Mafura seed oil: Improvement in oxidative stability

BY

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DECLARATION

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

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February 2016

DEDICATION

I dedicate this dissertation to my parents, Firmino Chemane and Guilhermina Chitsembe, my brothers Hélder and Arcénio Chemane and my nephews Ilker and Kheynner Chemane. This work would never have been possible without your support and constant presence in my life. I am so thankful for all the words and prayers. You are really my pillar. Thank you so much for the never ending love. You are really a gift from God to me. I love you very much…

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ABSTRACT

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Trichilia emetica seeds are processed for their oil by rural communities in the South of Mozambique. Traditionally, the oil is extracted by boiling the seed pulp in an aqueous medium for 5 h and recovering the clear yellow oil. The extended heating period and the use of water for oil extraction may reduce the oil quality.

Mafura oil was extracted using a laboratory method that mimics the traditional method. Acid value (titrimetry), iodine value (Wijs method), saponification value (titrimetry) and fatty acid composition (gas chromatography) were determined in comparison with sunflower and olive oils. Oxidative stability (OS), (peroxide and anisidine values) of mafura oil stored at (65ºC) over a 14 day period was also determined in comparison with sunflower and olive oils. The effect of refining, solvent extraction (hexane and ethanol) and the effect of incorporation of freeze dried crude phenolic extract (CPE) from red condensed tannin sorghum bran (at 1000 and 2000 ppm) compared with tertiary-butylhydroquinone (TBHQ) (200 ppm) on OS of mafura oil during storage was determined. Whole grain, bran and CPE were analysed for total phenolic content (TPC), (Folin-Ciocalteu assay) and antioxidant activity, (ABTS radical scavenging). Phenolic composition of the CPE was determined using High Performance Liquid Chromatographic.

Traditionally extracted crude mafura oil (CMFO) had the highest acidity. Crude and refined mafura oils had the lowest iodine values and the highest saponification values compared with sunflower oil and the olive oils. CMFO was rich in palmitic acid, sunflower oil in linoleic acid and the olive oils were rich in oleic acid. CMFO was the least stable during storage as shown by high anisidine values. Solvent extraction (but not refining) improved the OS of CMFO.

Mafura oil treated with CPE was more oxidatively stable than the control and this seemed to be dose-dependent. CPE at 2000 ppm was less effective in inhibiting the formation of primary oxidation products than TBHQ, however, it was as effective in reducing secondary oxidation. The CPE had higher TPC and antioxidant activity than whole grain and sorghum bran. The CPE contained phenolic acids and high levels of (+)-catechin and (-)-epicatechin. The ability of the CPE to improve the OS may be related to the action of the phenolic compounds as primary antioxidants in scavenging free radicals.

This study shows that the traditional method of mafura oil extraction decreases its OS due to extensive heating in an aqueous medium. The CPE may potentially be used as a natural antioxidant to prevent oxidation of mafura oil due to the antioxidant properties of the phenolic compounds present in the sorghum bran.

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CHAPTER 1

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and problem statement

Mafura (*Trichilia emetica* Vahl) is widespread in sub-Saharan African countries such as South Africa, Swaziland, Zimbabwe, Cameroon, Sudan, Uganda (Vermaak et al., 2011) and Mozambique (Adinew, 2014). Mafura seeds are edible and people in rural communities in the South of Mozambique use them for consumption as a dessert as well as to extract oil (locally called "Munhantsi"). The oil is used in its crude form for the preparation of various foods (such as grilled chicken, boiled cassava, eggs and beans). Mafura oil is extracted by boiling the pulp of the seed in an aqueous medium for a long period of time (Matakala et al., 2005). The extended heating period and the use of water for oil extraction may reduce the oil quality (Guillén and Cabo, 2002). In addition, the oil is usually stored in transparent closed glass or plastic bottles which can also contribute to a decrease in the oxidative stability of the oil.

The quality of edible oils is greatly affected by oxidation which is one of the causes of deterioration (Shahidi and Zhong, 2010a). It alters the properties of the oils, giving unpleasant flavour and aroma (Moure et al., 2001, Velasco and Dobarganes, 2002) thus lowering their shelf life. Furthermore, this degradation affects their chemical and nutritional properties (Martin-Polvillo et al., 2004, Marquez—Ruiz et al., 2013) and safety due to the formation of oxidation products (Muik et al., 2005). Oxidation products are formed due to reactions of molecular oxygen with the double bonds of mono- and polyunsaturated fatty acids present in the fats, oils and lipid-containing foods (Frega et al., 1999) These reactions lead to the formation of free radicals and the onset of auto-oxidation (Muik et al., 2005) which occurs via a free radical chain reaction (Tan et al., 2002, Shahidi and Zhong, 2010a).

To delay the onset of rancidity during storage of edible vegetable oils, potential strategies that could be utilized include protection from light, storage at refrigerated temperatures, packaging in airtight containers, refining (Lindley, 1998) and the use of antioxidants (Morelló et al., 2004) which may be natural or synthetic (Hraš et al., 2000). Synthetic antioxidants such as tertiary-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) have been used in stabilizing food lipids

because they are effective and cheaper than natural antioxidants (Duh et al., 1997). However, concerns about the safety and toxicity of synthetic antioxidants have been raised (Ito et al., 1986, Shahidi, 1997, Moure et al., 2001, Aluyor and Ori-Jesu, 2008, Matthäus, 2010). Therefore, there is a growing interest in the potential use of antioxidants from natural sources (Matthäus, 2010, Mohdaly et al., 2011) because they are considered to be safer since they occur in plants and are deemed more desirable than their synthetic counterparts (Frankel, 1993, Hraš et al., 2000).

Extracts from plant sources (prepared using various types of aqueous or organic solvents) possess antioxidant properties due to the presence of phenolic compounds. They are able to inhibit oxidative deterioration by scavenging free radicals, and, therefore, may be used as natural antioxidants to delay or inhibit the oxidative degradation of lipids (Iqbal et al., 2008, Mohdaly et al., 2011). In this regard, sorghum (*Sorghum bicolor* (L.) Moench) a drought-tolerant cereal (FAO, 1995) may be of significance. It is well known to contain phenolic compounds such as phenolic acids, flavonoids and tannins, and therefore, phenolic extracts from sorghum grains may be potentially used as antioxidants to prevent the oxidative degradation of lipids (Awika and Rooney, 2004, Dykes and Rooney, 2006, Dykes et al., 2014).

Information on processing and physico-chemical properties of mafura oil is very limited and it appears that so far, a comprehensive study on the oxidative stability of mafura oil has not been conducted. Knowledge about the oil quality, could promote its consumption, utilization and commercialization especially by small scale processors in rural communities in Mozambique. Improvements in production and stabilization of the mafura oil could significantly contribute to household food security of this area. Furthermore, this could generate more income and improve livelihoods of small scale Mozambican mafura oil processors due to the fact that in some seasons other cash crops have lower productivity. Smallholders rely on their own production to sustain their food needs which depend largely on the variable rainfall.

1.2 Literature review

1.2.1 Mafura tree

Mafura (*Trichilia emetica* Vahl.) belongs to the Meliaceae family (Germano et al., 2005, Germano et al., 2006, Komane et al., 2011, Vermaak et al., 2011). The tree grows in forests along the coast as well as in open riverine-alluvial lowland rainforests (Beentje et al., 1994 according to Kioko et al., 2006, Vermaak et al., 2011) and is widespread in Tropical Africa (Diallo et al., 2003, Germano et al., 2005, Germano et al., 2006, Mashungwa and Mmolotsi, 2007, Konaté et al., 2015). It can be found in South Africa, Swaziland, Cameroon, Sudan, Uganda, Tanzania (Fupi and Mork, 1982), Mali (Diallo et al., 2003, Germano et al., 2006), Zimbabwe (Grundy and Campbell, 1993) and South of Mozambique (Adinew, 2014). In each country, the fruit is known by various common names such as "natal mahogany" in South Africa (Jøker, 2003) and mafura or "tihulhu" in Mozambique.

Trichilia emetica is an evergreen tree which grows to a height of about 20-35 m (Komane et al., 2011). The bark is dark grey-brown (Mashungwa and Mmolotsi, 2007) with dark glossy leaves (Komane et al., 2011, Vermaak et al., 2011). Green pear-shaped fruits with tendency to turn brown as the fruit ripens (Komane et al., 2011) and appear between January and April (in Mozambique). The furry fruit contains a shell that splits into 3 parts when the seeds are ripe and inside the shell there are seeds with orange to red pulp (Komane et al., 2011) and a large black spot (Daniel and McCrae, 1908). Seeds with a colourless pulp can be found in Mozambique. Fruits and seeds of *Trichilia emetica* are shown in Figure 1.

Figure 1 - Flowering twig (1), fruits (2), seed (3) and kernels (4) of *Trichilia emetica* Vahl. (Mashungwa and Mmolotsi, 2007).

All parts of the mafura tree have a wide range of uses in traditional medicine. The roots are ground into powder and used to treat cirrhosis, onchocerca, ascaris stomach-aches and dysmenorrhoea (Diallo et al., 2003, Mashungwa and Mmolotsi, 2007). The powder is also used as a purgative when it is mixed with milk (Diallo et al., 2003). An extract of the bark prepared by soaking in warm water is used as emetic for treating intestinal ailments, as a purgative and also to treat hepatitis and pneumonia (Mashungwa and Mmolotsi, 2007). The root extract is used for prevention of infertility in women (Mashungwa and Mmolotsi, 2007). The leaves are used to treat malaria, wounds and hypertension (Diallo et al., 2003). The oil obtained from the fleshy seed envelope is used to prevent rheumatism and to treat skin diseases such as leprosy and bone fractures (Mashungwa and Mmolotsi, 2007).

In rural areas of Mozambique, mafura seeds are processed extensively for their oil mainly by informal, small scale women entrepreneurs. The seeds are firstly sun-dried and stored in sisal bags inside huts until the oil extraction period (which is at the end of the season). Mafura seeds are considered dried when the pulp no longer attaches to the kernels.

1.2.2 Mafura oil

Mafura oil (Figure 2) is obtained from *Trichilia emetica* seeds. The seeds consist of an oily shell-like husk (makes up 23% of the seed) and a kernel (77% of the seed). The kernels contain a brownish fat (55 – 65%) and 13% protein (Fupi and Mork, 1982). Mafura oil can be extracted either from the kernel or from the pulp (Daniel and McCrae, 1908, Fupi and Mork,

1982, Mashungwa and Mmolotsi, 2007). The oil extracted from the pulp is pale yellow (liquid at ordinary temperature) with no bitterness or emetic properties and can be used for cooking purposes (Daniel and McCrae, 1908, Fupi and Mork, 1982, Mashungwa and Mmolotsi, 2007).

The oil extracted from ground kernels is solid at fairly high temperature, pale brown to brown in colour and is not suitable for consumption because it is bitter and has emetic properties (Daniel and McCrae, 1908, Fupi and Mork, 1982, Mashungwa and Mmolotsi, 2007). Adinew (2014) also reported that mafura oil obtained from crushed kernels needs to be purified to make it suitable for consumption. As reported by Daniel and McCrae (1908), the oil obtained from the kernels was used for greasing the skin of the natives in Portuguese East Africa (that is, Mozambique). It is suggested that the brownish colour may be as a result of Maillard browning reactions that take place during storage and processing or may also be due to the degradation of sugars (glucose and fructose) (Fupi and Mork, 1982).

Fupi and Mork (1982), Grundy and Campbell (1993) and Adinew (2014) raised concerns regarding the bitter taste, emetic properties, brown colour (which gives the oil low aesthetic appeal) and high acid value of *Trichilia emetica* seed oil for human consumption. The research carried out in this study is an attempt to address some of these concerns.

Figure 2 - Mafura oil extracted in Mozambique (Zavala district, Inhambane province).

1.2.2.1 Physico-chemical characteristics of mafura oil

Mafura oil is a vegetable edible oil with high nutritive value which can provide some nutrients such as vitamins, fatty acids (with high content of palmitic and oleic acids and low content of linoleic, linolenic and stearic acids) and minerals (Engelter and Wehmeyer, 1970). Grundy and Campbell (1993) and Mashungwa and Mmolotsi (2007) reported the properties of oil extracted from *Trichilia emetica* seeds. Table 1 gives the physico-chemical properties of Mafura oil in comparison with the more commonly known sunflower and olive oils.

