radical compounds (Madhavi *et al.*, 1996). Radical coupling is a process with low enthalpy of activation but the occurrence of termination reactions is controlled by the concentration of radicals as well as stereochemistry that causes radicals to collide with the correct orientation (Shahidi & Wanasundara, 1992; Madhavi *et al.*, 1996).

The importance of termination reactions is indicated by the formation of polymers in edible oils heated to elevated temperatures. Decomposition of hydroperoxides occurs readily and spontaneously at 160°C and the concentration of peroxy radicals becomes relatively high under these conditions, thus leading to the formation of polymers (Madhavi *et al.*, 1996). Compounds such as alcohols, aldehydes, alkyl formates, ketones and hydrocarbons are also produced by the decomposition of hydroperoxides (Coupland & McClements, 1996). These are referred to as secondary oxidation products. The carbonyl compounds in particular are responsible for off-flavour development or rancidity in oils (Frankel, 1984). Lipid oxidation is a major problem in the edible oil industry. However, antioxidants can be used to retard or delay the onset of lipid oxidation in oils.

### **1.2.7 Antioxidants in food**

Antioxidants are a group of chemicals capable of extending the shelf life of food that contain lipids (Madhavi *et al.*, 1996). They are used to retard the development of unpleasant flavour caused by the oxidation of unsaturated fatty acids (Tsaliki, Lagouri, & Doxastakis, 1999). They retard oxidation of lipids by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Shahidi & Wanasundara, 1992). Currently the food industry uses synthetic antioxidants such as BHA, BHT and TBHQ to retard lipid oxidation (Shahidi & Wanasundara, 1992).

Regulatory laws are used to govern the use of antioxidants in food products. According to the South African regulations, most synthetic antioxidants are allowed at a concentration of 200 mg/kg in fats and oils (Foodstuffs, Cosmetics & Drug, Act 54 of 1972). Antioxidants should meet the following requirements before they are used in fatty food products (Madhavi *et al.*, 1996):

- They should be safe.

- They should not impart colour, odour or flavour.
- They should be effective at low concentrations.
- They should be soluble in fat.
- They should be stable in the finished product (carry through effect).

#### 1.2.7.1 Classification of antioxidants according to mechanism of action

Food antioxidants may be classified on the basis of their function as primary, synergistic and secondary antioxidants (Rajalakshmi & Narasimhan, 1996).

##### 1.2.7.1.1 Primary antioxidants

These antioxidants are believed to retard oxidative reactions by donating hydrogen to free radicals from their phenolic hydroxyl groups, thereby converting them to stable radicals (Madhavi *et al.*, 1996). Antioxidants such as BHA, BHT, TBHQ and gallates, are examples of this group (Rajalakshmi & Narasimhan, 1996). These antioxidants are also known as synthetic antioxidants (Shahidi & Wanasundara, 1992)

Primary antioxidants either delay or inhibit the initiation step by reacting with free radicals or the propagation step by reacting with hydroperoxy or alkoxy radicals ( $L^\bullet$ ,  $LO^\bullet$ ,  $LOO^\bullet$ ).

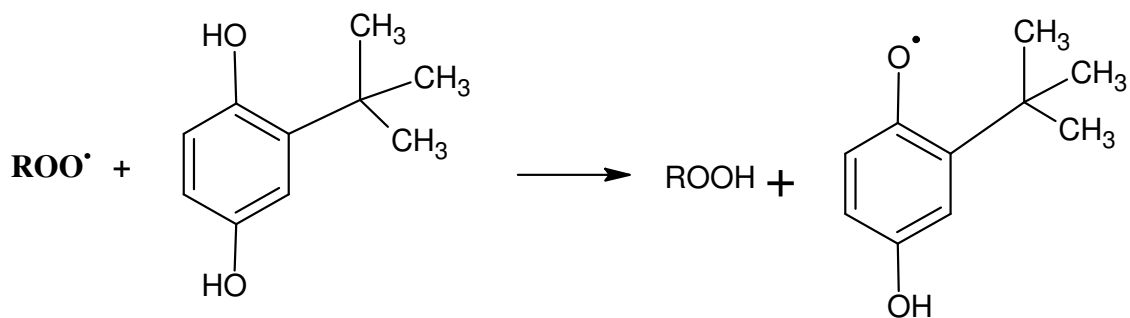


The resulting antioxidant free radical ( $A^\bullet$ ) continues to interfere with the propagation reactions through the formation of stable peroxy antioxidant compounds (LOOA, LOA) (Shahidi & Wanasundara, 1992; Rajalakshmi & Narasimhan, 1996).



Where, AH is the antioxidant molecule and  $A^\bullet$  is the antioxidant free radical.

The antioxidant free radical is relatively stable due to resonance delocalisation. It also lacks positions suitable for attack by molecular oxygen. Figure 1.6 shows resonance stabilisation in the TBHQ radical.



**Figure 1.6.** Resonance stabilization in the TBHQ radical

#### 1.2.7.1.2 Secondary antioxidants

This group can be classified broadly as oxygen scavengers or chelators. They act as reducing agents by transferring hydrogen atoms (Dziezak, 1986). Ascorbic acid, ascorbyl palmitate and Vitamin E, act as oxygen scavengers by reacting with free oxygen and removing it in a closed system (Gordon, 1990). Chelators such as citric acid and phosphates are not antioxidants as such but they effectively act as synergists with both primary and oxygen scavengers. The chelating action is promoted by the presence of an unshared pair of electrons in their molecular structures. They form stable complexes with pro-oxidant metals such as iron and copper (Dziezak, 1986; Rajalakshmi & Narasimhan, 1996).

Although primary and secondary antioxidants are currently used to retard lipid oxidation, there is concern about their safety and accumulation in the body (Frankel, 1996). This has led to the exploration of the potential use of phenolic compounds from plant sources as antioxidants in edible oils.

#### 1.2.8 Use of phenolic extracts from plant sources as antioxidants in lipids

As a result of concern for the safety of synthetic antioxidants (Ito, Hirose, Fukushuma, Tsuda, Shirai & Tatematsu, 1986; Lindenschmidt, Tryka, Goad & Witschi, 1986; Kahl & Kappus, 1993; Whysner, Wang, Zang, Iatropoulos & Williams, 1994; Frankel, 1996; reviewed by Moure *et al.*, 2002), there is a growing interest in the potential use of phenolic extracts from plant sources as antioxidants in lipids. The potential use of phenolic extracts from different plant sources such as sorghum, herbs and spices have been studied extensively (reviewed by Moure *et al.*, 2001). Extracts of spices such as rosemary and sage are now available commercially

for use as natural antioxidants (Bandoniene, Pukalskas, Venskutonis & Gruzdiene, 2000).

Sage extracts at a concentration of 0.02% in rapeseed oil during accelerated oxidation storage conditions were found to be more effective as antioxidants than BHT at the same concentration (Bandoniene *et al.*, 2000). The antioxidant activity may be due to phenolic compounds in the extracts, as they are able to donate hydrogen atoms from their phenolic hydroxyl groups. Phenolic compounds which are responsible for the antioxidant activity of sage extracts have been identified as rosmanol, carnosol and carnosic acid (Madsen & Bertelsen, 1995). According to Aruoma, Halliwell, Aeschbach, & Loligers (1992), these compounds contain orthodihydroxyl groups in the aromatic ring thus they possess good peroxy and hydroxyl radical scavenging activity.

Hras, Hadolin, Knez & Bauman (2000) found that the addition of rosemary extracts lowered the final peroxide value of sunflower oil after 11 days from 200 (control sample), to 120 meq/kg. The ability of the extracts to retard lipid oxidation could be due to the presence of phenolic compounds. The phenolic compounds responsible for the antioxidant activity of rosemary extracts have been identified as carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol and methyl carnosate (Cuvelier, Berset & Richard, 1994; Thorsen & Hildebrandt, 2003).

De Leonardis, Macciola & Di Rocco (2003), found phenolic extracts from sunflower seeds to be more effective than BHA in stabilising cold-pressed sunflower oil at 30°C. Sunflower seeds are known to contain phenolic compounds such as chlorogenic acid and smaller quantities of caffeic acid, cinnamic and coumaric acid, which possess antioxidant activity (De Leonardis, Macciola & Di Rocco, 2003).

Phenolic extracts from olive fruits were found to retard the oxidation of sunflower oil (Farag *et al.*, 2003). Compounds such as oleuropein, catechin and tyrosol, verbascoside and dialdehydic form of oleuropein aglycone have been identified in olive extracts and could contribute to antioxidant activity (Del Rio, Baidez, Botia & Ortuno, 2003)

Ginger extracts were also found to greatly inhibit oxidation of sunflower oil (Rehman, Salariya & Habib, 2003). A significant difference was not found between the ability of synthetic antioxidants and ginger extracts to retard sunflower oil oxidation. The principal active phenolic compounds in ginger extracts have been identified as gingerols and shogaols (Kikuzaki & Nakatani, 1993; Zancan, Marques, Petenate & Meireles, 2002).

Navy bean hull extracts inhibited oxidation of sunflower and soy oil (Onyeneho & Hettiarachchy, 1991). The extracts had a higher antioxidant effect than the combined effect of BHT, BHA & Rosemary AR. The antioxidant activity of the extracts could be due to the presence of phenolic compounds in the extracts.

Durum wheat bran extracts exhibited strong antioxidant activity when compared to the control (oil without additive) (Onyeneho & Hettiarachchy, 1992). Phenolic acids such as ferulic, vanillic, syringic, caffeic and p-coumaric acid were identified in the durum wheat bran extracts. These compounds act together in exerting antioxidant effects in soy oil (Onyeneho & Hettiarachchy, 1992).

Sorghum phenolic extracts inhibited oxidation of sunflower oil (Sikwese, 2005). The extracts were able to retard oxidation because of the ability of phenolic compounds to scavenge and stabilise lipid radicals by donating hydrogen atoms (Sikwese, 2005).

### **1.2.9 Analytical methods for phenols, antioxidant activity and oil oxidative quality**

#### **1.2.9.1 Analytical methods for phenols**

Different methods for analysis and quantification of phenolic compounds have been developed. They may be classified into two groups: those that analyse total phenol content and those that target a specific group of phenolic compounds. Methods such as the Folin- Ciocalteu phenol assay (Singleton & Rossi, 1965) and the Ferric ammonium citrate method (ISO, 1988) are employed in quantification of total phenol content (as total reducing phenolic groups) whereas methods such as the vanillin-HCl assay (Price, Van Scoyoc & Butler, 1978) are specific for catechins and proanthocyanidins. A number of factors are known to influence analysis of phenolic compounds. These factors include the chemical nature of the phenolic compounds,



extraction methods, sample particle size and the assay method itself (Naczk & Shahidi, 2004).

#### *1.2.9.1.1 Folin-Ciocalteu method*

The Folin-Ciocalteu method (Singleton & Rossi, 1965) quantifies the total concentration of phenolic hydroxyl groups present in the sample being assayed (Waterman & Mole, 1994). The assay is based on a reduction-oxidation reaction during which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman & Mole, 1994). The method is simple however, it is not specific and it detects all phenolic groups in extracts including those found in extractable proteins (Waterman & Mole, 1994). Moreover, it is susceptible to interference with reducing substances such as ascorbic acid (Naczk & Shahidi, 2004). Notwithstanding these demerits, the Folin-Ciocalteu method is widely used and provides a reasonably good and reliable estimate of the concentration of total reducing phenolic groups.

#### *1.2.9.1.2 Ferric ammonium citrate method*

The Ferric Ammonium Citrate method (ISO 1988) is based on the ability of phenolic compounds in alkaline conditions to reduce ferric ion to ferrous. Under these conditions the reaction results in the formation of a blue-green colour. The absorbance of the reaction products at 525 nm is linearly related to concentration of the phenolic compounds (Daiber, 1975). The Ferric ammonium citrate method is not specific as it not only responds to phenols but also other reducing agents such as ascorbate (Beta, Rooney, Marovatsanga & Taylor, 1999). It is also not very sensitive especially at low tannin concentrations (Deshpande, Cheryan & Salunkhe, 1986). However, it is simple (Deshpande, Cheryan & Salunkhe, 1986) and offers advantages for samples such as sorghum where it distinguishes between condensed-tannin containing and condensed-tannin free sorghum types (Daiber, 1975). The method also gives a reasonably good estimation of total reducing phenolic groups and exhibits similar trends in values as the Folin-Ciocalteu method.

#### *1.2.9.2 Analytical methods for antioxidant activity*

Antioxidants may function either by scavenging free radical species that promote oxidation or by preventing formation of products of lipid oxidation such as peroxides

and carbonyl compounds. Most assays for antioxidant activity are based on the ability of the antioxidant to perform the above-mentioned functions. The Trolox equivalent antioxidant capacity (TEAC) assay measures the free radical scavenging ability of the antioxidant (Rice-Evans, Miller & Paganga, 1996; Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003a). To determine the power of an antioxidant in oil, its ability to prevent formation of peroxides (by using the peroxide value assay) or aldehydes (by using the anisidine value or thiobarbituric acid value assays) may be determined (Antolovich, Prenzler, Patsalides, McDonald & Robards, 2002). The peroxide value, anisidine value and thiobarbituric acid value assays may also be used to determine the oxidative quality or stability of oils. (Guillen & Cabo, 2002).

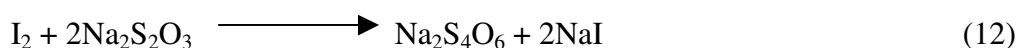
#### *1.2.9.2.1 Trolox equivalent antioxidant capacity (TEAC) assay*

The TEAC assay measures the ability of an antioxidant to scavenge free radicals generated either in the aqueous or lipophilic phase (Awika *et al.*, 2003a). The method involves targeting specific radicals such as hydroxyl radicals, superoxide radical, (Antolovich *et al.*, 2002) or the ABTS<sup>•+</sup> radical cation (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). For ABTS<sup>•+</sup>, the antioxidants reduce the radical depending on the antioxidant activity, concentration of the antioxidant and duration of the reaction. The extent of discolouration as percentage inhibition of ABTS<sup>•+</sup> is determined as a function of concentration and time, which is calculated relative to the reactivity of Trolox, a water-soluble vitamin E analogue as a standard under the same conditions (Re *et al.*, 1999). The method is preferred for its simplicity and speed of analysis. It can be used over a wide pH range and can be used to study effects of pH on antioxidant mechanism (Lemaska, Szymusiak, Tyrakowska, Zielinski, Soffer & Rietjens, 2001). Moreover, the ABTS radical is soluble in both aqueous and organic solvents and is not affected by ionic strength, thus it can be used in multimedia to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Prior, Wu & Schaich, 2005). A drawback to the use of this method is that the end-point detection varies and has been based on measurement of fluorescence inhibition, oxygen uptake and absorbance (Antolovich *et al.*, 2002).

#### *1.2.9.2.2 Peroxide value*

This technique measures the initial products of lipid oxidation. It is based on the principle that hydroperoxides (the primary products of oil oxidation) in the presence

of acetic acid act as oxidizing agents and liberate iodine from potassium iodide (reactions 10 and 11), (Christian, 1986). The amount of iodine liberated is measured titrimetrically using standard thiosulphate solution (reaction 12) and is directly proportional to the amount of hydroperoxides in the sample. The peroxide value is usually expressed in terms of milliequivalents of peroxides per kilogram of fat (AOCS Method Cd 8-53, 1985).



In this case, the antioxidant activity of the substance of interest is judged by its ability to prevent formation of hydroperoxides or lower the peroxide value. Limitations to this method include poor sensitivity and selectivity, possible addition of iodine across unsaturated bonds leading to low results, oxidation of iodide by dissolved oxygen and variations in reactivity of different peroxides (Antolovich *et al.*, 2002).

### 1.2.10 Gaps in knowledge

Despite the enormous information available on the use of different plants as sources of phenolic compounds, there is limited data available on cowpea phenolics and their antioxidant activity. There is lack of information on the effect of variety on the phenolics of cowpea and thus subsequently their antioxidant activity.

The use of cowpea phenolics in food systems still needs to be investigated. For instance, there is no information on the use of cowpea phenolics to retard oxidation of edible oil. Generally, most studies on the antioxidant activity of phenolic compounds have been carried out on extracted phenols. There is a need for an investigation into how the antioxidant activity of cowpea seed coat flour compares to that of extracted phenolic compounds when incorporated into a lipid food system such as vegetable oil. In rural areas, vegetable oils are used extensively and it is important to find cheap ways of stabilizing these oils against oxidation to prolong their shelf-life. People in rural areas do not necessarily have the means to prepare phenolic extracts from

cowpeas to be used as antioxidants in oil. It will be of great benefit if cowpea seed coats (readily obtainable in cowpea-consuming rural areas) could be used directly to stabilize oils against oxidation.

### 1.3 Hypotheses

- Due to varietal differences, the types and levels of phenolic compounds in the seed coats of *Bechuana white* and *Agriblue* cowpea varieties would differ and this will affect their antioxidant power.
- Due to their ability to act as reducing agents, phenolic compounds in the seed coats and in crude phenolic extracts from seed coats of *Bechuana white* and *Agriblue* cowpea varieties can be used as antioxidants in sunflower oil to retard formation of primary oxidation products, specifically hydroperoxides.
- The effectiveness as antioxidants in sunflower oil of seed coats or crude phenolic extracts from seed coats of *Bechuana white* and *Agriblue* cowpea varieties would differ due to differences in the extent of dispersion of phenolic compounds within the oil.

### 1.4 Objectives

#### Primary Objective

- To study and compare the effects of seed coats and crude phenolic extracts from seed coats of *Bechuana white* and *Agriblue* cowpea varieties on the oxidative stability of sunflower oil.

#### Secondary Objectives

- To determine the types and levels of phenols in *Bechuana white* and *Agriblue* cowpea varieties and their relation to antioxidant activity.
- To determine the effect of incorporation of seed coats and crude phenolic extracts from seed coats of *Bechuana white* and *Agriblue* cowpea varieties on formation of primary oxidation products (Peroxide Value) in sunflower oil in comparison with the synthetic antioxidant, TBHQ.

## CHAPTER 2

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### 2 RESEARCH

#### 2.1 Phenolic content and antioxidant activity of *Bechuana white* and *Agriblue* cowpea seed coats and their freeze-dried, crude phenolic extracts

##### 2.1.1 Abstract

Seed coats of cream-coloured *Bechuana white* and purple-coloured *Agriblue* cowpea varieties and the freeze-dried forms of their acetone extracts (CPE) were analysed for their total phenol contents using the Ferric Ammonium Citrate and Folin-Ciocalteu methods, free phenolic acid content using reverse-phase high performance liquid chromatography (HPLC) and antioxidant activity using the Trolox Equivalent Antioxidant Capacity assay. Contrary to the general trend that darker coloured cowpea varieties contain higher levels of phenols, the seed coat and CPE of cream-coloured *Bechuana white* had higher levels of total phenols than corresponding samples of the purple-coloured *Agriblue*. *Bechuana white* seed coat and CPE also had a higher antioxidant activity (free radical scavenging) than the seed coat and CPE of *Agriblue*. HPLC chromatograms showed that both cowpea varieties had similar types of phenolic acids namely, the cinnamic acid derivatives *p*-coumaric, ferulic and sinapic acids and the benzoic acid derivatives *p*-hydroxybenzoic acid, 3,4 dihydroxybenzoic, syringic, gallic and vanillic acid. However, these phenolic acids were present in higher amounts in *Bechuana white* than *Agriblue*, and may explain the higher free radical scavenging capacity of *Bechuana white* than *Agriblue*. For both cowpea varieties the free radical scavenging activity of the seed coat was lower than that of the CPE.

Keywords: Cowpea, Phenolic compounds, Seed coat and Antioxidant Activity.

## 2. 1.2 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp) is a legume that is widely cultivated in the tropics. It originates in Africa and Asia (Chavan, Kadam & Salunkhe, 1989). Cowpeas are a member of the starchy legumes, which are also known as pulses. Factors such as seed cultivar or variety and origin affect properties such as seed colour (ranging from white to purple to black) and seed composition (reviewed by Taiwo, 1998).

Cowpeas are consumed in different forms. In India, they may be cooked alone or processed into flour to make fried *dhals* (Tharanathan & Mahadevamma, 2003). In Nigeria they are either cooked alone and eaten with yam or maize or processed into a paste, which is either fried (*akara*) or steamed (*moin-moin*) or cooked with spices and condiments into bean soup (*gbegiri*) (Onyenekwe, Njoku & Ameh, 2000). During processing, the seeds are decorticated or dehulled (which is the removal of the seed coat) and the seed coats are then thrown away as waste. The purpose of dehulling is to improve the appearance, texture, aroma and taste (Phillips & Mcwaters, 1991) and to reduce the cooking time (Tharanathan & Mahadevamma, 2003) of the cowpeas.

The presence of antinutritional factors limits the utilization of cowpeas and legumes in general. Phenolic compounds are one group of some of the important antinutritional factors in cowpeas (Egounlety & Aworh, 2003; Giami, 2005). They are considered antinutrients due to their ability to form insoluble complexes with food nutrients such as proteins and minerals thus rendering these nutrients unavailable for absorption (Towo, Svanberg & Ndossi, 2003). However, recent research into phenolic compounds has focused on their beneficial characteristics such as bioactive properties. Phenolic compounds are concentrated in the seed coat (Preet & Punia, 2000) and play a major role in the physical and chemical defence system of the seeds when exposed to environmental factors (Troszynska, Estrella, Lopez-Amores & Hernandez, 2002). They protect against oxidative damage and thus can contribute to antioxidant activity (Troszynska *et al.*, 2002). Phenolic compounds are also reported to possess antibacterial, antiviral, antimutagenic and anticarcinogenic properties (Moure, Cruz, Franco, Dominguez, Sineiro, Dominguez, Nunez & Parajo, 2001).

The presence of various phenolic compounds in cowpeas is well known. These include flavonoids (e.g. quercetin, isorhamnetin and kaempferol) (Prakash & Joshi, 1979), phenolic acids (e.g. coumaric acid, ferulic acid, caffeic acid, hydroxybenzoic acid and protocatechuic acid) (Cai, Hettiarachchy & Jalaluddin, 2003) and tannins (Lattanzio, Cardinali, Linsalata, Perrino, & Ng, 1997). Various values have been reported for the total phenol and tannin content of cowpeas. Different standards are used and this makes comparison of the reported values difficult.

Cowpeas are consumed extensively in developing countries and where dehulling of the seeds is conducted, large amounts of seed coats are discarded as waste. These seed coats may therefore be considered potential sources of phenolic compounds for application as natural antioxidants in foods. The objective of this work was to determine the types and levels of phenolic compounds in seed coats of *Agriblue* (purple-coloured) and *Bechuana white* (cream-coloured) cowpea varieties and their antioxidant activities as measured by free radical scavenging.

### **2.1.3 Materials and methods**

#### 2.1.3.1 Materials

##### *2.1.3.1.1 Cowpea samples*

Two cowpea varieties, *Bechuana white* (cream-coloured) and *Agriblue* (purple-coloured) were obtained from Agricol in Potchefstroom, South Africa. The cowpeas were stored in a cold room at 4°C until required.

##### *2.1.3.1.2 Reagents for analysis*

Acetone was obtained from Radchem laboratory supplies (Johannesburg, South Africa); Folin-Ciocalteu reagent, sodium carbonate (anhydrous), potassium persulphate, 2,2'-Azinobis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich (Steinheim, Germany); tannic acid was obtained from Merck (Johannesburg, South Africa); di-sodium hydrogen orthophosphate (anhydrous) was obtained from Saarchem-Holpro Analytic (Pty) Ltd (Johannesburg, South Africa) and Sodium dihydrogen orthophosphate (anhydrous) was from Associated Chemical Enterprises (Pty) Ltd (Johannesburg, South Africa).

#### *2.1.3.1.3 Preparation of cowpea seed coats*

The seeds were conditioned by adding 186 g of water to 1000 g cowpea seeds for 2 h at room temperature. The initial moisture content of the cowpeas was 11 % and conditioning increased the moisture content to 25 %. The amount of water to be added in order to increase the moisture content of the cowpeas to 25 % was determined by performing a mass balance. After conditioning the seeds were dried overnight at 50°C. The purpose of the conditioning step was to make the seed coats friable to facilitate dehulling. The seeds were dehulled for 1.5 min using a rotary attrition dehuller (Sprecher+ Schuh IP65, France). The seed coats were then collected and milled using a laboratory hammer mill (Falling Number AB, Huddinge, Sweden) fitted with a 500 µm screen. The milled seed coats were vacuum packaged in polyethylene bags and stored in a box in a cold room at 4°C until further use.

#### *2.1.3.1.4 Preparation of freeze-dried crude phenolic extracts (CPE) from cowpea seed coats*

Milled cowpea seed coats (20 g) were extracted using 80 ml of 75% aqueous acetone (Kaluza *et al.*, 1980). The mixture was vortex mixed every 10 min for 2 h. This was followed by centrifuging at 3500 rpm for 6 min (25°C) using a Medifriger centrifuge (J.P Selecta, England). The sample residue was collected and re-extracted twice with 80 ml of the solvent while vortex mixing every 5 min for 10 min, centrifuged as above and decanted. The three supernatants were then mixed together. The extracts were concentrated in a Buchi Rotavapor RE 120 rotary evaporator (Laboratoriums Technik AG, Switzerland) at 35°C to remove acetone and freeze-dried to obtain the extracts in powder form. The yield of the freeze-dried extracts (soluble solids) from the cowpea seed coats was approximately 17.5% (*Agriblue*) and 20.0% (*Bechuana white*). The freeze-dried extracts were vacuum packaged in polyethylene bags, and stored at – 20°C.



### 2.1.3.2. Methods

#### 2.1.3.2.1 *Determination of total phenols of cowpea seed coats and CPE*

The cowpea seed coats were analysed for total phenol content using the Folin-Ciocalteu method (Singleton & Rossi, 1965) as described by Waterman and Mole (1994) and the Ferric Ammonium Citrate (FAC) method (ISO, 1988). Total phenol content of the CPE was analysed using the Folin-Ciocalteu method.

##### 2.1.3.2.1.1. Folin-Ciocalteu Method

The Folin-Ciocalteu method quantifies the total concentration of phenolic hydroxyl groups present in the sample being assayed (Waterman & Mole, 1994). The reaction is a reduction-oxidation in which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman & Mole, 1994).

The seed coats were extracted with 75% aqueous acetone (v/v) (Kaluza, McGrath, Roberts & Schroeder, 1980) at a sample-to-solvent ratio of 1:50 (w/v) for 2 h while vortex mixing every 5 min. After extraction, the suspension was centrifuged for 5 min at 4500 rpm. A 1 ml sample of the supernatant was mixed with 60 ml distilled water in a 100 ml volumetric flask. Then, 5 ml Folin-Ciocalteu reagent was added to the volumetric flask, followed by addition of 15 ml sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution (20 g/100 ml). This was thoroughly mixed and the volumetric flasks were then made up to 100 ml with distilled water. The flasks were then stoppered and mixed thoroughly by inverting several times. After incubation for 2 h at room temperature, the absorbance was read at 760 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). Tannic acid was used as a standard.

The CPE (0.02 g) was redissolved in 15 ml 75% aqueous acetone and 0.5 ml of the reconstituted sample was used to determine total phenols using the Folin-Ciocalteu method as described above.

##### 2.1.3.2.1.2. Ferric Ammonium Citrate Method

Under alkaline conditions, phenolic compounds reduce ferric ion ( $\text{Fe}^{3+}$ ) in the ferric ammonium citrate reagent to ferrous ion ( $\text{Fe}^{2+}$ ) (ISO, 1988). The absorbance of the

reaction products at 525 nm is linearly related to concentration of the phenolic compounds (Daiber, 1975).

Finely milled seed coats (0.25 g) were extracted with 5 ml dimethylformamide (DMF) for 1 h while vortex mixing every 5 min (Daiber, 1975). The suspension was centrifuged at 4500 rpm for 5 min. The sample residue was discarded and the supernatant was used to evaluate total phenols. The following reagents were mixed in a test tube: distilled water (5 ml), 1 ml of a solution consisting of 1% (w/v) Carboxymethylcellulose (CMC) and 0.2% (w/v) ethylenediaminetetraacetate (EDTA) in distilled water, 0.2 ml DMF extract or tannic acid (as a standard), 0.2 ml of 1.75% (w/v) FAC (Ferric Ammonium citrate) reagent and 0.2 ml of 28.8% (w/v) ethanolamine. After thoroughly mixing the contents, the test tubes were capped and allowed to stand for 30 min at room temperature. The absorbance was read at 525 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA).