Table 1 - Physico-chemical properties of mafura oil (Engelter and Wehmeyer, 1970)[1] and (Vermaak et al., 2011)[2], (Adinew, 2014)[3], sunflower oil (Anjum et al., 2006) and olive oil (Satue et al., 1995)[4], (Dimberu and Belete, 2011)[5] and (Kostik et al., 2012)[6].

Properties	Mafura oil	Sunflower oil	Olive oil
Oil content (%)	65.8[3] (kernel)	35.8	-
Palmitic acid (%)	38.3[1]	8.06	11.5[5]
Stearic acid (%)	2.20[1]	4.04	2.00[5]
Oleic acid (%)	48.5[1]	30.8	78.4[5]
Linoleic acid (%)	10.4[1]	53.9	7.00[5]
Linolenic acid (%)	1.00[1]	-	0.00[5]
Melting point (^oC)	30.0[1]	-	-
Refractive Index	1.45 (25^oC)[2]	1.46 (40^oC)	-
Density	0.91 g/cm^3 (20^oC)[2]	0.88 mg/mL (24^oC)	-
Acid value (mg KOH/g oil)	8.13[3]	-	7.29[6]
FFA (% as oleic acid)	-	0.80	0.30[5]
Saponification value (mg KOH/g of oil)	180[3]	189	234[6]
Iodine value (g I_2/100 g oil)	60.2[3]	138	-
Peroxide value	0.56 (mg O_2/g)[3]	3.77 (meq/kg oil)	15.6 (meq/kg oil)[4]
p-Anisidine value	-	3.24	-

1.2.3 Methods of mafura oil extraction

1.2.3.1 Traditional extraction

Mafura oil is extracted traditionally by small scale processors in the South of Mozambique and the process has been described by Matakala et al., (2005). After harvesting, the seeds are sorted, cleaned and sun-dried for about 3 weeks. Then the seeds are stored inside huts where they are preserved using smoke treatment which prevents insect and fungi attack. Thereafter, the seeds are placed in bags until the oil extraction period which is at the end of the season (the seeds are stored for approximately 3 months). Dried seeds are sorted again in order to remove any more rotten seeds. Then the seeds are washed and soaked in cold tap water and covered with leaves of the mafura tree (which helps to soften the seeds) for two days. This mixture of seeds soaked in water is then filtered and the pulp is removed by manual pressing and mixed with water. The kernel is separated from this mixture by filtration and discarded. The liquid pulp is then boiled in clay pots on firewood for at least 3 hours. During boiling a clear emulsion which contains oil appears at the top. The emulsion is skimmed off and placed into a small clay pot which undergoes further boiling for about 2 hours. After this time the mafura oil is obtained as a clear oil layer.

Fupi and Mork (1982) reported that the natives in Tanzania extract mafura oil by boiling ripe whole seeds (composed of kernels and the pulp) for 10 – 15 min, followed by drying in the sun and then agitating in water. The oil obtained originates from the shell and is clear and yellow. According to Daniel and McCrae (1908), the natives in Portuguese East Africa (Mozambique) obtained a clear yellow mafura oil by boiling whole seeds.

1.2.3.2 Solvent extraction

Hexane is widely used for oil extraction due to its high efficiency and production of high oil yield. The use of hexane for oil extraction is based on the principle that a non-polar solute dissolves in a non-polar solvent (Johnson and Lusas, 1983). According to Fupi and Mork (1982) and Adinew (2014) mafura oil can be extracted from ground kernels in a Soxhlet extractor using hexane. The oil is recovered after the hexane has been evaporated off.

1.2.4 Oil refining process

Crude vegetable oils contain various substances such as free fatty acids, mono-, di- and triglycerides, phosphatides, pigments, sterols, tocopherols (Aluyor et al., 2009, Vidrih et al., 2010), traces of metals, flavonoids, tannins and glycolipids (O'Brien, 2004). Some substances such as phospholipids, free fatty acids and metals may act as pro-oxidants and thus compromise the oil quality (Lin et al., 1997, Čmolík and Pokorný, 2000), and lower its shelf-life. In order to produce plant oils of acceptable quality and oxidative stability, crude vegetables oils are taken through a refining process where non-triglyceride materials are removed (Ojeh, 1981, Gordon and Rahman, 1991, Mariod et al., 2012).

Although pro-oxidant substances are removed in each stage of refining, antioxidants may also be removed (Jung et al., 1989). Desirable substances such as tocopherols, phenolic compounds and other minor components that act as natural antioxidants (Sherwin, 1976, Gordon and Rahman, 1991, Yoon and Kim, 1994, Shahidi and Zhong, 2010a) may be removed during refining and this has a negative impact on the oxidative stability of the refined oil. Several authors have reported that refining decreases the oil stability. For instance, Jung et al. (1989) reported that the refining process decreased soybean oil oxidative stability in the following order: crude > deodorized > degummed > refined > bleached oil. Yoon and Kim (1994) reported that crude rice bran oil was more oxidatively stable than its refined oil and observed the following order of oil stability: crude ≥ degummed > bleached = deodorized > alkali-refined oil. Nevertheless, good refining practices such as minimizing contact of the oil with air by using hermetic separators as well as avoiding metallic contaminants (especially iron and copper) may produce good quality oil with good flavour, aroma, colour and stable to oxidation (Sherwin, 1978, Lindley, 1998).

Oil refining may be conducted in two main ways: either by alkali refining or physical refining (Lindley, 1998, Verleyen et al., 2002, Aluyor and Ori-Jesu, 2008) also called steam refining (Cvengros, 1995). Alkali refining involves degumming, neutralization, bleaching and deodorization (Ojeh, 1981, Gordon and Rahman, 1991, Cvengros, 1995, Lin et al., 1997, Mariod et al., 2012) whereas physical refining consists of degumming, bleaching and steam distillation (Tandy and McPherson, 1984, Lindley, 1998). The major difference between the two methods resides in the way free fatty acids are removed (conducted during the

neutralization step for alkali refining and during the steam distillation step for physical refining) (Cvengros, 1995).

According to Fupi and Mork (1982), mafura oil with improved and acceptable flavour was obtained after refining in four consecutive steps: degumming (with 0.14% acetic anhydride and 3% water), neutralization (with 1 N NaOH + 10% excess followed by repeated washings with 0.25 N NaOH, brine and water), bleaching (with 1.5-3.0% Tonsil 0FF + 0.3% active carbon) and deodorization at 220-240^{o}C and 1-2 mmHg for 3 h.

1.2.4.1 Degumming

Degumming is the first step in the refining process during which phosphatides (mostly lecithin and cephalin) (Lin et al., 1997, Mariod et al., 2012) and other polar lipids (mucilaginous gums) (Jung et al., 1989, Čmolík and Pokorný, 2000) are removed by adding water or an acid to the oil (Čmolík and Pokorný, 2000). There are two types of degumming, water degumming and acid degumming (Subramanian and Nakajima, 1997, Verleyen et al., 2002). In the former, phospholipids are removed by mixing the oil with water whereas in the latter, the oil is mixed with citric acid (Verleyen et al., 2002), phosphoric, acetic, oxalic, nitric or boric acid (Ohlson and Svensson, 1976). Water reacts with phosphatides producing hydrated gums (insoluble in oil) which are then removed (Weiss, 1983, Rosenthal et al., 1996) by separators or decanters (Čmolík and Pokorný, 2000). In acid degumming, phosphoric acid forms non-dissociable phosphates with calcium, magnesium or iron (Čmolík and Pokorný, 2000). Ohlson and Svensson (1976) reported that phosphoric acid and oxalic acid are the best degumming agents for rapeseed oil, although phosphoric acid degumming has the disadvantage of producing the undesirable phosphate waste in streams.

1.2.4.2 Neutralization

In this step, free fatty acids, the remaining phospholipids (Jung et al., 1989, Lin et al., 1997), metal ions, soaps (Shahidi and Zhong, 2010a) and chlorophyll are removed (Jung et al., 1989). During neutralization, sodium hydroxide is added to the oil and free fatty acids are neutralized with production of soaps which are then removed during centrifugation (Lin et al., 1997).

1.2.4.3 Bleaching

Bleaching is the process during which the undesirable oil colour is removed or reduced by an adsorption process (Čmolík and Pokorný, 2000). The adsorbents mostly used are hydrated aluminium silicates known as bleaching clays (Rossi et al., 2003), activated carbon and silica-based products (Sabah et al., 2007). Chlorophyll and carotenoids are the pigments present in the oil which are removed. Residual fatty acid salts (Jung et al., 1989) and peroxides (primary oxidation products) are also removed (Shahidi and Zhong, 2010a).

1.2.4.4 Deodorization

Deodorization (also known as vacuum steam distillation) (Cowan, 1976) is the last step in the refining process during which volatile compounds, carotenoids and other residual pigments, free fatty acids, tocopherols, sterols and secondary oxidation products are removed to improve flavour quality and stability of the oil (Jung et al., 1989, Shahidi and Zhong, 2010a).

1.2.5 Deterioration of vegetable oils

Vegetable oils deteriorate with time through lipolysis or oxidation (Robards et al., 1988). Lipolysis or hydrolytic rancidity is the hydrolysis of the ester linkages of the triglyceride molecule through the action of lipase (enzymatic hydrolysis) or the presence of moisture and heat (non-enzymatic hydrolysis), resulting in the formation of free fatty acids (Buransompob et al., 2003, Osawa et al., 2007). The free fatty acids formed are responsible for undesirable flavours and aromas especially when they are polyunsaturated (Osawa et al., 2007). Oxidation is the major cause of degradation of edible oils. It causes modifications in the physical properties of the oil such as texture, appearance, flavour, aroma (McClements and Decker, 2000, Velasco and Dobarganes, 2002, Shahidi and Zhong, 2010a, Vidrih et al., 2010) viscosity, density, solubility (Šimon et al., 2000) as well as formation of potentially toxic oxidation compounds (Kubow, 1992) which can lead to development of diseases such as cancer (Kanazawa et al., 2002, Choe and Min, 2006, Shahidi and Zhong, 2010a). Lipid oxidation causes rancidity (Frankel, 1993) and this reduces the acceptability of the oil to consumers (Min and Boff, 2002, Choe and Min, 2006).

Lipid oxidation (also known as auto-oxidation) can be initiated through different mechanisms such as: the action of atmospheric oxygen, the presence of UV-light and a sensitizer or through the action of an enzyme (Naz et al., 2004, Wasowicz et al., 2004). Auto-oxidation occurs during processing and storage in three stages: initiation, propagation and termination via a free radical chain reaction mechanism (Gray, 1978, Shahidi and Zhong, 2010a).

Initiation: In the initiation stage a free radical is produced when an allylic hydrogen atom is removed from an unsaturated lipid under conditions such as heat, light/ionizing radiation and presence of catalysts such as metal ions and metallo-proteins (Reaction 01) (Sherwin, 1976, Gunstone, 2004, Wasowicz et al., 2004, Kamal-Eldin, 2006, Shahidi and Zhong, 2010a)**.**

$$RH \xrightarrow{\text{initiator}} R^{\cdot} + H^{\cdot} \qquad (01)$$

Weakly bound hydrogen atoms (hydrogen atoms whose removal requires the least energy) are the most labile or most susceptible to removal from carbon during this stage (Frankel, 1985). The degree of unsaturation of fatty acids is of importance (Frankel, 1985). In monounsaturated fatty acids the loss of hydrogen atoms takes place at the carbon next to the double bond or at a tertiary carbon atom. These bonds are weaker and easier to break due to lower carbon-hydrogen bond energy. In polyunsaturated fatty acids, the most labile hydrogen atoms are in the methylene groups between two double bonds (Wasowicz et al., 2004).

Propagation: The free radical produced in the initiation stage reacts with atmospheric oxygen and produces peroxyl radicals (ROO·) (Reaction 02). These radicals are highly reactive and immediately react with other unsaturated lipids to produce hydroperoxides (ROOH) and regenerate the free radical (R·) (Reaction 03) (Wasowicz et al., 2004, Shahidi and Zhong, 2010a). Hydroperoxides are primary oxidation products which indicate the first stages of the oxidation process (Frankel, 2014). Lipid hydroperoxides are unstable and in the presence of metals or at high temperatures, they can easily decompose to alkoxy and peroxyl radicals (RO· and ROO·, respectively) (Reactions 04 and 05). Thereafter, alkoxy radicals undergo homolytic carbon-carbon scission and form secondary oxidation products such as aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, among others (Choe and Min, 2006, Shahidi and Wanasundara, 2008).

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot} \quad (02)$$

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot} \quad (03)$$

$$ROOH \xrightarrow{\text{heat / UV light}} RO^{\cdot} + {}^{\cdot}OH \quad (04)$$

$$ROOH \xrightarrow{\text{oxidizing metals}} ROO^{\cdot} + H^{+} \quad (05)$$

Secondary oxidation products such as 2,4-decadienal, 2,4-nonadienal, 2,4-octadienal, 2-heptanal or 2-octenal give desirable characteristic deep-fried flavour (Warner et al., 2001) whereas hexanal, heptanal, octanal, nonanal and 2-decenal contribute to undesirable odours, therefore, they are considered as the products that give off-flavours to the foods (Shahidi and Wanasundara, 2008).

Termination: The alkyl and peroxyl radicals produced in propagation undergo dimerisation and form stable products (non-radical products) (Reactions 06, 07 and 08) preventing further reactions (McClements and Decker, 2000, Gunstone, 2004, Kołakowska, 2010, Frankel, 2014).

$$R^{\cdot} + R^{\cdot} \longrightarrow R\text{-}R \quad (06)$$

$$R^{\cdot} + ROO^{\cdot} \longrightarrow ROOR \quad (07)$$

$$ROO^{\cdot} + ROO^{\cdot} \longrightarrow ROOR + O_2 \quad (08)$$

Stable products can also be formed by the action of antioxidants which donate a hydrogen atom to the radicals formed in the propagation step (section 1.2.5.1.4.5).

1.2.5.1 Factors that influence oxidative stability of vegetable oils

Oxidative stability is an important quality parameter for oils and fats (Muik et al., 2005, Bozan and Temelli, 2008). It is a measure of the resistance of the oil to oxidation which depends on the composition of the oil, how it has been processed and on the external conditions to which the oil has been subjected, which may be controlled, to some extent, by edible oil processors (Guillén and Cabo, 2002, Matthäus, 2010).

More specifically, the oxidative stability of vegetable oils can be affected by factors such as the fatty acid composition, the oil purification process, presence of light and moisture (Naz et al., 2004, Bozan and Temelli, 2008, Matthäus, 2010), the storage temperature, the presence of oxygen (Choe and Min, 2006, Matthäus, 2010, Vidrih et al., 2010), the minor unsaponifiable compounds in the oil (phenolic compounds, tocopherols, chlorophylls, metals), and the minor saponifiable compounds in the oil (free fatty acids, phospholipids) (Velasco and Dobarganes, 2002, Kamal-Eldin, 2006, Matthäus, 2010, Shahidi and Zhong, 2010a).

The oxidative stability may be improved, to some extent, by controlling the external conditions (light, temperature, oxygen, water) to which the oil is subjected, using antioxidants (natural or synthetic) or through genetic modification or conventional breeding (Matthäus, 2010). The fatty acids can be modified through genetic modification producing high-oleic edible oils with high oxidative stability because oleic acid is a monounsaturated fatty acid and less prone to oxidation. However, it is important to take into account that it is only possible to delay the oxidative deterioration but not to avoid it (Matthäus, 2010).

1.2.5.1.1 Fatty acid composition

Oils with higher levels of polyunsaturated fatty acids such as linolenic acid (Figure 3) are less oxidatively stable (Gray, 1978, Frega et al., 1999) than oils rich in monounsaturated fatty acids. Polyunsaturated fatty acids (PUFA) are most susceptible to oxidation due to the fact that they contain a high degree of unsaturation (Shahidi and Zhong, 2010a). The number of double bonds determines the rate of oxidation since the chemical reactions take place at the double bonds (Morelló et al., 2004).

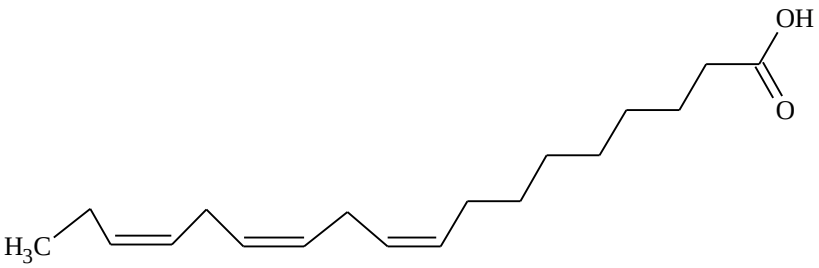


Figure 3 - α-Linolenic acid (ALA) (Shahidi and Zhong, 2010a).

1.2.5.1.2 Oil processing

Processing conditions may affect the oxidative stability and the shelf life of edible oils. Extensive extraction conditions, as well as intensive heat treatment, affect the oil quality through the production of oxidation products that are responsible for off-flavours (Matthäus, 2010). During oil extraction some undesirable substances that may impair the oil quality are also extracted, therefore, crude oils undergo a refining process. However, the refining process may affect the oxidative stability of the edible oils (section 1.2.4). Initial oilseed quality, improper handling and storage of the oilseeds as well as the composition of the crude oil as a result of how it has been extracted may affect the quality of the finished oil (Matthäus, 2010).

1.2.5.1.3 Environmental conditions

Temperature is an important factor that may accelerate the oxidative degradation of edible oils (Matthäus, 2010). The higher the temperature of storage, the higher the rate of auto-oxidation of edible oils, especially at temperatures above 60 ^{o}C (Sherwin, 1978). As the storage temperature increases, the decomposition of hydroperoxides into secondary oxidation products increases, thus lowering the quality of the oil (Choe and Min, 2006). According to Matthäus (2010) for every temperature increase of 10^{o}C, the rate of the oxidative reaction doubles and the storage stability is reduced to half.

The presence of ultraviolet (UV) or near-ultraviolet light also affects oil stability (Sherwin, 1978). Therefore, packaging materials used for oils are important. Transparent glass or plastic bottles can lead to oil oxidation due to exposure to light. It is, therefore, necessary to use opaque containers or incorporate substances that absorb UV light thus protecting the oils from oxidative deterioration (Azeredo et al., 2003, Matthäus, 2010). Ultraviolet light absorbers that can be used for food packaging include Tinuvin 326 (2-(3’-tert-butyl-2’-hydroxy-5’-methylphenyl)-5-chlorobenzo-triazole) (Pascall et al., 1995) or Tinuvin 234 (2-(2-hydroxy-3,5-di(1,1-dimethylbenzyl)phenyl) benzotriazole) (Azeredo et al., 2003).

Oxidation reactions can only take place in the presence of oxygen, especially when the oil is in contact with oxygen at elevated temperature. Therefore, it is important to avoid contact of the oil with oxygen or to decrease the storage temperature (Matthäus, 2010). The headspace in an oil storage container should be reduced in order to prevent oxidation. This is because

when the oxygen partial pressure in the headspace is high, a high amount of oxygen is dissolved in the oil thus increasing the oxidation of the oil (Andersson, 1998 according to Choe and Min, 2006). Also high levels of oxygen can lead to the formation of volatile compounds during storage which can be minimized by lowering the dissolved free oxygen in oil (Min and Wen, 1983b). The type of oxygen also influences the oxidation of the oil. The rate of lipid oxidation with singlet oxygen ($^1O_2$) is higher than with triplet oxygen ($^3O_2$). This is because $^1O_2$ reacts directly with lipids while $^3O_2$ reacts with the radical state of lipids. $^3O_2$ easily reacts with lipid radical because it contains 2 unpaired orbitals in the molecule whereas $^1O_2$ does not contain unpaired orbitals, so it is a non-radical and readily reacts with compounds with high electron densities such as the double bonds of unsaturated fatty acids (Choe and Min, 2006).

1.2.5.1.4 Minor components present in oil

Minor components (such as free fatty acids, metals, phospholipids, thermally oxidized compounds, antioxidants) affect the oxidative stability of edible oils by either acting as prooxidants or antioxidants. Prooxidants such as metal ions (e.g. Fe^{2+} and Cu^{2+}) accelerate the oxidation rate of the oils by acting as catalysts or by inhibiting the antioxidant action whereas antioxidants (such as tocopherols) inhibit or retard oil oxidation through radical scavenging (Martin-Polvillo et al., 2004).

1.2.5.1.4.1 Free fatty acids

Free fatty acids are present in crude edible oil as a result of hydrolysis (or lipolysis) in the presence of moisture (Narayan et al., 1988, O'Brien, 2004), exposure to light and heat (Pearson, 1976, O'Brien, 2004) or by the action of lipase enzyme (Robards et al., 1988, Ramezanzadeh et al., 1999, Naz et al., 2004, Liauw et al., 2008). Lipolysis involves breaking of ester bonds between glycerol and fatty acids with the formation of free fatty acids that can contribute to off-flavours (O'Brien, 2004). The free fatty acids tend to be present in low amounts in purified oils because they are removed during the refining process (Choe and Min, 2006). Free fatty acids act as prooxidants (Miyashita and Takagi, 1986, Mistry and Min, 1987) and accelerate the oil oxidation rate by decreasing the surface tension of edible oils and increasing the diffusion rate of oxygen from the headspace (Mistry and Min, 1987). Free fatty

acids react with oxygen producing peroxyl radicals which in reaction with unsaturated lipids produce hydroperoxides (section 1.2.5).

1.2.5.1.4.2 Metals

Iron and copper are transition metals that may enter the oil during its extraction from the raw material (Choe and Min, 2006, Matthäus, 2010) or may be introduced from the processing equipment (Sherwin, 1978). They are removed partly during the refining process (section 1.2.4), however, residual metal ions act as prooxidants and lower the oxidative stability of oils (Min and Wen, 1983a). Cobalt and nickel also have deteriorating effects on the stability of the oil (Benjellourr et al., 1991). Metals increase the rate of oil oxidation due to the reduction of activation energy of the initiation step in the autoxidation down to 63~104 kJ/mol (Jadhav and others 1996 according to Choe and Min, 2006). Metals catalyse oxidation of oils in trace amounts (at concentrations less than 1 ppm) (Sherwin, 1978) through degradation of primary oxidation products and formation of alkyl radicals (Reactions 09 and 10) initiating the chain reaction of auto-oxidation (section 1.2.5) (Benjellourr et al., 1991, Matthäus, 2010).

$$Me^{n+} + ROOH \longrightarrow RO^{\cdot} + Me^{(n+1)+} + OH^{-} \quad (09)$$

$$Me^{(n+1)+} + ROOH \longrightarrow ROO^{\cdot} + Me^{n+} + H^{+} \quad (10)$$

1.2.5.1.4.3 Phospholipids

Phospholipids such as phosphatidylethanolamine, phosphhatidylcholine, phosphatidylinositol, among others, are present in crude oils. They are partly removed during degumming (section 1.2.4.1). They may act as both prooxidants and antioxidants depending on their concentration and on the presence of metals (Choe and Min, 2006). Phospholipids act as antioxidants inhibiting the prooxidant activity of the metals due to their ability to chelate metal ions, therefore, reducing the oil oxidation (Velasco and Dobarganes, 2002). In the absence of metals, phospholipids act as prooxidants decreasing the surface tension of edible oil and increasing the diffusion rate of oxygen from the headspace, therefore accelerating the oil oxidation (Choe and Min, 2006).

1.2.5.1.4.4 Thermally oxidized compounds

Thermally oxidized compounds are compounds produced during oil processing due to the high temperature used in processing steps (Choe and Min, 2006). The oxidized compounds produced include cyclic and noncyclic carbon-to-carbon-linked dimers and trimers, hydroxydimers and dimers and trimers joined through carbon-to-oxygen linkage (Choe and Min, 2006). They act as prooxidants and accelerate the rate of oil oxidation by decreasing the surface tension and increasing the levels of oxygen (Choe and Min, 2006).

1.2.5.1.4.5 Antioxidants

Antioxidants are substances naturally present in oils or intentionally added with the aim of retarding or delaying lipid oxidation (Wasowicz et al., 2004). However, they are not able to improve the quality of an oxidized product. Antioxidants inhibit oxidative deterioration by scavenging free radicals (Reactions 11, 12 and 13) (lipid alkyl or peroxy radicals), quenching singlet or triplet oxygen, decompose peroxide (Djeridane et al., 2006) chelating transition metals and inactivating sensitizers (Choe and Min, 2006). They are classified as primary, secondary and synergist antioxidants (Eskin and Przybylski, 2000). Primary antioxidants (such as tocopherols (Figure 5), BHT, BHA, TBHQ, PG (Figure 6) and phenolic compounds (Figure 7)) are able to stop the radical chain reaction by donating a hydrogen atom to a lipid radical (Sherwin, 1976, Shahidi and Zhong, 2010a).

$$R^{\cdot} + AH \longrightarrow RH + A^{\cdot} \qquad (11)$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot} \qquad (12)$$

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \qquad (13)$$

The antioxidant radical produced is stabilized by resonance by delocalization of the unpaired electron around the phenol ring (Figure 4) and, therefore, they have low reactivity and do not propagate the oxidation reaction (Kamal-Eldin, 2006, Shahidi and Zhong, 2010a).