#### 2.1.3.2.1.3. Reverse-phase high performance liquid chromatographic analysis (HPLC) of free phenolic acid composition of CPE

**Sample preparation:** The CPE (0.5 g) of each of the two cowpea varieties were re-dissolved in a 100 ml solution of acetonitrile: ultra fine distilled water acidified with *o*-phosphoric acid to pH 2.6 (10:90) and filtered using 0.45 µm PTFE filters. The filtered samples were then subjected to HPLC analysis in duplicate.

**HPLC conditions:** The HPLC apparatus used consisted of a Waters 1525 binary HPLC pump equipped with a Waters 2487 dual wavelength absorbance detector using Breeze™ software (Waters, Milford, MA, USA). Separation was achieved on a Phenomenex C18 reverse-phase column (250 mm × 4.6 mm, 100 Å pore size, 5-µm particle size) (Phenomenex, Torrance, CA, USA). Sample sizes of 20 µl were injected. The column was held at 25°C during the run and the flow rate was 1 ml/min. Elution of the free phenolic acids was monitored at 280 nm. The mobile phases used were solvent A, (ultra fine distilled water acidified with *o*-phosphoric acid to pH 2.6) and B, (acetonitrile). The gradient program was as follows: 93 to 80% solvent A in the first 20 min, 80 to 77% solvent A from 20 to 29 min, 77 to 20% A from 29 to 30 min, held isocratic at 20% A from 30 to 35 min and then increased to 93% A from 35 to 40 min.

**Standard calibration:** A set of standard solutions of concentrations 0.01, 0.1, 1, 10, 20, 50 and 100 ppm consisting of mixtures of the following phenolic acids: gallic, 3,4-dihydroxybenzoic, *p*-hydroxybenzoic, vanillic, syringic, caffeic, *p*-coumaric, ferulic and sinapic, were prepared in acetonitrile: ultra fine distilled water acidified with *o*-phosphoric acid to pH 2.6 (10:90). A 20- $\mu$ l aliquot of each standard solution was injected into the HPLC column. Calibration curves were obtained for each phenolic acid by plotting peak areas versus concentrations. Regression equations were obtained from the calibration curves for each individual phenolic acid. Identification of the phenolic acids was done by comparing the retention time of the unknown with those of the standard phenolic acids and further confirmed by spiking the unknown samples with authentic compounds.

**Calculation of phenolic acid contents:** Phenolic acid contents of the samples were calculated using the following equation:  $PA \text{ (mg/ 100 g CPE)} = [(y - c)/m](100/5)$   
Where PA = phenolic acid content, y = peak area of sample, c and m = y-intercept and slope for the individual phenolic acids respectively.

#### 2.1.3.2.2. Determination of antioxidant (free radical scavenging) activity

The free radical scavenging activity of the seed coats and CPE was determined using the Trolox Equivalent antioxidant capacity (TEAC) assay as described by (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003). TEAC measures the ability of antioxidants to scavenge the 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) ABTS<sup>•+</sup> radical cation chromogen. The reduced ABTS<sup>•+</sup> concentration by a certain amount of antioxidant is related to that of Trolox, the water-soluble vitamin E analogue and this gives the TEAC value of the antioxidant (Awika *et al.*, 2003). The ABTS<sup>•+</sup> radical cation was produced by mixing equal volumes of 8 mM ABTS and 3 mM potassium persulphate prepared using distilled water and allowed to react for 12 h at room temperature before use. A phosphate buffer solution (pH 7.4) prepared by mixing 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.2 M Na<sub>2</sub>HPO<sub>4</sub> and 150 mM NaCl, and 1 l of distilled water was used to dilute the ABTS<sup>•+</sup> solution. The ABTS<sup>•+</sup> solution (2.9 ml) was mixed with either 75% aqueous acetone extracts of cowpea seed coats (0.1 ml) and 75% aqueous acetone CPE (0.1 ml) or Trolox (dissolved in 75% aqueous acetone) (0.1 ml) and allowed to react for 30 min (samples) and 15 min (Trolox standard). The absorbance of the samples and standard was measured at 734 nm using a Lambda EZ150

spectrophotometer (Perkin Elmer, USA). The results were expressed as  $\mu\text{M}$  Trolox equivalents/g of sample, dry weight basis.

#### 2.1.3.2.3. Statistical Analysis

Duplicate samples were evaluated during each analysis and the experiment was repeated three times. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences between treatments. ANOVA was performed using STATISTICA program for Windows version 6.1. Least significant difference (LSD) tests were conducted to determine differences among means at  $p < 0.05$ .

#### 2.1.4. Results and discussion

**Table 2.1.1** Total phenol (TP) content of *Agriblue* and *Bechuana white* cowpea seed coats as determined by the Ferric Ammonium Citrate (FAC) and Folin-Ciocalteu (FC) methods

Sample	TP (FAC) <sup>1</sup>	TP (FC) <sup>1</sup>
<i>Agriblue</i>	50.2 a (1.36)	41.5 a (0.76)
<i>Bechuana white</i>	62.0 b (1.27)	60.1 b (1.80)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

<sup>1</sup>Expressed as mg tannic acid equivalents/g cowpea seed coat, on dry basis

**Table 2.1.2** Total phenol (TP) content of freeze-dried crude phenolic extracts (CPE) from *Agriblue* and *Bechuana white* cowpea seed coats as determined by the Folin-Ciocalteu method

Sample	TP <sup>1</sup>
<i>Agriblue</i>	433.3 a (0.80)
<i>Bechuana white</i>	558.5 b (0.12)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

<sup>1</sup>Expressed as mg tannic acid equivalents/g CPE on dry basis

Table 2.1.1 shows that the seed coats of the cream-coloured *Bechuana white* cowpea variety contained higher levels of total phenols than the purple-coloured *Agriblue* variety using both the Ferric Ammonium Citrate (FAC) and Folin-Ciocalteu (FC) methods. This observation appears to differ from the generally observed trend in literature where darker-coloured legume grains tend to contain higher concentrations of phenolic compounds than lighter-coloured grains. Chang, Collins, Bailey and Coffey (1994) reported higher concentrations of phenolic compounds in coloured cowpea varieties than the white varieties. The dark coloured seed coat of lima beans, pigeon peas, African yam bean and jackbean were also found to contain significantly higher tannin content than the lighter coloured seed coat (Oboh, Muzquiz, Burbano, Cuadrado, Pedrosa, Ayet & Osagie, 1998). The Folin-Ciocalteu method quantifies the total amount of phenolic hydroxyl groups present in the sample being assayed (Waterman & Mole, 1994) based on the ability of the phenolic hydroxyl groups to reduce the phosphotungstic-phosphomolybdate complex in the Folin-Ciocalteu reagent. The Ferric ammonium citrate method is based on the ability of the phenolic compounds in alkaline conditions to reduce Ferric ion ( $\text{Fe}^{3+}$ ) to Ferrous ion ( $\text{Fe}^{2+}$ ) (Daiber, 1975). Essentially, both methods quantify phenolic compounds based on their reducing power. The higher amount of total phenols in *Bechuana white* than *Agriblue* could therefore be because *Bechuana white* may contain some phenolic compounds that may not necessarily contribute to the colour of the seed coat, but with higher reducing power than the phenolic compounds found in *Agriblue*. Liquid chromatographic analysis was conducted on the CPE of *Bechuana white* and *Agriblue* cowpeas in order to determine their phenolic composition. The results are discussed later.

The levels of total phenols found in the the seed coats of the two cowpea varieties used in this study were 4.2 and 5.0% for *Agriblue* using the Folin-Ciocalteu and Ferric ammonium citrate methods respectively and 6.0 and 6.2% for *Bechuana white*. These are higher than reported levels for total phenols in whole cowpeas, which are of the order of 0.1 – 0.2% (Giami & Okwechime, 1993); 0.8 – 0.9% (Preet & Punia, 2000) and 0.03 – 0.4% (Cai *et al.*, 2003). The high phenol content in the seed coats is because phenolic compounds in legumes are known to be concentrated in the seed coat (Preet & Punia, 2000) where they play a major role in the physical and chemical defense system of the seeds when exposed to environmental factors such as oxidative

damage and microbial infections thus contributing to antioxidant and antimicrobial activity (Troszynska *et al.*, 2002).

Total phenol content reported for seed coats and whole seeds of other legumes also support this observation. Barroga, Laurena and Mendoza (1985) reported the total phenol content of whole mung beans as 0.4% and for mung bean seed coats, 3.4%. The total phenol contents of whole beans, dehulled beans and bean seed coats were 0.2%, 0.2% and 7.8% respectively (Cardador-Martinez, Loarca-Pina & Oomah, 2002). Other factors such as variety also have an effect on the levels of total phenols in cowpeas and other legumes (Morrison, Asiedu, Stuchbury, & Powel, 1995; King & Young, 1999; Cai *et al.*, 2003).

As shown in Table 2.1.2, preparation of freeze-dried extracts from the seed coats of the cowpeas concentrated the phenols further by a factor of about 10. Total phenol levels for the freeze-dried extracts (determined using the Folin-Ciocalteu method) were 43.3% for *Agriblue* and 55.9% for *Bechuana white* compared to 4.1% and 6.0% respectively for the seed coats. Similar results have been reported in sorghum by Sikwese (2005). Freeze-drying of a crude phenolic extract prepared from the bran of a condensed tannin sorghum (also with 75% aqueous acetone as extracting solvent) produced a yield of 10% soluble solids (compared to 17.5% for *Agriblue* and 20% for *Bechuana white*) and further concentrated the phenol content from 10% in the bran to about 58% in the CPE.

**Table 2.1.3** Antioxidant (free radical scavenging) activity of seed coats and freeze-dried crude phenolic extracts (CPE) from seed coats of *Agriblue* and *Bechuana white* cowpeas as determined by the Trolox equivalent antioxidant capacity (TEAC) assay

Sample	Seed coats ( $\mu\text{M TE}/100 \text{ mg}$ )	CPE ( $\mu\text{M TE}/100 \text{ mg}$ )
<i>Agriblue</i>	3.46 a (0.16)	21.10 a (1.05)
<i>Bechuana white</i>	4.40 b (0.66)	22.99 b (1.43)

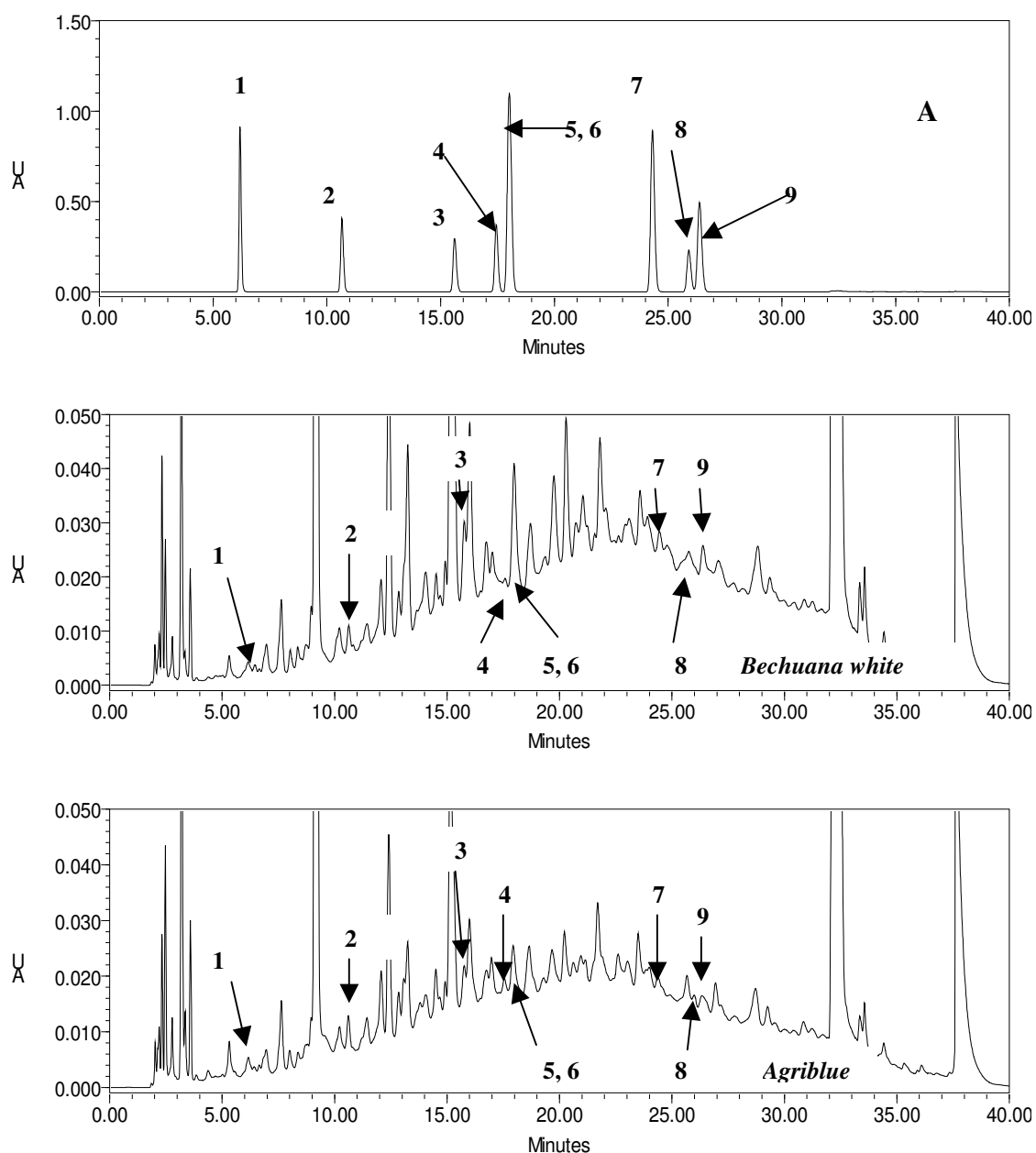
Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

TE, Trolox equivalents

Table 2.1.3 shows that the seed coats and CPE of *Bechuana white* (with higher contents of total phenols from Tables 2.1.1 and 2.1.2) had higher antioxidant activity as measured by radical scavenging capacity than *Agriblue*. The free radical scavenging activity results obtained in Table 2.1.3 reflects the observed levels of total phenols obtained in Tables 2.1.1 and 2.1.2. The higher the levels of total phenols, the higher the free radical scavenging activity. Phenolic compounds scavenge free radicals through hydrogen or electron donation from their phenolic hydroxyl groups (Murthy, Singh & Jayaprakasha, 2002). The number of hydroxyl groups in the aromatic ring influences the ability of the phenolic compounds to act as radical scavengers. Thus the higher the number of phenolic hydroxyl groups the greater the radical scavenging activity (Wang, Li, Rangarajan, Shao, LaVoie, Huang & Ho, 1998).