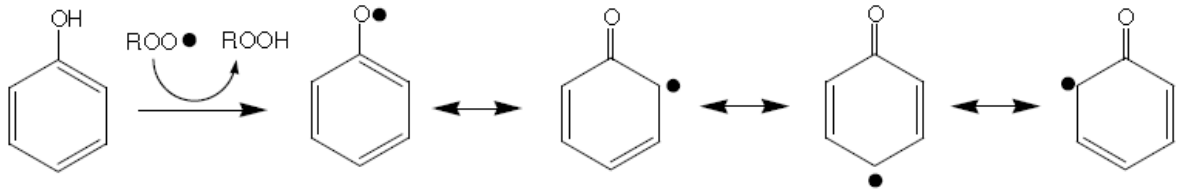


Figure 4 - Resonance stabilization of an antioxidant radical (Choe and Min, 2006).

The relatively stable antioxidant radicals react to form stable non-radical compounds (Reactions 14, 15 and 16) (Shahidi and Zhong, 2010a).

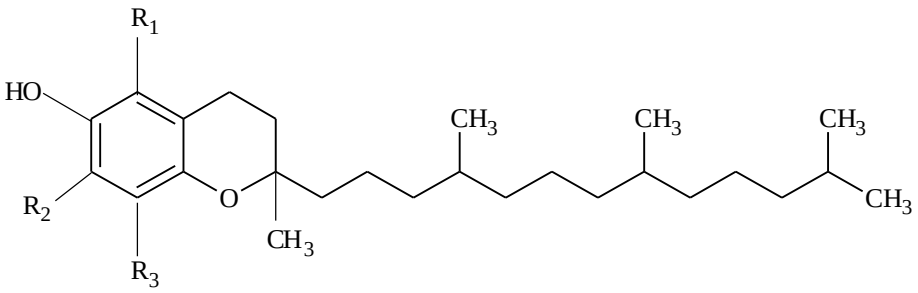
$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA \qquad (14)$$

$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA \qquad (15)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow AA \qquad (16)$$

Vegetable oils contain tocopherols as their main antioxidants. Tocopherols act as primary antioxidants and are able to retard lipid oxidation by donating a hydrogen atom to lipid radicals and forming more stable non-radicals (Shahidi and Zhong, 2010a). During oil processing and refining, some tocopherol is lost but a large proportion remains in the oil (Frankel, 1996). Morelló et al. (2004) reported that after 12 months of storage of virgin oil in the dark, α-tocopherol was no longer present in the oil.

The effectiveness of tocopherols is related to their isomers (α-, β-, γ- and δ- tocopherols, figure 5) and also their concentration in the oils (Choe and Min, 2006, Kamal-Eldin, 2006). α-Tocopherol has the highest antioxidant activity (Ferrari et al., 1996, Bozan and Temelli, 2008). Tocopherols are efficient antioxidants at low concentrations but tend to have decreased efficiency at high concentrations (Kamal-Eldin, 2006, Bozan and Temelli, 2008). Therefore, tocopherols are considered to have pro-oxidant activity as their concentration increases (Cillard et al., 1980, Kamal-Eldin, 2006).



α-tocopherol: $R_1 = R_2 = R_3 = CH_3$ β-tocopherol: $R_1 = R_3 = CH_3$; $R_2 = H$

γ-tocopherol: $R_1 = H$; $R_2 = R_3 = CH_3$ δ-tocopherol: $R_1 = R_2 = H$; $R_3 = CH_3$

Figure 5 - Chemical structure of tocopherols (Shahidi and Zhong, 2010a).

Secondary antioxidants inhibit the formation of free radicals through various mechanisms (Sikwese, 2008, Shahidi and Zhong, 2010a). They act as reducers and chelators of metals (e.g. citric acid), oxygen scavengers and reducing agents (e.g. ascorbic acid), singlet oxygen quenchers (carotenoids) (Reische et al., 2002 according to Wąsowicz et al., 2004), and substances able to recover primary antioxidants (ascorbic acid) (Wasowicz et al., 2004).

Synergistic antioxidants function by enhancing the activity of primary antioxidants naturally present or the ones added into the food lipid (Sherwin, 1976). These include ascorbic acid and citric acid (Eskin and Przybylski, 2000). They act through different mechanisms such as regeneration of primary antioxidants by donating hydrogen atoms to phenoxyl radical (Sherwin, 1976, Eskin and Przybylski, 2000), chelation of prooxidant metals and inhibition of peroxide decomposition (Sherwin, 1976). However, these acid-antioxidants exhibit strong antioxidant effect only if used in combination with another primary antioxidant such as tocopherol (Sherwin, 1976).

1.2.6 The use of natural and synthetic antioxidants in vegetable oils

Oxidative deterioration of vegetable oils may be prevented or decreased by using natural or synthetic antioxidants. Some synthetic antioxidants (Figure 6) most commonly used are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ) and propyl gallate (PG) (Ito et al., 1986, Shahidi, 1997, Moure et al., 2001, Aluyor and Ori-Jesu, 2008, Matthäus, 2010). These antioxidants are widely used in food industries due to their effectiveness in prevention of lipid oxidation. However, some concerns regarding their safety and toxicity have been raised and they are also linked to possible carcinogenic

effects (Whysner et al., 1994, Wanasundara and Shahidi, 1998, Matthäus, 2010). Therefore, there is a growing interest in the potential use of antioxidants from natural sources (Iqbal and Bhanger, 2007, Matthäus, 2010, Mohdaly et al., 2011).

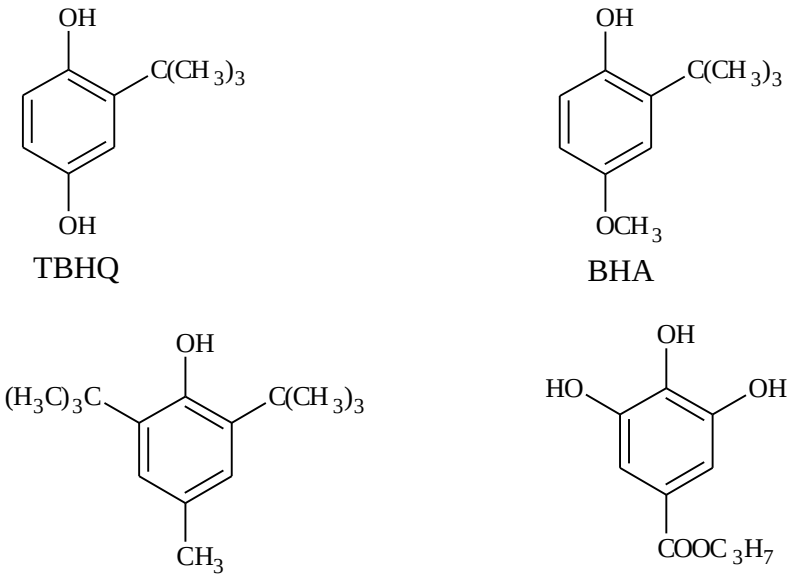


Figure 6 - Chemical structures of some synthetic antioxidants (TBHQ – tert-butylhydroxyquinone, BHA – butylated hydroxyanisole, BHT – butylated hydroxytoluene, PG – propyl gallate) used to prevent lipid oxidation (Sikwese and Duodu, 2007).

Phenolic extracts from plant sources such as herbs, seeds, fruits, cereals and roots have been used to stabilize oils against oxidation due to the antioxidant properties of phenolic compounds (Kähkönen et al., 1999, Iqbal et al., 2008, Mohdaly et al., 2011). The antioxidant activity is due to their redox properties which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers and metal chelators (Rice-Evans et al., 1995 according to Kähkönen et al., 1999). Phenolic compounds are a wide variety of compounds found in plants whose structures are based on one or more hydroxyl substituents bonded onto an aromatic ring (Waterman and Mole, 1994) and may be divided into three main groups based on their size: phenolic acids, flavonoids and tannins (Dykes et al., 2014).

Phenolic acids (Figure 7 A and 7 B) are derivatives of benzoic and cinnamic acids, respectively and may be present in plants in free or bound forms (e.g. esterified to cell wall components) (Waniska, 2000, Hahn et al., 1984 according to Dykes, 2006). Flavonoids

(Figure 7 C) are phenolic compounds with a C6-C3-C6 structural skeleton (Waterman and Mole, 1994). The most common classes of flavonoids found in plants are flavones, flavanones, flavonols, flavanols and anthocyanins (Hahn et al., 1984 according to Sikwese, 2008). Tannins in plants fall into two major categories. These are hydrolysable tannins (they break down into phenolic acids and sugars by hydrolysis with acid, alkali or hydrolytic enzymes) and condensed tannins (Figure 7 D) which are polymers of flavan-3-ol and/or flavan-3,4-diol and yield anthocyanins upon heating in acidic media and, therefore, are called proanthocyanidins (Awika and Rooney, 2004).

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal in the world and it is especially widespread in Africa (Awika and Rooney, 2004, Dykes and Rooney, 2006). It is a drought-tolerant cereal that grows in semi-arid tropics of Africa and Asia (FAO, 1995). This crop is a source of energy, vitamins, proteins and minerals for the people in these regions (FAO, 1995) and it is reported to contain a large number of bioactive compounds including phenolic compounds (Awika and Rooney, 2004, Dykes and Rooney, 2006, Dykes et al., 2014). These phenolics have been reported to have antioxidant activity. Therefore, phenolic extracts from sorghum may be used as natural antioxidants in food lipids to prevent lipid oxidation.

There are different types of sorghum which vary in their phenolic composition. Sorghum is classified into type I, type II and type III (Awika and Rooney, 2004). All sorghum types contain phenolic acids and most contain flavonoids, but only type II and type III contain tannins (Waniska, 2000, Awika and Rooney, 2004, Dykes and Rooney, 2006). In type II sorghums the condensed tannins are present in the pigmented testa while in type III sorghums, the tannins are present in the pigmented testa and pericarp and in addition, the type III sorghums contain more tannins than type II (Waniska, 2000, Taylor and Duodu, 2009) Type I sorghums do not contain pigmented testa (Dykes and Rooney, 2006). Tannins in types II and III sorghums are extracted using different solvents due to the way tannins are deposited in the testa layer (Dykes and Rooney, 2006). Tannins of type II sorghum are deposited in the vesicles within the testa layer and are extracted with acidified methanol whereas tannins of type III sorghum are deposited along the cell wall of the testa and are extracted with either methanol or acidified methanol (Dykes and Rooney, 2006).

The presence of tannins is not related to the colour of the sorghum grain. Some white sorghum varieties may contain tannin while some red, brown or black sorghum varieties may not contain tannins (Waniska, 2000, Awika and Rooney, 2004, Dykes and Rooney, 2006, Taylor and Duodu, 2009).

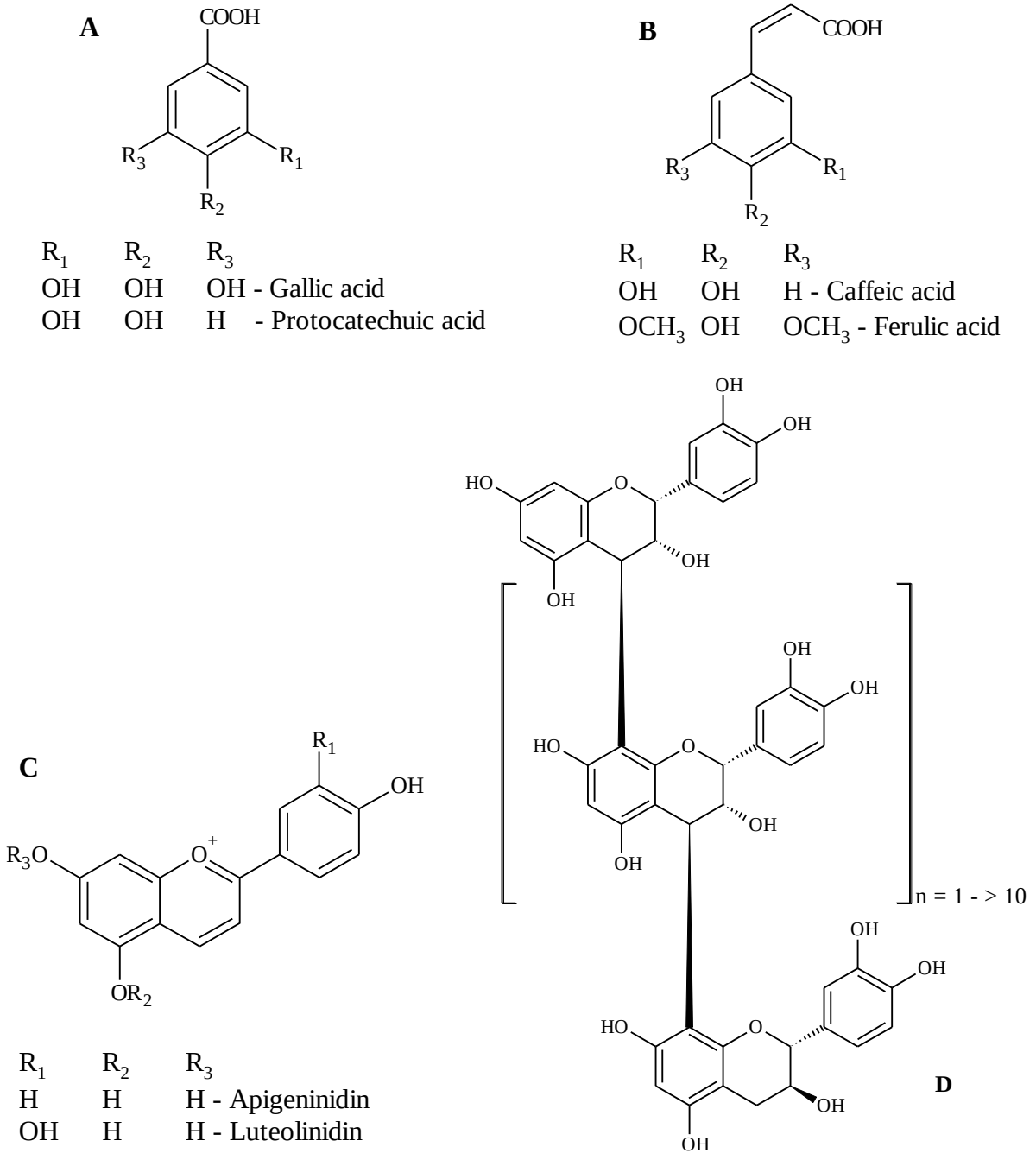


Figure 7 - Some phenolic compounds found in sorghum (A – Phenolic acid derivatives of benzoic acid, B – Phenolic acid derivatives of cinnamic acid, C – 3-deoxyanthocyanidins (the most common anthocyanins in sorghum reported by Awika and Rooney (2004), D – Proanthocyanins (condensed tannins most common in sorghum reported by Awika and Rooney (2004) and Dykes and Rooney (2006)).

Sikwese and Duodu (2007) reported that phenolic extracts prepared from condensed tannin sorghum bran were able to inhibit oxidation of sunflower oil during 14 days of storage. The oil with added phenolic extracts showed reduced formation of primary oxidation products compared to the oil with no addition of phenolic extract even though both samples showed an increase in peroxide value during storage. Anisidine values remained almost the same over the 14 day storage period, suggesting that the phenolic extracts prevented the formation of secondary oxidation products and thus protected the oil against oxidation.

1.2.7 Factors that influence the antioxidant capacity of phenolic compounds

1.2.7.1 Concentration

The antioxidant capacity of phenolic compounds in terms of their ability to scavenge free radicals depends on their concentration (Rice-Evans et al., 1996, Milić et al., 1998). Generally, at lower concentrations, phenolic compounds exhibit lower radical scavenging activities as compared to their presence in high concentration (Milić et al., 1998). It was reported that gallic acid at a concentration of 2 mmol was more effective in scavenging lipid alkoxyl radical than when present at a lower concentration of 0.5 mmol (Milić et al., 1998).

1.2.7.2 Structure-antioxidant activity relationships of phenolic compounds

A very important determinant of the ability of a phenolic compound to exert antioxidant effects is its chemical structure. The antioxidant activity of phenolic acids depends on the number and position of the hydroxyl groups (-OH) in relation to the carboxyl functional group (-COOH). Hydroxyl groups enhance the antioxidant activity of the phenolic acids. The more phenolic hydroxyl groups on phenolic acids the higher the antioxidant activity (Rice-Evans et al., 1996, Milić et al., 1998, Robards et al., 1999, van den Berg et al., 2000) because it provides more sites for radical scavenging (Sikwese, 2008). Gallic acid, (Figure 7A) which contains three hydroxyl groups exhibits a higher inhibition effect on free radical formation than caffeic acid which possesses two hydroxyl groups (Figure 7B) (Milić et al., 1998).

Substitution of hydroxyl groups in the ortho-, meta- and para-positions in relation to the carboxyl functional groups affects the antioxidant activity of the phenolic acid (Rice-Evans et al., 1996). Mono hydroxybenzoic acids substituted in the ortho- or para-positions exhibit no

antioxidant activity whereas hydroxyl groups in the meta-position have the ability to donate hydrogen to free radicals, thus exhibiting antioxidant activity (Rice-Evans et al., 1996). Benzoic acid derivatives di-substituted with hydroxyl groups in the ortho- and meta-positions in relation to the carboxyl group exhibit higher antioxidant activity than those di-substituted in the meta-, para-positions or both ortho-positions (Rice-Evans et al., 1996). Increasing the hydroxylation results in higher antioxidant activity, whereas methoxylation (substitution of the –OH for –OCH_3) decreases the antioxidant activity. In the case of the hydroxycinnamic acids, they exhibit higher antioxidant activity than the corresponding hydroxybenzoic acids due to the presence of the group –CH=CH-COOH. The ethylenic group enhances the reducing properties of the –OH group ensuring greater hydrogen donating ability and stabilization of the free radicals (Rice-Evans et al., 1996).

In the case of flavonoids, the structure and the nature of substitutions on rings B and C affects the antioxidant activity (Jovanovic et al., 1994, Balasundram et al., 2006). Unsaturation in the C ring between C-2 and C-3 conjugated with a 4-oxo group confers high antioxidant activity to flavonoids due to the electron delocalization across the molecule for stabilization of the aryloxyl radical after hydrogen donation (Rice-Evans et al., 1996, Pietta, 2000). This was demonstrated by Rice-Evans et al. (1996) which reported that quercetin which contains a 2,3-double bond in the C ring and the 4-oxo function had higher antioxidant activity than catechin which has a saturated heterocyclic ring.

1.2.8 Methods for determination of oxidative stability of oils

The quality of oils may be determined by the measurement of their oxidative stability during storage (Guillén and Cabo, 2002, Tan et al., 2002, Bozan and Temelli, 2008). To assess the extent of oxidation, oils and fats are subjected to conditions that induce oxidation such as high temperature, presence of light, metals, oxygen, among others in order to accelerate the deterioration process and reduce the stability test period (Frankel, 1993, Frega et al., 1999, Guillén and Cabo, 2002). The oxidative stability can be assessed through various methods such as peroxide value (Chu and Hsu, 1999, Abou-Gharbia et al., 2000, Rudnik et al., 2001), anisidine value (Abou-Gharbia et al., 2000, Guillén and Cabo, 2002), oxidative stability index (OSI) (Chu and Hsu, 1999, Tan et al., 2002), differential scanning calorimetry (DSC) (Rudnik et al., 2001, Tan et al., 2002), conjugated diene hydroperoxides and volatile compounds (Abdalla and Roozen, 1999), among others. These methods are related to the

measurement of the concentration of primary and secondary oxidation products in which the most commonly used are peroxide and anisidine values (Muik et al., 2005).

1.2.8.1 Peroxide value

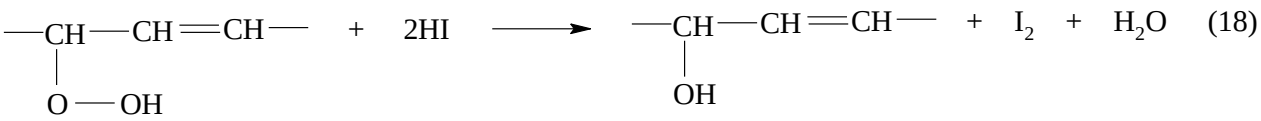
Peroxide value is the most common method used to determine the concentration of primary oxidation products (hydroperoxides) (Guillén and Cabo, 2002, O'Brien, 2004, Bozan and Temelli, 2008) which are formed by the reaction between unsaturated fatty acids and lipid radicals, as mentioned earlier (Matthäus, 2010). The peroxide value describes the oxidative deterioration in the early stages and under mild conditions (at temperatures above 100^{o}C the primary oxidation products only exist transiently) (Bozan and Temelli, 2008). During storage, the peroxide value increases to a maximum and then decreases (O'Brien, 2004, Bozan and Temelli, 2008) due to decomposition of hydroperoxides into other substances. Therefore, the peroxide value on its own is not enough to determine and quantify oxidative rancidity of food lipids (Matthäus, 2010). It is however regarded as a good guide to the quality of oils (Rossell, 1986) in the sense that high levels of peroxide value indicate that the formation of primary oxidation products has started but not that the oil is rancid (O'Brien, 2004). Therefore, in order to have reliable information regarding the oxidative stability of the oil, peroxide value measurement should be combined with other analyses (Matthäus, 2010).

According to Matthäus (2010), refined oils with good quality should have peroxide value between 0.5 to 1 mEq O_2/ kg oil and virgin oils can be higher, up to 3 mEq O_2/ kg oil. Rossell (1986) similarly reported that freshly refined oils should have a peroxide value of up to 1 mEq O_2/ kg oil whereas fats that have been stored for some time after refining may have a peroxide value of up to 10 mEq O_2/ kg oil. According to Abramovic and Abram (2005), PV of 20 mEq/kg oil is the upper limit for unrefined oils. O'Brien (2004) reported that for soybean oil, a peroxide value of 1 or less indicates freshness; 1 to 5 indicates low oxidation; 5 to 10 indicates moderate oxidation; > 10 indicates high oxidation and > 20 indicates poor flavour.

The concentration of hydroperoxides may be measured using several analytical procedures. It can be measured using colorimetry (Rossell, 1986), iodometric titration (Rossell, 1986, AOCS, 1989, O´Brien, 2004), chemiluminescence, polarographic analysis or High Performance Liquid Chromatographic (HPLC) (Matthäus, 2010). The most commonly used

is the iodometric titration method which measures the concentration of peroxides that oxidize potassium iodide to iodine (Rossell, 1986, AOCS, 1989, O´Brien, 2004). The peroxides (ROOH) react with potassium iodide (KI) in the presence of acetic acid (CH_3COOH) liberating iodine (I_2) (Reactions 17 and 18) which is then titrated with sodium thiosulphate ($Na_2S_2O_3$) (Reaction 19) (Sikwese, 2008, Matthäus, 2010).

$$2KI + 2CH_3OOH \longrightarrow 2HI + 2CH_3COO^-K^+ \quad (17)$$

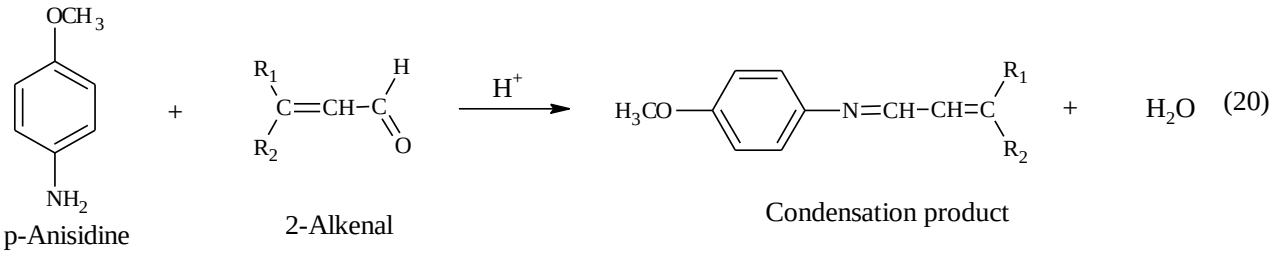


(18)

$$I_2 + 2Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2NaI \quad (19)$$

1.2.8.2 Anisidine value

Anisidine value is used to determine the concentration of α-, β-unsaturated aldehydes principally 2-alkenals and 2,4-dienals formed during oxidation of food lipids (Rossell, 1986, Guillén and Cabo, 2002, O'Brien, 2004, Ramadan and Mörsel, 2004, Muik et al., 2005). The secondary oxidation products are measured spectrophotometrically in the UV region (at 350 nm) (Guillén and Cabo, 2002, O'Brien, 2004, Muik et al., 2005). The aldehydic compounds present in the oil react with p-anisidine in the presence of acetic acid, producing yellowish condensation products (Reaction 20) whose colour intensity depends on the amount of the aldehydic compounds present in the oil and on their structure (Robards et al., 1988, O'Brien, 2004). Dubois et al. (1996) reported that anisidine value may also be determined using Fourier transform infrared spectroscopy (FTIRS) in thermally stressed oils. According to Rossell (1986) the anisidine value of good oils should be less than about 10. Secondary oxidation products are responsible for off-flavours specifically hexanal, therefore, anisidine value gives a reliable information regarding the oxidative stability of the oils and complements peroxide value (Robards et al., 1988).