A number of studies have found high positive correlations ( $r > 0.9$ ) between levels of total phenols of various samples and antioxidant activity (Frankel, Waterhouse & Teissedre, 1995; Simonetti, Pietta & Testolin, 1997; Sun, Chu, Wu & Liu, 2002; Awika *et al.*, 2003; De Beer, Joubert, Gelderblom & Manley, 2003). However, in other cases, lower correlations have been reported between phenolic content of specific phenolic fractions (Cardador-Martinez *et al.*, 2002) or specific phenolic compounds (Frankel *et al.*, 1995) and antioxidant activity. This suggests that though phenolic compounds in general may be responsible for antioxidant activity, this is dependent on the types of phenolics found in the sample (Rice-Evans, Miller & Paganga, 1996; Larrauri, Sanchez-Moreno & Saura-Calixto, 1998; Sroka & Cisowski, 2003). Antioxidant activity of phenolic compounds is influenced by their chemical structures (Rice-Evans, Miller & Paganga, 1996).



**Figure 2.1.1** HPLC chromatograms of phenolic acid standards (100 ppm) (A), and freeze-dried crude phenolic extracts (CPE) of *Bechuana white* and *Agriblu* cowpea varieties. Detector was set at 280 nm for the standards and CPE.

1 – Gallic acid; 2 – 3,4-dihydroxybenzoic acid; 3 – *p*-hydroxybenzoic acid; 4 – Vanillic acid; 5, 6 – Syringic and Caffeic acid\*; 7 – *p*-coumaric acid; 8 – Ferulic acid; 9 – Sinapic acid.



\* Syringic and caffeic acid eluted very close to each other under the gradient conditions used. In the samples, the peaks numbered 5, 6 are reported as syringic acid.

Figure 2.1.1 shows the HPLC chromatograms of the phenolic acid standards and CPE of *Agriblue* and *Bechuana white* cowpea seed coat at 280 nm. The profiles of the chromatograms for both varieties were similar and showed the presence of various free phenolic acids (such as vanillic, caffeic, coumaric and sinapic acids). However, the identity of a number of peaks could not be established. The majority of phenolic acids exist in plants as structural components of the plant such as cellulose, proteins, lignin (Andreasen, Christensen, Meyer, Hansen, 2000; Lam, Kadoya, Iiyama, 2001) with only a minor fraction existing in the free form (reviewed by Robbins, 2003). The insoluble bound phenolic acids can only be extracted by organic solvent after saponification (Krygier, Sosulski, & Hogge, 1982; Maillard, & Berset, 1995; Andreasen, *et al.*, 2000; Liyana-Pathirana, & Shahidi, 2006) or by enzymatic treatment (Meyer, Jepsen, & Sorensen, 1998; Andreasen, Christensen, Meyer, & Hansen, 1999; Landbo, & Meyer, 2001) in order to break the ester linkages and release the phenolic acids (reviewed by Robbins, 2003). Therefore the identified phenolic acids in this work are predominantly free phenolic acids. In agreement with work done by Cai *et al.*, (2003), a very small proportion of cinnamic acid derivatives such as ferulic and *p*-coumaric acids may occur in free form and may be extracted with organic solvents such as methanol (or acetone in this work).

**Table 2.1.4** Free phenolic acid content of crude phenolic extracts (CPE) from seed coats of *Agriblue* and *Bechuana white* cowpeas as determined by HPLC

Phenolic acid	Retention time	<i>Bechuana white</i> (mg/100g CPE)	<i>Agriblue</i> (mg/100g CPE)
Gallic	6.14	17.0 a (2.69)	18.9 a (1.01)
3,4 DHBA	10.62	51.2 a (3.38)	36.4 b (5.09)
PHBA	15.74	197.2 a (1.39)	99.0 b (3.63)
Vanillic	17.56	94.7 a (2.81)	21.4 b (0.03)
Syringic	17.93	107.9 a (1.13)	50.6 b (2.52)
<i>p</i> -coumaric	24.39	65.9 a (4.61)	16.0 b (0.01)
Ferulic	25.79	72.0 a (0.45)	47.4 b (7.42)
Sinapic	26.34	120.5 a (10.4)	5.9 b (0.08)

Means within a row with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

3,4 DHBA (3,4 dihydroxybenzoic acid)

PHBA (*p*-hydroxybenzoic acid)

The free phenolic acid content of CPE from seed coats of *Bechuana white* and *Agriblue* as determined by HPLC are shown in Table 2.1.4. CPE from both cowpea varieties contained free phenolic acids belonging to the family of cinnamic acid derivatives (ferulic, *p*-coumaric and sinapic acids) and benzoic acid derivatives (vanillic, gallic, 3,4 dihydroxybenzoic, syringic and *p*-hydroxybenzoic acid). Even though the two-cowpea varieties had similar types of free phenolic acids, *Bechuana white* contained higher levels of these compounds than *Agriblue*. Of all the identified free phenolic acids, PHBA (*p*-hydroxybenzoic acid) was the most abundant phenolic acid in both cowpea varieties. Phenolic acids such as 3,4 DHBA (protocatechuic acid), syringic, ferulic acids have been previously reported in cowpeas (Sosulski & Dabrowski, 1984; Cai *et al.*, 2003). The levels of the phenolic acids reported in this study were higher than those reported in the literature. Cai *et al.* (2003) reported the levels of 3,4 DHBA and ferulic acid in the whole flour of various cowpea varieties to range between 0.4-3.6 mg/100g and 0.6-6.2 mg/100g respectively. Sosulski and Dabrowski (1984) reported levels of 3,4 DHBA and syringic acid in cowpea seed coat to be 0.8 and 1.3 mg/100g respectively. From Table 2.1.4, these occurred in much higher amounts. This is a further demonstration of the concentration effect of freeze-drying the extracts. In this study HPLC was performed on the CPE.

The HPLC chromatograms show that *Bechuana white* CPE contained higher levels of free phenolic acids belonging to the family of cinnamic acid derivatives and benzoic acid derivatives. Cinnamic acid derivatives generally have higher antioxidant activity than benzoic acid derivatives (Rice-Evans, Miller & Paganga, 1996). This is because the  $-\text{CH}=\text{CH}-\text{COOH}$  group in cinnamic acid derivatives brings about greater H-donating ability and greater radical stabilization compared to the carboxylate group in benzoic acid derivatives (Rice-Evans, Miller & Paganga, 1996). As a result, it would be expected that, based on their free phenolic acid content, CPE from *Bechuana white* would have higher reducing ability and radical scavenging capacity than CPE from *Agriblue*. This is in fact what was observed in Table 2.1.2 and 2.1.3. These observations suggest that phenolic extracts from darker-coloured cowpea varieties may not always have higher phenolic content and antioxidant activity than from

lighter-coloured cowpea varieties. The phenolic profile of an extract is more important in determining its antioxidant activity.

### 2.1.5 Conclusions

Seed coats and freeze-dried forms of their acetone extracts (CPE) prepared from the cream-coloured *Bechuana white* cowpea variety have higher levels of total phenols than corresponding samples of the purple *Agriblue* variety. This is contrary to the generally observed trend in literature where darker-coloured cowpea varieties are reported to contain higher levels of total phenols than lighter-coloured ones. The *Bechuana white* seed coats and CPE have a higher antioxidant (free radical scavenging) activity than the corresponding *Agriblue* samples, a similar trend to their levels of total phenols. HPLC shows that *Bechuana white* CPE contains higher levels of free phenolic acids with relatively higher antioxidant activities such as the cinnamic acid derivatives *p*-coumaric, ferulic and sinapic acids than *Agriblue* and explains the higher free radical scavenging capacity of *Bechuana white* CPE.

### 2.1.6. References

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## **2.2 Effect of *Bechuana white* and *Agriblue* cowpea seed coats and their crude phenolic extracts on the oxidative stability of sunflower oil**

### **2.2.1 Abstract**

The effect of seed coats and freeze-dried crude phenolic extracts (CPE) prepared from *Bechuana white* and *Agriblue* cowpeas on the oxidative stability of sunflower oil was determined by measuring the peroxide value of the oil at two-day intervals during storage at 65°C over a 16-day period. The seed coat and the CPE were added to the sunflower oil at concentrations of 1800 ppm and 900 ppm and their effect compared to that of the synthetic antioxidant tertiary butyl hydroquinone (TBHQ) added to the oil at 200 ppm. The seed coats and CPE from the two-cowpea varieties were able to reduce the formation of hydroperoxides in sunflower oil as shown by low peroxide values compared to the control (oil without additive). The seed coat and CPE of *Bechuana white* was more effective in reducing the formation of hydroperoxides in sunflower oil than their *Agriblue* counterparts. Even though the seed coats and CPE both cowpea varieties were able to retard lipid oxidation, their activity was much lower than that of TBHQ. This may be because the seed coats and CPE were added as crude extracts while TBHQ was used as a pure compound. The efficiency of the seed coat and CPE from both cowpea varieties in retarding sunflower oil oxidation was dependent on their concentration. The CPE of both cowpea varieties performed better than the seed coat in retarding oxidation of sunflower oil. This is because phenolic compounds in the seed coat are bound to cell wall components while those in the CPE have been extracted and so are in a freer form to exert their antioxidant effect.

Keywords: Cowpea, Phenolic extracts, Seed coat, Lipid oxidation, Antioxidant activity, Peroxide value, and Sunflower oil

### 2.2.2 Introduction

Lipid oxidation is a major cause of food spoilage (Yagi, 1990). Oxidation of lipids results in the production of rancid odours and undesirable flavours due to their exposure to environmental factors such as light, air and temperature (Shahidi & Wanasundara, 1992). The lipid oxidation process occurs in three stages that include initiation, propagation and termination (Madhavi, Singhal & Kulkarni, 1996; Stauffer, 1996). The process is initiated by free radicals during exposure to these environmental factors (Hamilton, Kalu, Prisk, Padley & Pierce, 1997). The oxidation process primarily produces hydroperoxides, which have no taste and odour. As the oxidation reaction proceeds, the hydroperoxides are broken down into carbonyls and other compounds, which are responsible for the rancid flavours in oxidised lipids (Gordon, 1991 according to Hras, Hadolin, Knez, & Bauman, 2000). Furthermore, lipid oxidation products are potentially toxic and may lead to adverse effects such as production of carcinogens, mutagenesis and ageing (Yagi, 1990).

In the edible oil industry, synthetic phenolic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertiarybutyl hydroquinone (TBHQ) are widely used to retard oxidation of lipids thereby increasing the shelf life of edible oil (Abdalla & Roozen, 1999). They retard oxidation of lipids by reacting with free radicals (Shahidi & Wanasundara, 1992) thereby interrupting the propagation phase of lipid oxidation (Birch, Fenner, Watkins & Boyd, 2001). Even though synthetic antioxidants are effective in retarding oxidation of lipids, there is concern about their safety and toxicity in relation to their metabolism and accumulation in body organs and tissues. There are several studies indicating increased mutagenesis and carcinogenesis associated with synthetic antioxidants (Ito, Hirose, Fukushuma, Tsuda, Shirai & Tatematsu, 1986; Lindenschmidt, Tryka, Goad & Witschi, 1986; Kahl & Kappus, 1993; Moure, Cruz, Franco, Dominguez, Sineiro, Dominguez, Nunez & Parajo, 2001; Malecka, 2002). As a result of this and consumer preference for natural food ingredients, there is a growing interest in the potential use of phenolic extracts from plant sources as natural antioxidants in lipids (Duh, Du & Yen, 1999). Several plant extracts exhibiting antioxidant properties have been studied and proposed to be used as antioxidants against lipid oxidation (Moure *et al.*, 2001).

The antioxidant activity of certain plant extracts such as rosemary extracts has been established and is available commercially (Birch *et al.*, 2001).

Cowpeas (*Vigna unguiculata* (L.) Walp) are an important part of the staple diet of many developing countries. (Aykroyd & Doughty, 1964). They are a rich and low-cost source of proteins (Egounlety & Aworh, 2003). Traditionally cowpeas are dehulled in order to improve acceptability by consumers (Phillips & McWaters, 1991) and reduce cooking time (Tharanathan & Mahadevamma, 2003). After dehulling, the seed coats are then thrown away as waste. Cowpeas contain phenolic compounds mainly in the seed coat (Preet & Punia, 2000), which possess antioxidant activity (Duenas, Fernandez, Hernandez, Estrella & Munoz, 2005). This antioxidant activity of phenolic compounds is due to their ability to donate hydrogen from their phenolic hydroxyl groups (Murthy, Singh & Jayaprakasha, 2002). Such phenolic compounds, which have been reported in cowpeas, include flavonoids (e.g. quercetin, isorhamnetin and kaempferol) (Prakash & Joshi, 1979), phenolic acids (e.g. sinapic, ferulic, hydroxybenzoic, syringic, caffeic and protocatechuic acids) (Cai, Hettiarachchy & Jalaluddin, 2003; Sosulski & Dabrowski, 1984) and tannins (Lattanzio, Cardinali, Linsalata, Perrino & Ng, 1997).

The seed coats produced during the processing of cowpeas may therefore be considered potential sources of phenolic compounds for use as natural antioxidants in edible oils. The objective of this study was to determine the effect of cowpea seed coats and their phenolic extracts on the oxidative stability of bulk sunflower oil compared to that of the synthetic antioxidant, TBHQ.

## **2.2.3 Materials and methods**

### 2.2.3.1 Materials

#### 2.2.3.1.1 Samples

- Sunflower oil (5 l) without added antioxidants was obtained from Continental Oil Mills (Johannesburg, South Africa). The 5 l bottle was flushed with nitrogen and stored in the cold room at 5°C until required.

- Two cowpea varieties, *Bechuana white* (cream-coloured) and *Agriblue* (purple-coloured) were obtained from Agricol in Potchefstroom, South Africa. The cowpeas were stored in a cold room at 4°C until required.