(20)

1.2.8.3 Total oxidation value

Total oxidation value (Totox) has also been used to determine oxidative stability and it combines the present state of the oil (peroxide value) and the history of the oil (anisidine value). Therefore, it provides information regarding progression of the formation of primary and secondary oxidation products. Total oxidation value is defined as the anisidine value plus twice the peroxide value (Reaction 21) (Rossell, 1986, Matthäus, 2010).

$$\text{Totox value} = \text{Anisidine value} + 2 \times (\text{Peroxide value}) \qquad (21)$$

According to Matthäus (2010) there are no official standards that define the limit of the total oxidation value, therefore, a rule of thumb suggested by Rossell (1986) is applicable, in which a good quality oil should have a totox value less than 10. Frankel (2014) reported that the totox value of good quality oil should be up to 4.

1.3 Hypotheses

- Traditionally extracted crude mafura oil will be more oxidatively stable than sunflower oil and less stable than olive oil. Fats and oils rich in saturated fatty acids are less prone to oxidative deterioration than the ones rich in polyunsaturated fatty acids (Tan et al., 2002, Jacobsen et al., 2008). Unsaturation makes the fats and oils susceptible towards oxidation because the oxidation takes place between the double bonds and the molecular oxygen (Muik et al., 2005, Kolanowski et al., 2007). This leads to the formation of hydroperoxides which are unstable and easily decompose to secondary oxidation products, the ones responsible for rancidity (Kolanowski et al., 2007).

- Crude mafura oil will have higher oxidative stability than refined mafura oil during 14 days of storage under accelerated oxidative conditions. Some of the steps during the refining process such as degumming and neutralization also remove various minor components that act as antioxidants in the crude oil such as tocopherols (Ramadan and Mörsel, 2004, Shahidi and Zhong, 2010a).

- Mafura oil extracted with organic solvents (hexane or ethanol) will have higher oxidative stability than mafura oil extracted using the traditional process during storage under accelerated oxidative conditions over 14-days. The organic solvent-extracted mafura oil will contain higher levels of antioxidant compounds such as tocopherols which are extractable with hexane (Shahidi and Zhong, 2010a) and phenolic compounds which are extractable with ethanol (Krygier et al., 1982) compared with mafura oil extracted using the traditional process. These antioxidant compounds will offer better protection against oxidative deterioration of the organic solvent-extracted mafura oil.

- Mafura oil containing phenolic extracts from sorghum bran will be more oxidatively stable and will have lower peroxide values and anisidine values than mafura oil without extracts during 14 days of storage under accelerated oxidative conditions. Phenolic compounds in the extracts will act as primary antioxidants and scavenge free radicals (Fernandez et al., 2002) preventing the formation of secondary oxidation products.

1.4 Objectives

- To determine the oxidative stability of traditionally extracted crude mafura oil in comparison with commercially available sunflower and olive oils.

- To determine the effect of refining and solvent extraction of mafura oil on its oxidative stability during storage

- To determine the antioxidative effect of phenolic extracts from red condensed tannin sorghum bran on oxidative stability of mafura oil.

CHAPTER 2

2. RESEARCH

2.1 Effect of refining and method of extraction on the oxidative stability of mafura oil.

2.1.1 Abstract

Mafura (*Trichilia emetica* Vahl.) seeds are extracted traditionally for their oil by small scale processors in the South of Mozambique using a process that involves boiling the seed pulp in an aqueous medium for 5 h. However, this extraction procedure may lead to reduced oxidative stability of the oil due to the lengthy exposure to high temperature in an excess amount of water. In this study, the effect of refining and method of extraction on oxidative stability of mafura oil stored under accelerated oxidative conditions was investigated. Palmitic acid was the predominant fatty acid in mafura oil unlike sunflower oil (linoleic acid) and olive oil (oleic acid). Traditionally extracted crude mafura oil had high acid value indicating high levels of free fatty acids which appeared to decrease on refining the oil. Traditionally extracted crude mafura oil was the least oxidatively stable as indicated by its high anisidine values during storage compared to sunflower oil and olive oil. Refining did not improve the oxidative stability of mafura oil. However, solvent extraction of mafura oil (using hexane or ethanol) improved its oxidative stability. This study shows that the traditional method of extraction of mafura oil decreases its oxidative stability due to extensive heating in an aqueous medium.

Key words: Mafura oil, oxidative stability, peroxide value, anisidine value, refining, solvent extraction.

2.1.2 Introduction

Mafura oil (called "munhantsi" in Mozambique) is extracted from mafura (*Trichilia emetica* Vahl.) seeds in the South of Mozambique (Matakala et al., 2005). This tree belongs to the Meliaceae family (Germano et al., 2005, Germano et al., 2006, Komane et al., 2011, Vermaak et al., 2011) and it is widespread in sub-Saharan Africa (Vermaak et al., 2011). All parts of the mafura tree (such as roots, bark, leaves) are used in Senegal, Mali and Zimbabwe to treat or prevent diseases (Diallo et al., 2003, Mashungwa and Mmolotsi, 2007). Mafura oil is rich in fatty acids such as palmitic acid and oleic acid (Engelter and Wehmeyer, 1970, Grundy and Campbell, 1993, Mashungwa and Mmolotsi, 2007, Vermaak et al., 2011) which could potentially make the oil relatively stable to oxidative deterioration at room temperature (Saga et al., 2011).

Matakala et al., (2005) reported that rural communities in Mozambique extract mafura oil by boiling the pulp of the sun-dried seeds mixed with water for about 5 h. This method is labour intensive and the yield of the oil is very low. It is also reported that the natives in Tanzania obtain the oil through boiling the whole mafura seeds (Fupi and Mork, 1982). Mafura oil has also been extracted using organic solvents such as hexane (Fupi and Mork, 1982, Khumalo et al., 2002, Adinew, 2014). Hexane is a non-polar solvent and is widely used for oil extraction due to its high efficiency and high oil yield (Weiss, 1983, Li et al., 2014). Ethanol (a polar solvent) has also been used as an extraction solvent for oils. In spite of the low oil yield due to its relative polarity compared to n-hexane and low selectivity for oils, ethanol has been used due to its ability to extract phenolic compounds which are known as antioxidants and may increase the oxidative stability of the edible oils (Hron Sr et al., 1982, Hron et al., 1994, Ferreira-Dias et al., 2003).

Edible oils undergo oxidative deterioration during storage leading to the formation of primary and secondary oxidation products which are responsible for rancidity (Guillén and Cabo, 2002, Muik et al., 2005). The oxidative stability (resistance to oxidation) is an important quality parameter and it can be determined by the measurement of peroxide and anisidine values (Gray, 1978, Guillén and Cabo, 2002). Peroxide value measures volumetrically the concentration of primary oxidation products which are formed in the early stages of oxidation under mild-conditions and anisidine value measures spectrophotometrically in the ultraviolet region the concentration of secondary oxidation products (Guillén and Cabo, 2002, Muik et

al., 2005), which are principally aldehydic compounds such as 2-alkenals and 2,4-dienals (Tompkins and Perkins, 1999) and saturated aldehydes (Dubois et al., 1996).

The oxidative stability of edible oils depends on the composition of the fatty acids present in the oil as well as the external conditions to which the oil is subjected such as light, temperature and oxygen (Guillén and Cabo, 2002, Matthäus, 2010). Oil oxidation may be prevented by controlling the conditions in which the oil is stored, through the use of antioxidants (Matthäus, 2010) and also through the refining process (Lin et al., 1997, Mariod et al., 2012) during which substances that may act as prooxidants such as free fatty acids (Bhosle and Subramanian, 2005) (which are formed in the presence of moisture, heat and/or enzymes), phospholipids (García et al., 2006) and metals (Wiedermann, 1981, Choe and Min, 2006) as well as substances that may act as antioxidants such as tocopherols and phenolic compounds are removed (Koski et al., 2003, Tasan and Demirci, 2005).

The method of extraction could also affect the oxidative stability of edible oils. For instance, as stated earlier, during the traditional extraction of mafura oil, there is an extensive heating process (boiling of the pulp in excess water for up to 5 h) (Matakala et al., 2005). The high temperatures during boiling, combined with the excess moisture could potentially degrade the mafura oil through a combination of lipolysis and oxidative deterioration and, therefore, reduce its oxidative stability (Narayan et al., 1988, O'Brien, 2004).

The aim of this study was to determine the oxidative stability of mafura oil as influenced by two main parameters namely, refining and method of extraction.

2.1.3 Materials

2.1.3.1 Mafura fruit, sunflower oil and olive oil samples

Ripe mafura fruits (Figure 8) grown in the 2013/2014 season were obtained from Mozambique (Gaza province, Manjacaze district and Inhambane province, Zavala district). Refined sunflower oil, extra-virgin olive oil and olive oil blend (mixture of refined and virgin olive oil) were obtained from a local supermarket in Pretoria, South Africa.

Figure 8 - Mafura fruits used in this study.

2.1.3.2 Reagents

Acetic acid (glacial), chloroform, diethyl ether, ethanol (absolute), hydrochloric acid, phenolphthalein, potassium hydroxide pellets, potassium iodide, sodium hydroxide pellets, sodium thiosulphate pentahydrate and starch (soluble) were obtained from Merck (Johannesburg, South Africa). Acetonitrile, p-anisidine, 4-hydroxybenzoic, 2,2,4-trimethlypentane (isooctane) and Wijs solution were obtained from Merck (Darmstadt, Germany).

2.1.3.3 Traditional extraction of crude mafura oil

Crude mafura oil (Figure 9B), was extracted from the pulp of dried mafura seeds. Ripe mafura fruits were peeled. The seeds were sun-dried for 3 weeks and stored at -20^{o}C until required for oil extraction. For oil extraction, dried mafura seeds were sorted, washed and soaked in tap water for 3 days. Then the pulp was manually removed and the seed kernels were discarded. The pulp was mixed with tap water and filtered to obtain a milky and oily filtrate. The filtrate was boiled for 2.5 h in an aluminium pot. The emulsion appearing at the top of the boiling filtrate was skimmed off and placed into another aluminium pot and boiled for a further 30 min to obtain an oil which looked light brown in colour (Figure 9B).

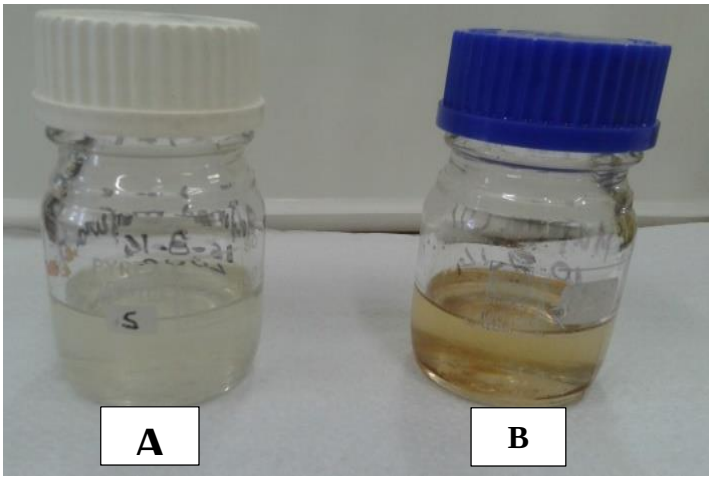


Figure 9 - Refined (A) and crude (B) mafura oils.