#### 2.2.3.1.2 Reagents

Acetone was obtained from Radchem laboratory supplies (Johannesburg, South Africa); acetic acid, chloroform, sodium thiosulphate, TBHQ, and soluble starch were obtained from Merck (Johannesburg, South Africa).

#### 2.2.3.1.3 Preparation of cowpea seed coats

Seed coats from *Bechuana white* and *Agriblue* cowpeas were prepared as described in section 2.1.3.1.3.

#### 2.2.3.1.4 Preparation of freeze-dried crude phenolic extracts (CPE) from cowpea seed coat

Freeze-dried crude phenolic extracts (CPE) from *Bechuana white* and *Agriblue* cowpeas were prepared as described in section 2.1.3.1.4.

#### 2.2.3.2. Methods

##### 2.2.3.2.1 Determination of the peroxide value

The oxidative stability of the sunflower oil during storage was determined before and after incorporation with the cowpea seed coats, CPE and TBHQ using the peroxide value method. The peroxide value is a measure of the concentration of peroxides in the oil and is used as an indicator of the initial stages of lipid oxidation (O'Brien, 2004). The assay is based on the principle that primary products of oil oxidation (peroxides) have the ability to liberate iodine from potassium iodide in equivalent amounts to the peroxides. The iodine is then titrated with standard sodium thiosulphate, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (Christian, 1986). The results are usually expressed in terms of milliequivalents of peroxides per kilogram of fat (AOCS Method Cd 8-53, 1985).

The seed coats and CPE were added to oil (150 g) at two concentrations, 900 and 1800 ppm (mg additive per kg oil) in 250 ml Schott glass bottles that were covered with aluminium foil in order to protect them from light. To facilitate dispersion, the seed coats and CPE were redissolved in 75 % aqueous acetone (approximately 1 ml) before being introduced into the sunflower oil. TBHQ as a standard was added at a concentration of 200 ppm (Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972) and oil without antioxidants was used as a control. The bottles were thoroughly mixed using a magnetic stirrer for 25 min before storing in a Labcon forced circulation oven type FSOE 16 (Labcon Pty Limited, Rooderport, South Africa) at 65°C for 16 days. The peroxide values of the oil samples were determined at 2-day intervals over the 16-day storage period and expressed as milliequivalent peroxide/kg oil. Prior to peroxide value determinations all samples were mixed for 5 min using a magnetic stirrer.

#### *2.2.3.2.2 Statistical Analysis*

Triplicate samples were evaluated during each analysis and the experiment was repeated three times. Analysis of variance (ANOVA) was used to determine whether there were significant differences between each treatments using STATISTICA program for Windows version 6.1 (Tulsa, Oklahoma, U.S.A, 2003). Least significant difference (LSD) tests were conducted to determine differences among means at  $p < 0.05$ .

## 2.2.4 Results and discussion

**Table 2.2.1:** Effect of cowpea variety on the peroxide value (expressed as milliequivalent peroxide/kg oil) of sunflower oil during storage at 65°C

Cowpea variety	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
<i>Bechuana white</i>	0.80 a (0.00)	1.96 a (0.09)	2.95 a (0.27)	4.13 a (0.75)	5.68 a (0.67)	7.18 a (0.56)	9.30 a (1.20)	11.20 a (1.35)	14.8 a (1.42)
<i>Agriblue</i>	0.80 a (0.00)	1.92 b (0.23)	3.11 b (0.09)	4.57 b (0.40)	6.04 b (0.61)	7.93 b (0.77)	9.99 b (0.98)	11.9 b (1.11)	15.4 b (1.41)
TBHQ	0.80 a (0.06)	1.07 c (0.12)	1.23 c (0.06)	1.43 c (0.06)	1.73 c (0.06)	1.93 c (0.12)	2.13 c (0.12)	2.27 c (0.06)	2.60 c (0.00)
Control	0.80 a (0.00)	2.23 d (0.06)	3.50 d (0.00)	5.20 d (0.00)	6.80 d (0.00)	9.27 d (0.06)	11.3 d (0.06)	14.4 d (0.00)	18.8 d (0.00)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

Table 2.2.1 shows the effect of *Bechuana white* and *Agriblue* cowpea varieties (seed coats and CPE) and TBHQ on the peroxide value of sunflower oil over the 16-day storage period. All the sunflower oil samples with additives (*Bechuana white*, *Agriblue* and TBHQ) showed activity against oxidation of sunflower oil as shown by their relatively lower peroxide values compared to the control oil, beginning especially from day 4. Sunflower oil with TBHQ stood out as the most stable sample with very low peroxide values throughout storage. Table 2.2.1 also shows that the *Bechuana white* cowpea variety was more effective in reducing formation of peroxides in sunflower oil than the *Agriblue* variety. By day 16, the peroxide value of sunflower oil samples containing *Bechuana white* (seed coat and CPE combined) was 14.8 milliequivalent peroxide/kg oil compared to 15.4 milliequivalent peroxide/kg oil for sunflower oil samples containing *Agriblue*.

The two cowpea varieties were able to reduce the formation of peroxides in sunflower oil (compared to the control) most likely due to the presence of phenolic compounds. The antioxidant activity of phenolic compounds is because of their ability to donate hydrogen atoms from their phenolic hydroxyl groups thus acting as radical scavengers and stabilizers (Frankel, 1996; Rice-Evans, Miller & Paganga, 1997; Naczki & Shahidi, 2004). The peroxide value assay is based on the principle that hydroperoxides in the presence of acetic acid act as oxidizing agents and liberate iodine from potassium iodide (Christian, 1986). The hydroperoxides are produced as the primary products of lipid oxidation. Lipid oxidation involves the formation of free radicals ( $R^\bullet$ ) by the reaction of atmospheric oxygen and the unsaturated lipid. The reaction is initiated by exposure of unsaturated lipids to light, irradiation, metal ions, heat, etc (Shahidi & Wanasundara, 1992; Nawar, 1996). The free radicals undergo reactions to produce hydroperoxy radicals ( $ROO^\bullet$ ) by reacting with atmospheric oxygen or by abstracting a proton from the  $\alpha$  position adjacent to a double bond in a polyunsaturated fatty acid. The lipid hydroperoxy radicals ( $ROO^\bullet$ ) enter into chain reactions with other molecules and produce lipid hydroperoxides (ROOH), which are measured in the peroxide value assay, and other lipid free radicals (Shahidi & Wanasundara, 1992; Jadhav, Nimbalkar, Kulkarni & Madhavi, 1996). The most probable mechanism by which the seed coats and CPE of the two cowpea varieties were able to reduce the formation of peroxides in sunflower oil (compared to the

control) could have been by donation of hydrogen atoms from their phenolic hydroxyl groups to free radical species such as hydroperoxy and alkoxy radicals (Madhavi *et al.*, 1996). This reduces the concentration of free radicals available for the propagation reactions that produce hydroperoxides (ROOH) and hence, lower peroxide values. In addition, antioxidant free radicals ( $A^\bullet$ ) are formed that could continue to interfere with the propagation reactions through the formation of stable peroxy antioxidant compounds (Shahidi & Wanasundara, 1992; Rajalakshmi & Narasimhan, 1996). The antioxidant free radical is relatively stable due to resonance delocalisation (Rajalakshmi & Narasimhan, 1996). It also lacks positions suitable for attack by molecular oxygen (Nawar, 1996). This explains the lower peroxides values of sunflower oil containing the two cowpea varieties (added as seed coats and CPE) compared to the control. Several workers have previously reported the antioxidant activity of plant phenolic extracts in edible oils (Onyeneho & Hettiarachchy, 1991; Onyeneho & Hettiarachchy, 1992; Bandoniene, Pukalskas, Venskutonis & Gruzdiene, 2000; Hras *et al.*, 2000; De leonardis, Macciola & Di Rocco, 2003; Farag *et al.*, 2003).

The lower effectiveness of the two cowpea varieties at reducing formation of hydroperoxides compared to TBHQ may be attributed to the fact that these additives were in a crude form while TBHQ was added as the pure compound. The two cowpea varieties (seed coat and CPE) were added to the oil in a crude form, without separating compounds with antioxidant activity from those with pro-oxidant activity, e.g. riboflavin and metal ions such as iron (Jadhav *et al.*, 1996), or no activity. Both riboflavin and iron are reported to be present in cowpea seed coats (Chavan, Kadam & Salunkhe, 1989).



Results presented in the previous chapter (2.1) showed that total phenol content of *Bechuana white* was 60.08 mg/g (seed coats) and 558.46 mg/g (CPE) compared to 41.49 mg/g (seed coats) and 433.30 mg/g (CPE) in *Agriblue*. Moreover, *Bechuana white* (22.99  $\mu$ M/100 mg) had higher free radical scavenging activity than *Agriblue* (21.10  $\mu$ M/100 mg) (section 2.1.4, Table 2.1.2 & 2.1.3). The HPLC results (section 2.1.4, Figure 2.1.1) of both cowpea extracts revealed that *Bechuana white* contained higher levels of free phenolic acids than *Agriblue*, which is a contributory factor to the higher free radical scavenging capacity of *Bechuana white*. Therefore sunflower oil samples containing both seed coats and CPE of *Bechuana white* would be expected to have relatively lower peroxide values than oil samples containing additives from *Agriblue*.

**Table 2.2.2:** Effect of concentration of *Bechuana white* seed coats and CPE on the peroxide value (expressed as milliequivalent peroxide/kg oil) of sunflower oil during storage at 65°C

Concentration	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
Seed coat 900 ppm	0.80 a (0.00)	2.00 a (0.00)	3.20 a (0.00)	4.83 a (0.06)	6.53 a (0.06)	8.00 a (0.00)	10.8 a (0.00)	12.9 a (0.06)	16.5 a (0.00)
Seed coat 1800 ppm	0.80 a (0.00)	2.00 a (0.06)	3.20 a (0.00)	4.67 b (0.06)	6.00 b (0.00)	7.00 b (0.00)	9.60 b (0.00)	11.6 b (0.01)	15.4 b (0.00)
CPE 900 ppm	0.80 a (0.00)	2.00 a (0.00)	2.80 b (0.00)	4.00 c (0.00)	5.40 c (0.00)	7.20 c (0.00)	9.20 c (0.00)	10.8 c (0.00)	14.4 c (0.00)
CPE 1800 ppm	0.80 a (0.00)	1.80 b (0.00)	2.59 c (0.01)	3.00 d (0.00)	4.80 d (0.00)	6.50 d (0.00)	7.60 d (0.00)	9.40 d (0.00)	12.8 d (0.00)
TBHQ	0.80 a (0.06)	1.07 c (0.12)	1.23 d (0.06)	1.43 e (0.06)	1.73 e (0.06)	1.93 e (0.12)	2.13 e (0.12)	2.27 e (0.06)	2.60 e (0.00)
Control	0.80 a (0.00)	2.23 d (0.06)	3.50 e (0.00)	5.20 f (0.00)	6.80 f (0.00)	9.27 f (0.06)	11.3 f (0.06)	14.4 f (0.00)	18.8 f (0.00)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

**Table 2.2.3:** Effect of concentration of *Agriblue* seed coats and CPE on the peroxide value (expressed as milliequivalent peroxide/kg oil) of sunflower oil during storage at 65°C

Concentration	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
Seed coat 900 ppm	0.8 a (0.00)	2.0 a (0.00)	3.20 a (0.08)	5.10 a (0.06)	6.60 a (0.00)	9.00 a (0.04)	11.0 a (0.00)	13.5 a (0.00)	16.7 a (0.02)
Seed coat 1800 ppm	0.8 a (0.06)	2.0 a (0.12)	3.20 a (0.02)	4.80 b (0.00)	6.40 b (0.04)	8.50 b (0.06)	10.0 b (0.03)	12.5 b (0.03)	16.3 b (0.04)
CPE 900 ppm	0.8 a (0.03)	1.8 b (0.06)	3.00 b (0.00)	4.60 c (0.03)	6.00 c (0.00)	8.00 c (0.00)	10.2 c (0.08)	13.0 c (0.00)	15.6 c (0.06)
CPE 1800 ppm	0.8 a (0.00)	1.8 b (0.00)	3.00 b (0.00)	4.00 d (0.00)	5.10 d (0.00)	6.80 d (0.00)	8.60 d (0.06)	12.0 d (0.08)	13.2 d (0.00)
TBHQ	0.80 a (0.06)	1.07 c (0.12)	1.23 c (0.06)	1.43 e (0.06)	1.73 e (0.06)	1.93 e (0.12)	2.13 e (0.12)	2.27 e (0.06)	2.60 e (0.00)
Control	0.80 a (0.00)	2.23 d (0.06)	3.50 d (0.00)	5.20 f (0.00)	6.80 f (0.00)	9.27 f (0.06)	11.3 f (0.06)	14.4 f (0.00)	18.8 f (0.00)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

The ability of the seed coat and CPE of both cowpea varieties to reduce formation of peroxides in sunflower oil was concentration-dependent as indicated by lower peroxide values with an increase in concentration of the additive (Tables 2.2.2 and 2.2.3). From day 6 onwards, sunflower oil containing seed coat or CPE of both cowpea varieties at a concentration of 1800 ppm had relatively lower peroxide values compared to a corresponding concentration of 900 ppm. At a higher concentration the additives are expected to perform better as they would contain more potential sites for antioxidant activity (e.g. more phenolic compounds to donate hydrogen atoms from their phenolic hydroxyl groups). Several authors have reported the antioxidant activity of phenolic extracts to be dependent on the concentration of the extract (Abdalla & Roozen, 1999; Bandoniene *et al.*, 2000; Birch *et al.*, 2001; Farag *et al.*, 2003).

**Table 2.2.4:** Effect of incorporation as seed coat or CPE (from *Bechuana white* and *Agriblue* cowpeas) on the peroxide value (expressed as milliequivalent peroxide/kg oil) of sunflower oil during storage at 65°C

Form of additive	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16
Seed coat ( <i>Agriblue</i> )	0.80 a (0.00)	1.90 a (0.33)	3.20 a (0.00)	4.83 a (0.28)	6.51 a (0.12)	8.43 a (0.48)	10.6 a (0.68)	12.5 a (0.59)	16.5 a (0.21)
CPE ( <i>Agriblue</i> )	0.80 a (0.00)	1.90 b (0.10)	3.02 b (0.04)	4.30 b (0.33)	5.56 b (0.51)	7.42 b (0.68)	9.39 b (0.89)	11.3 b (1.20)	14.4 b (1.31)
Seed coat ( <i>Bechuana white</i> )	0.80 a (0.00)	2.01 c (0.04)	3.20 c (0.00)	4.75 c (0.10)	6.27 c (0.29)	7.50 c (0.55)	10.2 c (0.66)	12.3 c (0.75)	15.9 c (0.60)
CPE ( <i>Bechuana white</i> )	0.80 a (0.00)	1.90 a (0.11)	2.30 d (0.11)	3.50 d (0.55)	5.10 d (0.33)	6.85 d (0.38)	8.40 d (0.88)	10.1 d (0.79)	13.6 d (0.88)
TBHQ	0.80 a (0.06)	1.07 d (0.12)	1.23 e (0.06)	1.43 e (0.06)	1.73 e (0.06)	1.93 e (0.12)	2.13 e (0.12)	2.27 e (0.06)	2.60 e (0.00)
Control	0.80 a (0.00)	2.23 e (0.06)	3.50 f (0.00)	5.20 f (0.00)	6.80 f (0.00)	9.27 f (0.06)	11.3 f (0.06)	14.4 f (0.00)	18.8 f (0.00)

Means within a column with different letters are significantly different ( $p < 0.05$ ).

Standard deviations are given in parenthesis

For each cowpea variety, sunflower oil containing CPE had lower peroxide values than sunflower oil containing seed coats during the storage period (Table 2.2.4). The CPE were prepared by solvent extraction from the seed coats (using aqueous acetone) followed by freeze-drying of the extract. The CPE were able to reduce the formation of peroxides in sunflower oil much better than the seed coats because for the same amount of CPE and seed coats added, the CPE would contain more phenolic compounds for hydrogen donation than the seed coats. From the previous chapter, it was shown that the total phenol content of the CPE was 558.46 mg/g (*Bechuana white*) and 433.30 mg/g (*Agriblue*) compared to 61.96 mg/g (*Bechuana white*) and 50.17 mg/g (*Agriblue*) for the seed coat (section 2.1.4, Table 2.1.1 & 2.1.2). Phenolic compounds are present in plants as components of the cell wall and compartmentalised within the plant cell vacuoles (Bengoechea, Sancho, Bartolome, Estrella, Gomez-Cordoves & Hernandez, 1997). Phenolic compounds such as hydroxycinnamic acids are linked to various cell components (Ishii, 1997). Phenolic acids such as ferulic and *p*-coumaric could be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in the form of dimers such as dehydroferulates and truxillic acid (Ishii, 1997; Wende, Waldron, Smith & Brett, 1999). Flavonoids often occur as glycosides rendering them less reactive towards free radicals (Rice-Evans, Miller & Paganga, 1997). In other words, phenolic compounds in the seed coat are present mostly in bound forms. However, a small proportion of cinnamic acid derivatives such as ferulic acid and *p*-coumaric acid may occur in free form in cowpeas and these are extracted with organic solvents such as methanol without a saponification step as observed by Cai *et al.* (2003). These authors reported the presence of ferulic acid and *p*-coumaric acid in some cowpea varieties (extracted with methanol), however in low amounts. The result obtained in this study with acetone extracts is similar to the above results reported by Cai *et al.*, (2003). The extracted phenolic compounds in the CPE would exist in a freer form compared to the seed coats and therefore would be expected to be more reactive towards free radicals. Extracted phenolics in the CPE would diffuse more easily into the sunflower oil and therefore exert relatively greater antioxidant effects compared to the seed coats.

### 2.2.5 Conclusions

Seed coats and CPE from *Bechuana white* and *Agriblue* cowpeas are able to reduce the formation of hydroperoxides in sunflower oil during storage at 65°C. The seed coat and CPE of *Bechuana white* are more effective in reducing the formation of hydroperoxides in sunflower oil during storage than their *Agriblue* counterparts. This is due to the higher content of total phenols and free phenolic acids in *Bechuana white*, making it more active in scavenging free radicals compared to *Agriblue*. The seed coats and CPE of both cowpea varieties exhibit lower ability to retard the formation of hydroperoxides in sunflower than THBQ. Purification of the CPE could improve their efficiency as antioxidant in the oil. For both cowpea varieties, the CPE have higher antioxidant activity than the seed coat. This is because phenolic compounds in the seed coat are linked to various cell wall components while those in the CPE are free to diffuse in the oil and exert antioxidant effects. To achieve maximum activity the phenolic extracts need to be extracted from their plant source. The antioxidant activity of the seed coat and CPE is related to the level of concentration. At a higher concentration the additives are expected to perform better as they would be more phenolic compounds available to donate hydrogen atoms from their phenolic hydroxyl groups.

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## CHAPTER 3

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### 3. GENERAL DISCUSSION

#### **3.1 Discussion of methods used**

This study was divided into two parts, firstly to determine the types and levels of phenols in cream-coloured *Bechuana white* and purple-coloured *Agriblue* cowpea varieties and their relation to antioxidant activity. The second part involved the determination of the effect of incorporation of seed coats and crude phenolic extracts (CPE) from seed coats of *Bechuana white* and *Agriblue* cowpea varieties on formation of primary oxidation products in sunflower oil.

Prior to determination of types and levels of phenolic compounds, the two varieties of cowpeas were conditioned (in water) and dehulled. Conditioning of the cowpea seeds was done to facilitate removal of the seed coat from the cotyledon. The seeds absorb water during the conditioning process, which results in the loosening of the seed coat from the cotyledon. The principle is the same as applied during milling of cereals such as maize and wheat. These cereals are conditioned in water to toughen the bran, thus making it easier to separate the bran from the endosperm and the germ (Matz, 1991; Posner & Hibbs, 1997; Atwell, 2001). Wheat is conditioned to 13.5-15.0 % for soft wheat and 15.5-16.5 % for hard wheat (Posner & Hibbs, 1997; Atwell, 2001). On the other hand maize is conditioned to 21 % moisture content (Matz, 1991). The initial moisture content of the cowpeas was 11 % and conditioning increased the moisture content to 25 %. The amount of water to be added in order to increase the moisture content of the cowpeas to 25 % was determined by performing a mass balance. The final moisture content of the conditioned seeds was verified using the air oven method (AOAC Official method 925.10, 2002). After conditioning, cowpea seeds were dried overnight in an oven at 50°C prior to decortication. This was done to remove excess moisture from the grain surface. The cowpea seed coats were decorticated using a rotary attrition dehuller, followed by collection and milling of the seed coats using a laboratory hammer mill fitted with a 500 µm screen. The yield of seed coats was 5.8 and 6.0 % for *Bechuana white* and *Agriblue*, respectively. For cowpeas, the seed coat makes up about 10.64 % of the total seed (Chavan, Kadam & Salunkhe, 1989). The

dehulling process applied here may therefore be considered fairly efficient considering that more than 50 % of the seed coat was removed. The efficiency of the dehulling process may be improved by conditioning the seeds for a longer time; however, it should be kept in mind that soaking of the seeds could result in leaching of phenolic compounds in the soaking water (Matuschek, Towo, & Svanberg, 2001; Shahidi, Chavan, Naczk, & Amarowicz, 2001).

Phenolic compounds in cowpeas are known to be concentrated in the seed coat (Preet & Punia, 2000). Phenols were extracted from the milled seed coats using 75% aqueous acetone (Kaluza *et al.*, 1980) for 2 h (Awika *et al.*, 2004) while vortex mixing every 5 min. Different solvents have been employed for the extraction of phenolic compounds. The type of solvent used for extraction influences the extraction yield of phenolic compounds and their antioxidant activity (Marinova & Yanishlieva, 2003). Solvents such as methanol (Cai, Hettiarachchy, & Jalaluddin, 2003), aqueous acetone (Morrison, Asiedu, Stuchbury, & Powel, 1995), ethanol (Onyeneho, & Hettiarachchy, 1991), water (Yilmaz, & Toledo, 2006) and dimethylformamide (Beta, Rooney, Marovatsanga, & Taylor, 1999) have been used for the extraction of phenolic compounds. However, there seem to be no agreement on the best solvent that gives optimum yield of phenolic compounds. Aqueous acetone was considered to be a better solvent for the extraction of various phenolic compounds than the alcoholic solvents such as aqueous methanol (Yilmaz & Toledo, 2006). It was also previously reported that 70 % acetone results in maximum extraction of total phenolic compounds (Kaluza *et al.*, 1980), condensed tannin (Chavan, Shahidi & Naczk, 2001) and anthocyanins (Kahkonen, Hopia & Heinonen, 2001) compared to methanol. Contrary to this, aqueous acetone, when used for extraction, has been reported to interact with anthocyanins and form pyrano-anthocyanins, which could result in reduction of extractable anthocyanins (Lu & Foo, 2001; Awika, Rooney & Waniska, 2004a; Awika, Rooney & Waniska, 2004b). However, it is not clear whether acetone reacts with other phenols such as phenolic acids in this manner. Results produced by Awika, Rooney and Waniska (2004a) have shown that despite the possible interactions of acetone with anthocyanins, aqueous acetone extracts from black sorghum bran still possess some antioxidant activity, even though less than extracts prepared with acidified methanol.

The majority of phenolic compounds are found in plants in bound form as glycosides linked by ester, ether, or acetal bonds to organic molecules such as glucose (flavonoids) (Rice-Evans, Miller & Paganga, 1997) and structural components of the plant such as cellulose, proteins, lignin (phenolic acids) (Andreasen, Christensen, Meyer, Hansen, 2000; Lam, Kadoya, Iiyama, 2001) with only a minor fraction existing in the free form (reviewed by Robbins, 2003). Some of the bound phenolic acids give rise to various phenolic derivatives, which are stored in vacuoles within the plant matrix (Rice-Evans, Miller & Paganga, 1997; reviewed by Robbins, 2003) and can therefore be extracted by organic or alcoholic solvents (reviewed by Robbins, 2003). These have been referred to as soluble phenolic acid esters. (Krygier, Sosulski & Hogge, 1982). However, other phenolic acids are bound to insoluble carbohydrates within the cell wall and may be extracted after saponification (Krygier, Sosulski, & Hogge, 1982; Maillard, & Berset, 1995; Andreasen, Christensen, Meyer, & Hansen, 2000; Liyana-Pathirana, & Shahidi, 2006) or by enzymatic treatment (Meyer, Jepsen, & Sorensen, 1998; Andreasen, Christensen, Meyer, & Hansen, 1999; Landbo, & Meyer, 2001) in order to break the ester linkages and release the phenolic acids (reviewed by Robbins, 2003). Therefore, 75 % aqueous acetone as an organic solvent would be expected to extract predominantly the free phenolic acids and the phenolic derivatives, which are stored in the vacuoles (soluble phenolic acid esters). Cai *et al.* (2003) reported the presence of cinnamic acid derivatives such as ferulic and *p*-coumaric acid in cowpea methanol extracts. This indicates that a small proportion of these phenolic acids may occur in free form and may be extracted with organic solvents without saponification. A similar result was obtained in this study where the CPE (prepared from 75% aqueous acetone extracts) contained small amounts of ferulic and *p*-coumaric acid. Thus the phenolic acids that were identified during HPLC analysis are expected to be in the free form.

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 phenol content and those that target specific groups of compounds. Two methods were employed for the purpose of measuring total phenols in this study, the Folin-Ciocalteu method (Singleton & Rossi, 1965) and the Ferric Ammonium Citrate (ISO 1988).

The Folin-Ciocalteu method is based on a reduction-oxidation reaction during which the phenolate ion is oxidized under alkaline conditions while reducing the phosphotungstic-phospho-molybdic complex in the reagent to a blue coloured solution (Waterman & Mole, 1994). However, its drawback is that it lacks specificity and it detects all phenolic groups in extracts including those found in extractable proteins (Waterman & Mole, 1994). Extractable proteins that contain the amino acid tyrosine (which contains a phenolic hydroxyl group) in significant amounts could have the potential of causing interferences with determination of phenols using the Folin-Ciocalteu method. Cowpea proteins are situated mainly in the cotyledons with only 4 % of the total protein found in the seed coat. The seed coat proteins contain approximately 3.6 g/16 g N (0.84 %) tyrosine (Phillips, 1982). Extraction of cowpea seed coats with aqueous acetone is not expected to result in extraction of appreciable amounts of seed coat proteins with significant levels of tyrosine to interfere with the determination of total phenols by the Folin-Ciocalteu method. The Folin-Ciocalteu method has been used extensively for the determination of total phenols in cereals (Vinson, Hao, Su, Zubik, 1998; Adom, & Liu, 2002; Adom, Sorrells & Liu, 2003) and legumes (Troszyńska, Estrella, López-Amòres & Hernández, 2002; Cai *et al.*, 2003; Jiratanan, & Liu, 2004).

The Ferric Ammonium Citrate method is based on the ability of phenolic compounds in alkaline conditions to reduce ferric ion to ferrous. Its draw back is that it does not only respond to phenols but also other reducing agents such as ascorbate (Beta, Rooney, Marovatsanga & Taylor, 1999). However, cowpeas do not contain ascorbate (Doblado, Zielinski, Piskula, Kozłowska, Munoz, Frias & Vidal-Valverde, 2005) and thus would not cause interferences with total phenol assays. The method is simple (Deshpande, Cheryan & Salunkhe, 1986) and as shown in this work, exhibits similar trends in values as the Folin-Ciocalteu method.

A number of analytical methods are used for the separation and determination of phenolic compounds (Zupfer, Churchhill, Rasmusson & Fulcher, 1998). For phenolic acids for instance, techniques such as thin-layer chromatography, gas-liquid chromatography, gas chromatography-mass spectrometry, capillary electrophoresis and high performance liquid chromatography (HPLC) are used (reviewed by Robbins, 2003). The choice of technique depends on the solubility properties and volatilities of the compounds to be separated (Harborne, 1984). HPLC, which was used in this



study, is perhaps the most widely used technique for both separation and quantification of the various groups of phenolic compounds namely, phenolic acids (Adom, & Liu, 2002; Rösch, Bergmann, Knorr, & Kroh, 2003), flavonoids (Romani, Pinelli, Mulinacci, Vincieri, Gravano, & Tattini, 2000; Chang, & Wong, 2004) and tannins (Peng, Hayasaka, Iland, Sefton, Høj, & Waters, 2001; Awika, Dykes, Gu, Rooney, & Prior, 2003b).