2.1.3.4 Preparation of refined mafura oil

Crude mafura oil was refined by using a laboratory scale degumming and neutralization process. Degumming was conducted according to the method described by Tsaknis et al. (1999) and Mariod et al. (2012). 65 g of crude mafura oil was placed in a 400 mL beaker and then 13 ml of boiling water was added to the oil and mixed with the aid of a glass rod for 10 min at 75^{o}C in a temperature-controlled water bath. The mixture was then cooled to room temperature (by placing the beaker containing the oil sample in a cold water bath) and centrifuged (Rotanta 460 R centrifuge, Germany) for 10 min at 3500 rpm, 25^{o}C, in tubes of 200 mL. The degummed supernatant oil layer was then decanted using a separating funnel. For neutralization, (Sathivel et al., 2003, Mariod et al., 2012) 50 g of the degummed oil was placed in a 400 mL beaker and mixed with 12.6 g of 9.5% of sodium hydroxide solution. The mixture was heated to 65^{o}C for 30 min with constant agitating using a shaking temperature-controlled water bath (Grant shaking water bath OLS 200). The mixture was then cooled to room temperature (as mentioned in degumming step) and kept undisturbed for 6 h. Then the mixture was centrifuged at 3500 rpm, 25^{o}C, for 10 min in 200 mL tubes. After centrifuging, the oil was decanted from the precipitated soap using a separating funnel. Then the oil was mixed with 50 mL ultra-pure water to wash out any remaining soap. Water and impurities were then removed from the refined oil (Figure 9A) by centrifuging at 3500 rpm, 25^{o}C, for 10 min (the washing step was repeated three times). The neutralized supernatant oil was then decanted using a separating funnel.

2.1.3.5 Solvent extraction of mafura oil

Two separate solvent extractions of mafura oil were carried out using two solvents namely n-hexane (Ferreira-Dias et al., 2003) and absolute ethanol (Kwiatkowski and Cheryan, 2002, Ferreira-Dias et al., 2003) with some modifications. After kernel removal, the dried pulp (as described in section 2.1.3.3) was washed with tap water and dried in a drying oven at 30^{o}C for 18 h. The dried pulp was ground using a blender to obtain a fine powder. 150 g of the dried and ground pulp was placed into a beaker and mixed with n-hexane in a ratio of pulp to solvent of 1:4 (m/v). The oil was extracted by agitating the mixture using a magnetic stirrer at room temperature for 5 h. The extraction mixture was filtered using Whatman No. 1 filter paper and the filtrate was left at room temperature in the fume hood for 12 h to allow evaporation of the hexane. The remaining hexane was separated from the oil using a vacuum rotary evaporator (Buchi Rotavapor RE-120, Laboratoriums Technik AG, Switzerland**)** at 30^{o}C. The same procedure was applied for oil extraction using absolute ethanol.

2.1.4 Methods

2.1.4.1 Determination of acid value

Acid value is a measure of the free fatty acids (FFA) (Atinafu and Bedemo, 2011) present in the oil. Acid value is defined as the number of milligrams of potassium hydroxide required to neutralise the free acidity in 1 gram of the oil (Pearson, 1976). It was determined using a titration method according to AOAC Official Method 969.17 (AOAC, 2000). In a 250 mL Erlenmeyer, 0.5 – 1.0 g of oil was accurately weighed and mixed with 3.0 – 4.0 mL of neutral ether-ethanol solution (mixture of equal parts of diethyl-ether and ethanol). Then the mixture was titrated with 0.1 M sodium hydroxide using phenolphthalein as indicator (the titration ended when the solution changed to pink).

2.1.4.2 Determination of iodine value

Iodine value was determined using Wijs method according to the AOAC Official Method 993.20 (AOAC, 2000). The oil sample reacts with an excess of Wijs solution (iodine monochloride). During the reaction, iodine adds quantitatively to double bonds in unsaturated fatty acids in the oil. Potassium iodide is then added and liberated iodine (unreacted iodine) is

titrated with standard sodium thiosulphate using starch solution as indicator. The iodine value is then determined by the amount of iodine (grams) that is absorbed by 100 g of the oil sample. In a 500 mL Erlenmeyer supplied with a glass stopper, 0.15 – 0.25 g of oil was accurately weighed and dissolved in 15 – 20 mL of chloroform. Using a graduated cylinder, exactly 25 mL of Wijs solution was added and the mixture was allowed to stand for 1 h in a dark place. Then 10 mL of 15% potassium iodide (freshly prepared) and 100 mL of distilled water were added. The mixture was then titrated with 0.5 M sodium thiosulphate using fresh starch solution (which was prepared by mixing 0.5 g of starch, 5 mL of distilled water and 50 mL of boiling distilled water) as indicator. The indicator was added shortly before the end point was reached (when the solution changed to colourless). A blank test using distilled water was also conducted.

2.1.4.3 Determination of saponification value

Saponification value was determined using a titrimetric method according to the AOAC Official Method 920.160 (AOAC, 2000). The oil is saponified by heating in excess alcoholic KOH under reflux. After saponification, unreacted KOH is titrated against standard HCl with phenolphthalein as indicator. The saponification value is then determined as the mg KOH needed to saponify 1 g of the oil. In a ground-glass joint Erlenmeyer flask, 1 – 2 g of oil was accurately weighed and mixed with exactly 25 mL alcoholic potassium hydroxide solution 0.5 M (28 g of potassium hydroxide was mixed in 1000 mL 95% ethanol). Then the beakers were connected to a Liebig condenser (13 – 15 mm diameter and 750 mm long) as reflux cooler. The oil was saponified by heating it under reflux on a boiling water bath for 30 min while mixing occasionally. Then the warm solution was titrated with 0.5 M HCl using phenolphatlein as indicator. The titration ended when the pink solution changed to colourless. A blank test was conducted.

2.1.4.4 Determination of fatty acid composition

Fatty acid composition (FAC) was determined by Gas chromatography (GC) according to AOCS Official Method Ce 2-66 (AOCS, 1989) by Precision Oil Laboratories, Tzaneen, Mpumalanga, South Africa.

2.1.4.5 Determination of oxidative stability

2.1.4.5.1 Accelerated oxidative storage

Crude mafura oil (traditionally and solvent extracted), sunflower oil, extra-virgin olive oil, olive oil blend and refined mafura oil were analysed for their oxidative stability through measurement of their peroxide values, anisidine values and total oxidation (totox) values. The oil samples were placed into screw-capped 100 mL Schott glass bottles and stored in a forced circulation oven (Labcon, type FSOE) at 65^{o}C for 14 days. Before storage, initial peroxide and anisidine values were determined on all the oil samples. A small amount of each oil sample was withdrawn and analysed every two days to assess the oxidative stability.

2.1.4.5.2 Determination of peroxide value

The peroxide value was determined according to the AOAC Official Method 965.33 (AOAC, 2000). In this method, a known quantity of the oil is dissolved in an acetic acid-chloroform solution and then reacted with saturated potassium iodide (KI) solution. Peroxides oxidize the KI and liberate iodine which is titrated with standard sodium thiosulphate solution. The amount of liberated iodine is proportional to the amount of peroxides. The peroxide value was expressed as milliequivalents of peroxides per kg of oil. 1.0 g of the oil was placed in a 250 mL Erlenmeyer and mixed with 6 mL of a mixture of 2 volumes of chloroform and 3 volumes of glacial acetic acid. Using a 100 µl micropipette, 0.1 mL of saturated potassium iodide was added and stirred for exactly 1 min using a magnetic stirrer. Then 6 mL of distilled water was added. The mixture was titrated with 0.01 M sodium thiosulphate until the yellow colour was almost discharged and then 1 mL of 1% starch solution (as indicator) was added. The mixture was titrated until the colour disappeared completely. A blank titration was conducted.

2.1.4.5.3 Determination of anisidine value

The International Union of Pure and Applied Chemistry (IUPAC) standard method 2.504 (IUPAC, 1979) was used to determine the anisidine values of the oil samples. In this method, a known quantity of the oil is dissolved in trimethylpentane and then reacted with p-anisidine solution. Aldehydes in the oil react with p-anisidine reagent under acidic conditions which

produces yellowish products (Shahidi and Wanasundara, 2008). To measure anisidine values, three solutions were prepared. Test solution (a) was prepared by dissolving 0.5 g of the oil in 25 ml of trimethylpentane in a volumetric flask. To prepare the reference solution, 5 mL of trimethylpentane was transferred to a centrifuge tube (previously covered with aluminium foil to protect the solution from light) and mixed with 1 mL of p-anisidine solution (which was prepared by dissolving 0.25 g of p-anisidine in 100 mL of acetic acid). Then the mixture was stirred with a vortex mixer. To prepare the test solution (b), 5 mL of test solution (a) was transferred to a centrifuge tube (also covered with aluminium foil) and 1 mL of p-ansidine solution was added and mixed on a vortex mixer. The absorbance of the test solution (a) was measured using trimethylpentane as the compensation liquid. The absorbance of the test solution (b) was measured exactly 10 min after its preparation using the reference solution as the compensation liquid. The absorbances were measured spectrophotometrically (Lambda EZ150 Spectrophotometer, Perkin-Elmer, USA) at 350 nm using a glass cuvette.

2.1.4.5.4 Determination of total oxidation value

The total oxidation (Totox) value was calculated using the formula: Totox value = AV + 2 PV where AV = anisidine value and PV = peroxide value (Gordon, 2001, Abramovic and Abram, 2005).

2.1.5 Statistical analyses

All experiments were conducted in duplicates and analyses (Acid value, Iodine value, Saponification value and Peroxide value) were conducted in triplicates. Anisidine value analyses were conducted in quadruplicates. The Analysis of variance (ANOVA) performed using STATISTICA (Statsoft, Tulsa, OK, USA) was conducted. Fisher´s Least Significance Difference test was used to compare mean values at a confidence level of 95%.

2.1.6 Results

2.1.6.1 Acid, iodine and saponification values

Acid value, iodine value and saponification value of the five oil samples analysed are shown in Table 2. Traditionally extracted crude mafura oil had the highest acid value (2.51 mg NaOH/g oil) while the olive oil blend and sunflower oil had the lowest acid values of 0.21 and 0.22 mg NaOH/g oil respectively. Refined mafura oil and extra-virgin oil had intermediate acid values of 0.79 and 0.68 mg NaOH/g oil respectively.

The olive oil samples (extra-virgin and blend) had higher iodine values (88.8 and 93.8 g I_2/100 g oil respectively) than the mafura oil samples (crude and refined; 77.1 and 70.2 g I_2/100 g oil respectively). Sunflower oil had the highest iodine value (101 g I_2/100g oil).

The mafura oil samples (crude and refined) had the highest saponification values (215 and 216 mg KOH/g oil) followed by sunflower oil (196 mg KOH/g oil) and olive oil blend (195 mg KOH/g oil). Extra virgin olive oil had the lowest saponification value (189 mg KOH/g oil).

Table 2 - Acid value, iodine value and saponification value of traditionally extracted mafura oil (crude and refined), sunflower oil and olive oil (extra-virgin and blend).

Samples	Acid value (mg NaOH/g oil)	Iodine value (g I_2/100g oil)	Saponification value (mg KOH/g oil)
Crude mafura oil	$2.51^{d} \pm 0.08$	$77.1^{b} \pm 4.14$	$215^{c} \pm 7.17$
Refined mafura oil	$0.79^{c} \pm 0.02$	$70.2^{a} \pm 1.29$	$216^{c} \pm 4.35$
Sunflower oil	$0.22^{a} \pm 0.02$	$101^{e} \pm 5.73$	$196^{b} \pm 4.98$
Extra-virgin olive oil	$0.68^{b} \pm 0.02$	$88.8^{c} \pm 3.52$	$189^{a} \pm 4.56$
Olive oil blend (refined and virgin olive oils)	$0.21^{a} \pm 0.01$	$93.8^{d} \pm 1.80$	$195^{ab} \pm 7.07$

Results are means ± standard deviation.

Means within the same column followed by a different letter are significantly different ($p<0.05$).