Generally chromatography entails the partitioning of sample molecules between a mobile phase and a stationary phase. During HPLC analysis, the different components of the sample will have different distribution equilibria depending on their solubility in the phases and/or molecular size (Bidleymeyer, 1992). This forms the basis for separation of the different components of the mixture. HPLC analysis may employ either reversed phase or normal phase systems. For reversed phase HPLC as used in this study, the stationary phase consists of a packing material surface that is relatively non-polar (such as the C18 column used in this study) with a relatively polar mobile phase (such as the acetonitrile and water solvents used here). This differs from the normal-phase, which consists of a polar stationary phase with non-polar solvents as the mobile phase (Bidleymeyer, 1992). HPLC may be conducted using two main elution systems, the gradient elution and the isocratic elution systems. Isocratic elution involves the elution of compounds in a sample using a mobile phase with constant flow rate and composition. On the other hand, gradient elution, which was used in this study, involves changing the composition of the mobile phase continuously during analysis (Bidleymeyer, 1992). Gradient elution is frequently used for the separation of complex mixtures of compounds that have a wide range of polarities and are not easily separated by an isocratic system within a reasonable time (Newton, 1982; Berridge, 1985; Bidleymeyer, 1992). The gradient system employed in this study involved a gradual increment in the amount of acetonitrile in the mobile phase with time. This had the effect of gradually decreasing the relative polarity of the mobile phase in order to separate and elute the different phenolic compounds in the acetone extracts from the two-cowpea varieties.

It must be noted that the chromatograms obtained had a drifting baseline due to lack of sample clean up prior to HPLC analysis. Crude extracts prepared as done in this study would contain not only phenolic acids but also flavonoid compounds and other

larger polyphenols. To obtain better chromatograms, sample clean up by solid phase extraction would be desirable.

Several methods are employed to measure the antioxidant activity of natural compounds in food and biological systems. Antioxidant activity assays measure the effects of the antioxidant in controlling the extent of oxidation (Antolovich *et al.*, 2002). For the purpose of this study the antioxidant activity was measured using the Trolox equivalent antioxidant capacity (TEAC). This assay measures the ability of an antioxidant to scavenge free radicals generated either in the aqueous or lipophilic phase (Awika *et al.*, 2003). The TEAC assay involves addition of antioxidants to the preformed radical cation ABTS<sup>•+</sup>. The antioxidants reduce the radical depending on the antioxidant activity, concentration of the antioxidant and duration of the reaction. The extent of discolouration as percentage inhibition of ABTS is determined as a function of concentration and time, which is calculated relative to the reactivity of Trolox, a water-soluble vitamin E analogue as a standard under the same conditions (Re, Pellegrini, Proteggente, Pannala, Yang & Rice-Evans, 1999). The method is preferred for its simplicity and speed of analysis. It can be used over a wide pH range and can be used to study effects of pH on antioxidant mechanism (Lemaska, Szymusiak, Tyrakowska, Zielinski, Soffer & Rietjens, 2001). Moreover, the ABTS radical is soluble in both aqueous and organic solvents and is not affected by ionic strength, thus it can be used in multimedia to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Prior, Wu & Schaich, 2005).

Oxidative stability is one of the most important indicators for maintaining the quality of edible oils (Tan, Man, Selemat & Yusoff, 2002). Several methods have been developed for measuring the oxidative stability of edible oils (Frankel, 1993). The oxidative stability methods are based on the determination of parameters related to the concentration of primary or secondary oxidation products in the oil (Guillen, Cabo, Ibargoitia & Ruiz, 2005). Oxidative stability tests are usually carried out under accelerated test conditions due to long test periods required in ambient temperatures (Abou-Gharbia, Shehata & Shahidi, 2000). The accelerated conditions are designed to speed up the oxidation process by exposing the oil samples to elevated temperatures in the presence of excess amounts of air or oxygen (Tan, Man, Selemat & Yusoff, 2002). During this study, sunflower oil samples (with or without antioxidants) were

kept in the dark at 65°C for a period of 16-days. The oxidation process was monitored by measuring the primary oxidation products using the peroxide value method. The method is based on the principle that hydroperoxides (the primary products of oil oxidation) in the presence of acetic acid act as oxidizing agents and liberate iodine from potassium iodide (AOCS Method Cd 8-53, 1985).

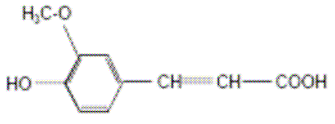
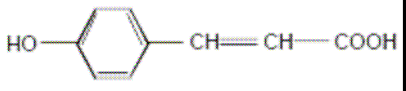
### 3.2 Discussion of results

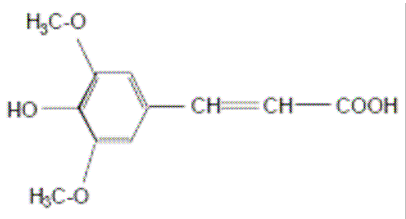
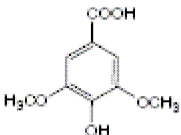
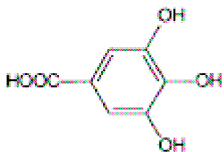
It is generally reported that the levels of total phenols in legumes is related to the colour of the seed coat with darker coloured legume grains containing higher levels of total phenols than the lighter coloured grains (Chang, Collins, Bailey and Coffey 1994; Oboh, Muzquiz, Burbano, Cuadrado, Pedrosa, Ayet & Osagie, 1998). In this study however, the cream-coloured *Bechuana white* had higher levels of phenolic compounds with relatively higher levels of antioxidant activity than the purple coloured *Agriblue* variety. The higher levels of total phenols and antioxidant activity in *Bechuana white* may be attributed to the presence of phenolic compounds with higher reducing power and free radical scavenging activity. Even though correlations were not done in this study, the results seem to agree with reports in literature that indicate that the levels of phenolic compounds can be positively correlated with the antioxidant activity. In other words, the higher the levels of total phenols, the higher the antioxidant activity (Velioglu, Mazza, Gao, & Oomah, 1998; Adom, & Liu, 2002; Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003b; Pellati, Benvenuti, Magro, Melegari, Soragni, 2004).

More importantly however, structure-activity relationships are the main factors that determine the antioxidant activity of phenolic compounds measured by free radical scavenging (Rice-Evans, Miller, & Paganga, 1996; Rice-Evans, Miller, & Paganga, 1997; Mathiesen, Malterud, Sund, 1997; Sroka, & Cisowski, 2003). The ability of any particular phenolic compound to scavenge free radicals depends on its structure. Therefore the types of phenolic compounds present in an extract would determine its resultant antioxidant activity (Rice-Evans, Miller & Paganga, 1996; Larrauri, Sanchez-Moreno & Saura-Calixto, 1998; Sroka & Cisowski, 2003). The HPLC chromatograms indicated that *Bechuana white* contained higher levels of free phenolic acids namely, the cinnamic acid derivatives *p*-coumaric, ferulic and sinapic

acids, and the benzoic acid derivatives, vanillic, gallic, 3,4 DHBA (dihydroxybenzoic acid), PHBA (*p*-hydroxybenzoic acid) and syringic acid. Structures of some of these phenolic acids and structural features that explain their antioxidant activity are shown in Table 3.1.1 below.

**Table 3.1.1** Some phenolic acids found in higher amounts in *Bechuana white* than in *Agriblue* and structural features that influence their antioxidant (free radical scavenging) activity

<b><u>Phenolic compound</u></b>	Structural features influencing antioxidant activity
<p><b>Ferulic acid (Cinnamic acid derivative)</b></p> 	<p>Electron donating <math>-\text{CH}=\text{CH}-\text{COOH}</math> group gives greater hydrogen donating ability and greater phenoxyl radical stabilisation by resonance compared to corresponding benzoic acid derivative (Rice-Evans <i>et al.</i>, 1996; Natella, Nardini, Felice, &amp; Scaccini, 1999).</p> <p>Electron donating methoxy group <i>ortho</i> to the OH group activates the ring (Rice-Evans <i>et al.</i>, 1996).</p> <p>OH group in the <i>para</i> position enhances antioxidant activity (Pannala <i>et al.</i>, 1998; Pannala <i>et al.</i>, 2001).</p>
<p><b>p-coumaric acid (Cinnamic acid derivative)</b></p> 	<p>Electron donating <math>-\text{CH}=\text{CH}-\text{COOH}</math> group gives greater hydrogen donating ability and greater phenoxyl radical stabilisation by resonance compared to corresponding benzoic acid derivative (Rice-Evans <i>et al.</i>, 1996; Natella, <i>et al.</i>, 1999).</p> <p>OH group in the <i>para</i> position enhances antioxidant activity (Pannala <i>et al.</i>, 1998; Pannala <i>et al.</i>, 2001).</p>

<p><b>Sinapic acid (Cinnamic acid derivative)</b></p> 	<p>Electron donating <math>-\text{CH}=\text{CH}-\text{COOH}</math> group gives greater hydrogen donating ability and greater phenoxyl radical stabilisation by resonance compared to corresponding benzoic acid derivative (Rice-Evans <i>et al.</i>, 1996; Natella, <i>et al.</i>, 1999).</p> <p>Two electron donating methoxy groups (<math>-\text{OCH}_3</math>) creates a hindered phenol (Pannala <i>et al.</i>, 2001), activates the ring (Morrison &amp; Boyd, 1992) and enhances electron donating properties in the <i>para</i> position (Pannala <i>et al.</i>, 2001).</p> <p>OH group in the <i>para</i> position enhances antioxidant activity (Pannala <i>et al.</i>, 1998; Pannala <i>et al.</i>, 2001).</p>
<p><b>Syringic acid (Benzoic acid derivative)</b></p> 	<p>Electron donating methoxy group (<math>-\text{OCH}_3</math>) in <i>ortho</i> position relative to the OH group activates the ring (Morrison &amp; Boyd, 1992) and enhances hydrogen donating ability (Zhou, Yin &amp; Yu, 2006).</p> <p>OH group in <i>para</i> position to the carboxylic group maintains the scavenging activity (Lu, Nie, Belton, Tang, &amp; Zhao, 2006).</p>
<p><b>Gallic acid (Benzoic acid derivative)</b></p> 	<p>Three <math>-\text{OH}</math> groups increase hydrogen donating ability (Rice <i>et al.</i>, 1996).</p> <p>OH group in <i>para</i> position to the carboxylic group maintains the scavenging activity (Lu, Nie, Belton, Tang, &amp; Zhao, 2006).</p>

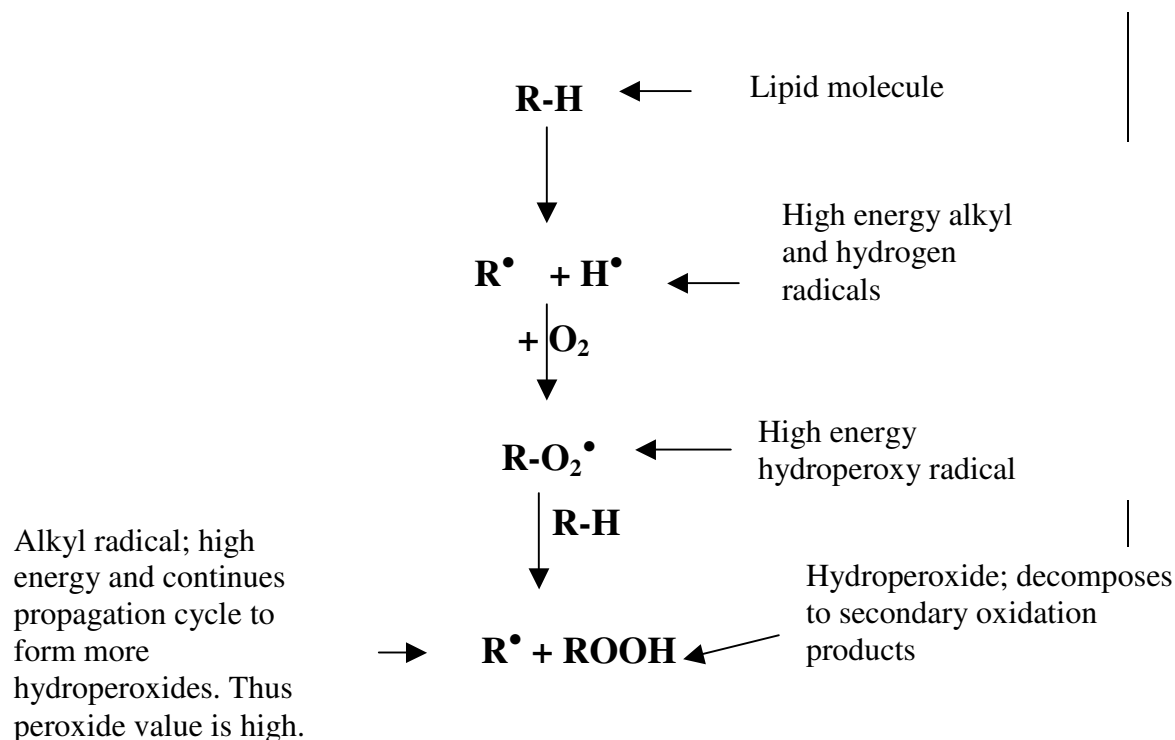
The presence of the electron donating  $-\text{CH}=\text{CH}-\text{COOH}$  group in cinnamic acid derivatives (Table 3.1.1) brings about greater hydrogen donating ability and greater

radical stabilisation than the carboxylate group in benzoic acid derivatives, thus increasing the antioxidant activity of cinnamic acid derivatives compared to benzoic acid derivatives (Rice-Evans, Miller & Paganga, 1996; Natella *et al.*, 1999). For phenolic acids, the presence of a hydroxyl group in the *para* position to the –CH=CH-COOH group (cinnamic acid derivatives) or the carboxylate group (benzoic acid derivatives) enhances antioxidant activity (Pannala, Razaq, Halliwell, Singh, & Rice-Evans, 1998; Pannala, Chan, O'Brien, & Rice-Evans, 2001). Electron donating methoxy groups activate the phenolic ring and enhance electron-donating properties (Rice *et al.*, 1996).

As previously reported in section 2.1.4, the levels of total phenols in the seed coat was 4.2 and 5.0% for *Agriblue* using the Folin-Ciocalteu and Ferric ammonium citrate methods respectively and 6.0 and 6.2% for *Bechuana white*. A number of studies have previously reported the levels of total phenols in cowpea cotyledons and whole seeds. There appears to be varietal differences in the levels of total phenols reported in cowpeas. Some reported levels of total phenols in whole cowpea grains are 0.103 % (Giami & Okwechime, 1993), 1.36 % (Vijayakumari *et al.*, 1998) 0.78-0.63 % (Preet & Punia, 2000), 0.35 % (Cai *et al.*, 2003), in white, brown, purple, varieties respectively. Factors such as cultivar, variety, growing conditions, processing and storage conditions are known to influence the amount of phenolic compounds present in food (Morrison, Asiedu, Stuchbury, & Powel, 1995; Vijayakumari *et al.*, 1998; King & Young, 1999). The type of solvent used for extraction could influence the amount of total phenols reported in a sample (Marinova & Yanishlieva, 2003; Naczka & Shahidi, 2004). The level of total phenols in the seed coat of both cowpea varieties used in this study were higher than levels previously reported for the cotyledons (0.02-0.07 %) (Laurena, Truong & Mendoza, 1984) and whole seed (0.03-0.9 %) (Giami & Okwechime, 1993; Preet & Punia, 2000; Cai *et al.*, 2003). This was expected because phenolic compounds are known to be concentrated in the seed coat (Preet & Punia, 2000).

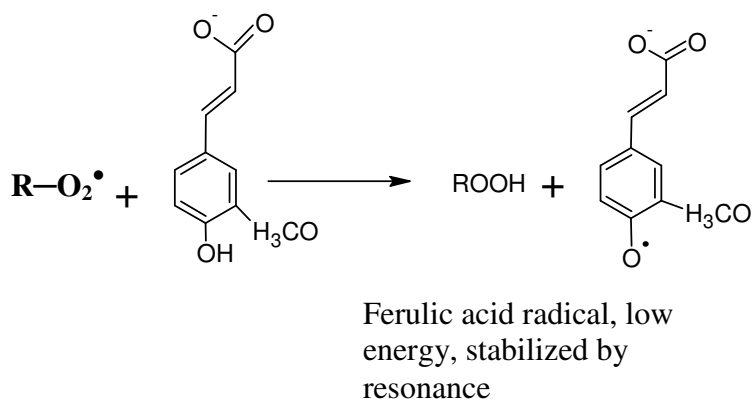
The second part of the study involved the determination of the effect of the seed coat and phenolic extracts from *Bechuana white* and *Agriblue* on the stability of sunflower oil. The oxidative stability of sunflower was determined using the peroxide value method.

The seed coat and CPE of the two-cowpea varieties (*Bechuana white* and *Agriblue*) were able to reduce formation of peroxides in sunflower oil during storage. This could be due to the presence of phenolic compounds in the seed coats and CPE. The antioxidant activity of plant extracts is due to the presence of phenolic compounds, which exert their antioxidant activity by donating hydrogen atoms from their phenolic hydroxyl groups (Frankel, 1996; Rice-Evans, Miller & Paganga, 1997; Farag, El-Baroty, & Basuny, 2003) to free radicals that may be generated in lipid systems. The products of these reactions are non-radical compounds and stable antioxidant free radicals. The chain propagation process in lipid oxidation is therefore curtailed. The mechanism by which the additives (seed coats and CPE) reduce formation of hydroperoxides leading to a lower peroxide value of the sunflower oil during storage may be explained by comparing the lipid oxidation process both in the absence and in the presence of the additive. These mechanisms are shown in Figures 3.1.1 and 3.1.2 below:



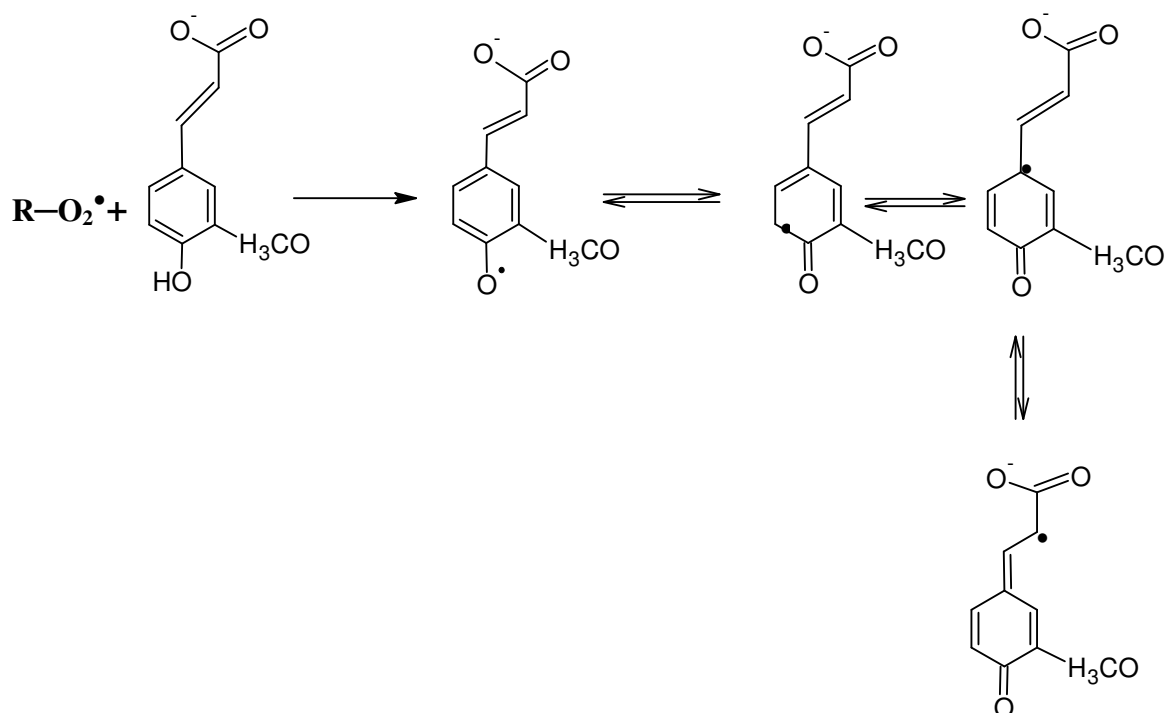
**Figure 3.1.1** Lipid oxidation in the absence of cowpea seed coats or CPE





**Figure 3.1.2** Reaction between ferulic acid (phenolic antioxidant present in cowpea seed coats or CPE) and hydroperoxy radical.

In the absence of additive (cowpea seed coats, CPE or TBHQ), hydroperoxy radicals react with lipid molecules to produce more high-energy radicals (alkyl radical  $R^\bullet$ ) and hydroperoxides (Figure 3.1.1). The high-energy alkyl radical continues the propagation process to form more hydroperoxides, which results in high peroxide values for the sunflower oil sample without additive. In the presence of additive however (Figure 3.1.2), the hydroperoxy radicals preferentially react with the phenolic antioxidant (e.g. ferulic acid in the seed coats or CPE) rather than another lipid molecule. This is because the activation energy required for reaction between the hydroperoxy radical and phenolic antioxidant is lower than activation energy for reaction with another lipid molecule. The antioxidant free radical formed (e.g. ferulic acid radical in Figure 3.1.2) is of low energy due to the fact that it is stabilised by resonance around the aromatic ring (Gordon, 1990; Nawar, 1996; Coultate, 2002). Therefore it does not propagate the chain reaction to form more highly reactive alkyl and hydroperoxy radicals. The net effect is that relatively lower amounts of hydroperoxides are formed in the sunflower oil samples with additive, leading to lower peroxide values. Possible resonance structures of the ferulic acid radical are shown in Figure 3.1.3.



**Figure 3.1.3** Possible resonance structures of ferulic acid phenoxyl radical

The ability of the seed coat and CPE to reduce formation of hydroperoxides in sunflower oil increased with an increase in the level of concentration, suggesting that at higher concentration more phenolic hydroxyl groups will be available to donate hydrogen atoms to free radicals thus stabilising sunflower oil.

The seed coat and CPE of *Bechuana white* variety were more effective than the *Agriblue* variety in reducing formation of peroxides in sunflower oil during storage. This was in agreement with the results of chapter 2.1, where *Bechuana white* had higher levels of total phenols and free radical scavenging activity than *Agriblue*. It was also established that the higher antioxidant activity (both free radical scavenging activity by TEAC assay and ability to retard hydroperoxide formation by peroxide value assay) is attributed to higher levels of phenolic acids such as ferulic, *p*-coumaric and sinapic acids in *Bechuana white* than *Agriblue*. As previously explained the chemical structures of these phenolic compounds allow them to act as better radical scavengers (Rice-Evans *et al.*, 1996) thus retarding the formation of hydroperoxides in sunflower oil.

Even though both the seed coat and CPE of both cowpea varieties were able to retard the formation of hydroperoxides in sunflower oil, the seed coats were relatively less

efficient than the CPE. Majority of phenolic compounds occur in plants in bound forms to various components giving rise to various phenolic acid derivatives, with some of the derivatives stored in vacuoles and can thus be extracted with organic solvents (Rice-Evans, Miller & Paganga, 1997; Robbins, 2003). Therefore the CPE phenolics occur in a freer form to exert antioxidant effects compared to the seed coat where they are confined within the cellular matrix and so do not diffuse out easily to exert antioxidant effects. This explains the higher antioxidant activity of the CPE than the seed coat. The antioxidant effects of the extracts could be further enhanced by saponification of the seed coats in order to release the insoluble, bound phenolics. It has been reported that the insoluble, bound phenolic fractions of wheat had highest free radical scavenging activity (Liyana-Pathirana & Shahidi, 2006).

This study demonstrated that cowpea seed coats have the potential to be used as a readily available source of antioxidants for application in sunflower oil. The CPE were more effective antioxidants than the seed coats. CPE and the seed coats from both cowpea varieties were however not as effective as the synthetic antioxidant TBHQ. Moreover, the reduction in peroxide values was rather minimal to be of economic or practical use. These findings suggest that using cowpea seed coats and CPE as antioxidants in sunflower oil in the manner and form in which they were used in this study may not be an efficient and cost-effective way of extending the shelf life of edible oils. The antioxidant activity of the CPE may be improved by purification to remove non-phenolic components that may be acting as pro-oxidants and saponification to release insoluble bound phenolics from the seed coats. Use of the seed coats themselves as antioxidants in edible oil will be important for rural communities because it presents a cheaper alternative. However, the effectiveness of the seed coats as antioxidants is hampered by the fact that the phenolics are confined within the cellular matrix and also occur mostly in bound forms. More research is needed to screen various cowpea varieties and identify those with relatively higher antioxidant activity in the seed coat.

## CHAPTER 4

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### 4. CONCLUSIONS AND RECOMMENDATIONS

Seed coats of the cream-coloured *Bechuana white* cowpea variety and the freeze-dried forms of their acetone extracts (CPE) contain more total phenols than the corresponding samples of the purple-coloured *Agriblue*. This differs from the generally observed trend that cowpeas with darker coloured seed coats contain higher levels of total phenols than those with light coloured seed coats. The above observation suggests that the levels of total phenols in cowpeas may not always be related to the colour of the seed coat. However, only two cowpea varieties were studied in this work. There is a need for an investigation of more cowpea varieties differing in colour of the seed coat in order to confirm this observation.

*Bechuana white* seed coats and CPE have higher antioxidant activity (determined as free radical scavenging ability) than corresponding samples of *Agriblue*. HPLC chromatograms show that both cowpea varieties have similar types of phenolic acids namely, the cinnamic acid derivatives *p*-coumaric, ferulic and sinapic acids and the benzoic acid derivatives gallic, vanillic, syringic, 3,4 DHBA and *p*-hydroxybenzoic acid. However, these compounds are present in higher amounts in *Bechuana white* than *Agriblue*. In addition, phenolic acids belonging to the family of cinnamic acid derivatives tend to have higher antioxidant activity than the benzoic acid derivatives. The higher level of these compounds in *Bechuana white* explains the higher antioxidant activity of *Bechuana white* than *Agriblue*.

All the additives (CPE and seed coat of *Bechuana white* and *Agriblue*), and TBHQ are able to inhibit the formation of hydroperoxides in sunflower oil. This is shown by lower peroxide values in sunflower oil with added additives compared to the control samples (oil without additive). This effect of the seed coat and CPE may be attributed to the presence of phenolic compounds, which donate hydrogen atoms from their hydroxyl groups thus reducing the formation of hydroperoxides in the oil. Even though the seed coat and CPE of both cowpea varieties are able to reduce the formation of hydroperoxides, their efficiency is less than that of TBHQ. This is because the seed coat and CPE were added in crude form while TBHQ is a pure

compound. Further studies to improve the antioxidant activity of the CPE in sunflower could include purification of the extracts to isolate phenolic compounds with appreciable antioxidant activity and/or saponification of the seed coats prior to extraction of the phenolic compounds to release the insoluble bound phenolic compounds thus increasing the antioxidant activity of the extracts.

CPE and seed coats of *Bechuana white* are more efficient in retarding oxidation of sunflower oil than the corresponding samples of *Agriblue*. This is in agreement with the higher levels of total phenols and free radical scavenging activity observed in *Bechuana white* than *Agriblue*. Moreover, the HPLC also showed that *Bechuana white* contains higher levels of free phenolic acids with relatively higher antioxidant activity than *Agriblue*. The amount and types of total phenols present in plants is influenced by factors such as variety. Therefore further studies could include screening of different cowpea varieties to identify those with higher levels of total phenols and antioxidant activity, which can be used as antioxidants in sunflower oil.

The seed coats of both cowpea varieties are less efficient in retarding the formation of hydroperoxides than CPE. Most phenolic compounds occur in plants in bound forms to various cellular components giving rise to various phenolic acid derivatives that are stored in vacuoles and are extracted with organic solvents such as acetone. Therefore the extracted phenolic compounds in the CPE are in a freer form to diffuse in the oil to exert antioxidant effects compared to the unextracted seed coat. Even though the seed coats are not as effective as CPE, they may be useful in rural areas where extraction of phenolics may not be possible due to economic reasons.

The antioxidant activity of the seed coat and CPE of both cowpea varieties is dependent on the level of concentration. The additives are expected to perform better at a higher concentration because more phenolic hydroxyl groups will be available to donate hydrogen atoms to the free radicals in the oil.

Even though the peroxide value method indicates the formation of primary oxidation products, it does not indicate the formation of secondary oxidation products. Therefore further studies are suggested to determine the effect of cowpea extracts on the formation of secondary oxidation products.

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