2.1.6.2 Fatty acid composition

The fatty acid profile of the oil samples are presented in Table 3. No myristic acid was detected in the olive oil samples and low levels (0.14 and 0.11 g/100 g oil) were present in the traditionally extracted crude mafura oil and sunflower oil samples respectively. The highest level of palmitic acid was found in crude mafura oil (43.7 g/100 g oil) and this was about 8 times greater than in sunflower oil and up to 4 times greater than in the olive oil samples. The olive oil samples were richer in cis oleic acid than the crude mafura oil and sunflower oil. The highest level of cis oleic acid was found in the olive oil blend (74.1 g/100 g oil) and this was about 3 times greater than in crude mafura oil, 2 times greater than in sunflower oil and 1.14 times greater than in extra-virgin olive oil. Sunflower oil had the highest level of cis linoleic acid (53.5 g/100 g oil) which was 2 times greater than in crude mafura oil and up to 6 times greater than in the olive oil samples. The highest level of n3 linolenic acid was found in sunflower oil (1.65 g/100 g oil) and this was about 6 times greater than in crude mafura oil and up to 3 times greater than in the olive oil samples. Small amounts of lignoceric acid and nervonic acid were detected only in sunflower oil but not in the other oil samples.

The contents of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids are presented in Table 4. Crude mafura oil was richest in saturated fatty acids by about 4 times that of sunflower oil and up to 3 times that of the olive oil samples. The olive oil samples were richest in monounsaturated fatty acids. Monounsaturated fatty acid content of the olive oil blend was about 3 times greater than in crude mafura oil, 2 times greater than in sunflower oil and 1.13 times greater than in extra-virgin olive oil. Sunflower oil was richest in polyunsaturated fatty acids by 2 times that of crude mafura oil and up to 6 times that of the olive oil samples.

Table 3 - Fatty acids profile of traditionally extracted crude mafura oil, sunflower oil and olive oils (extra virgin and blend).

Fatty acids	Percentage fatty acids (g/100 g oil)			
	Crude mafura oil	Sunflower oil	Extra virgin olive oil	Olive oil blend (refined and virgin olive oils)
Myristic acid (C14:0)	$0.14^{a} \pm 0.01$	$0.11^{abc} \pm 0.01$	ND	ND
Palmitic acid (C16:0)	$43.7^{e} \pm 0.66$	$5.80^{h} \pm 0.37$	$15.3^{f} \pm 0.52$	$11.8^{g} \pm 0.28$
Palmitoleic acid (C16:1)	$0.10^{a} \pm 0.04$	ND	$1.79^{c} \pm 0.09$	$1.04^{d} \pm 0.03$
Margaric acid (C17:0)	$0.29^{a} \pm 0.07$	$0.10^{abc} \pm 0.01$	$0.09^{a} \pm 0.01$	$0.11^{ab} \pm 0.01$
Glinkgolic acid (C17:1)	ND	ND	$0.11^{a} \pm 0.01$	ND
Stearic acid (C18:0)	$2.62^{b} \pm 0.06$	$5.49^{g} \pm 0.06$	$2.63^{d} \pm 0.07$	$2.71^{e} \pm 0.02$
cis Oleic acid (C18:1)	$25.6^{c} \pm 0.43$	$31.1^{i} \pm 0.10$	$64.8^{g} \pm 0.34$	$74.1^{h} \pm 0.06$
cis Linoleic acid (C18:2)	$27.0^{d} \pm 0.14$	$53.5^{j} \pm 0.04$	$13.9^{e} \pm 0.10$	$8.85^{f} \pm 0.01$
n6 Linolenic acid (C18:3)	ND	$0.05^{ab} \pm 0.07$	ND	$0.05^{ab} \pm 0.01$
n3 Linolenic acid (C18:3)	$0.27^{a} \pm 0.00$	$1.65^{f} \pm 0.01$	$0.65^{b} \pm 0.01$	$0.60^{c} \pm 0.03$
Arachidic acid (C20:0)	$0.22^{a} \pm 0.01$	$0.44^{d} \pm 0.01$	$0.51^{b} \pm 0.03$	$0.46^{c} \pm 0.04$
Behenic acid (C22:0)	$0.08^{a} \pm 0.01$	$1.03^{e} \pm 0.11$	$0.18^{a} \pm 0.01$	$0.18^{b} \pm 0.04$
Lignoceric acid (C24:0)	ND	$0.31^{cd} \pm 0.01$	ND	ND
Nervonic acid (C24:1)	ND	$0.10^{abc} \pm 0.02$	ND	ND

Results are means ± standard deviation.

Means within the same column followed by a different letter are significantly different ($p<0.05$).

ND – Not detected.

Table 4 – Content of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids of traditionally extracted crude mafura oil, sunflower oil and olive oils (extra virgin and blend).

Oil samples	Percentage of fatty acids g/100 g oil		
	Saturated fatty acids	Monounsaturated fatty acids	Polyunsaturated fatty acids
Crude mafura oil	47.05	25.70	27.27
Sunflower oil	13.28	31.20	55.20
Extra virgin olive oil	18.71	66.70	14.55
Olive oil blend (refined and virgin olive oils)	15.26	75.14	9.500

2.1.6.3 Oxidative stability of traditionally extracted crude mafura oil, sunflower oil and olive oils (extra-virgin and blend).

2.1.6.3.1 Peroxide value

In all oil samples analysed, the initial peroxide values at the beginning of the storage period (day 0) were lower than 20 mEq/kg oil. There was a progressive increase in peroxide values from the beginning to the end of the storage period (Figure 10). There were differences in the rates of increase of peroxide values. The rates of increase of peroxide values for traditionally extracted crude mafura oil and sunflower oil were similar. Likewise, the rates of increase of peroxide values of the olive oil samples (refined olive oil blend and extra-virgin olive oil) were similar although extra-virgin olive oil had relatively higher peroxide values. The rates of increase of the peroxide values of crude mafura oil and sunflower oil were higher than for the olive oil samples. The olive oil blend had the lowest peroxide values over the 14-day storage period ranging from 5 mEq/Kg oil on day 0 to 28.3 mEq/Kg oil on day 14. Extra-virgin olive oil had higher peroxide values (ranging from 15.2 mEq/Kg oil on day 0 to 49.7 mEq/Kg oil on day 14) than olive oil blend. After day 4, traditionally extracted crude mafura oil and sunflower oil had higher peroxide values than the olive oil samples, which increased sharply to 113 and 111 mEq/kg oil on day 14 respectively.

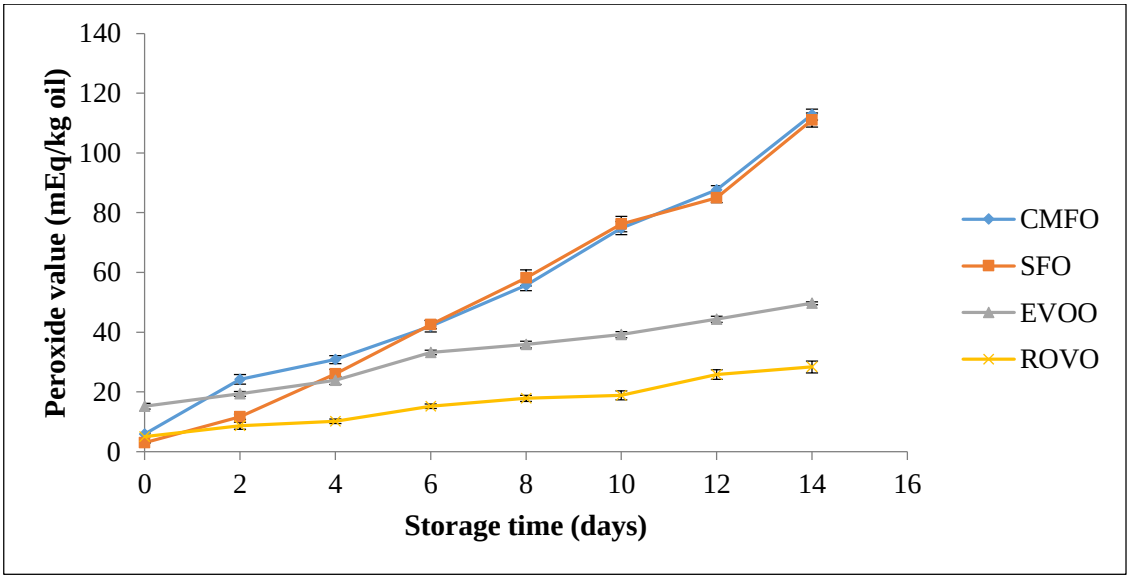


Figure 10 - Effect of storage time at 65°C for 14 days on peroxide value of traditionally extracted crude mafura oil (CMFO), sunflower oil (SFO), extra-virgin olive oil (EVOO) and olive oil blend (refined and virgin olive oils, ROVO). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.3.2 Anisidine value

The anisidine values of the olive oil samples over the 14 day storage period were fairly constant (Figure 11), ranging from 3.61 on day 0 to 3.24 on day 14 for extra-virgin olive oil and from 5.02 on day 0 to 5.10 on day 14 for olive oil blend. Up until day 6, the anisidine values of sunflower oil were similar to those of the olive oil samples and fairly constant ranging from 2.95 on day 0 to 4.02 on day 6. From day 8 onwards, the anisidine values of sunflower oil increased sharply until the end of the storage period ranging from 6.55 on day 8 to 17.0 on day 14. Over the 14 day storage period, traditionally extracted crude mafura oil had higher anisidine values than the sunflower and olive oil samples. Anisidine values of crude mafura oil ranged from 23.7 on day 0 to 24.9 on day 8. Thereafter, the anisidine values increased sharply for the rest of the storage period to 46.0 on day 14.

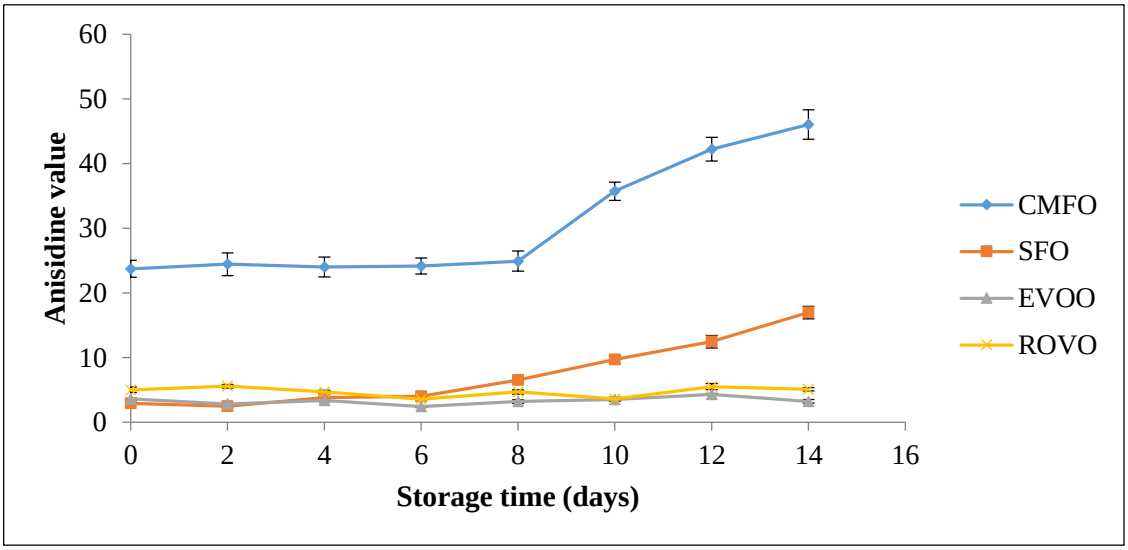


Figure 11 - Effect of storage time at 65ºC for 14 days on anisidine value of traditionally extracted crude mafura oil (CMFO), sunflower oil (SFO), extra-virgin olive oil (EVOO) and olive oil blend (refined and virgin olive oils, ROVO). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.3.3 Total oxidation value

The total oxidation values of traditionally extracted crude mafura oil, sunflower oil and olive oil samples are presented in Figure 12. There was a progressive increase on the total oxidation values of all oil samples analysed from the beginning to the end of the storage period. However, there were differences in the rate of increase for the oil samples. The rate of increase of total oxidation value for the olive oils was lower than for sunflower oil and crude mafura oil. From day 2 onwards, crude mafura oil always had higher total oxidation value than the other oil samples ranging from 72.8 on day 2 to 272 on day 14. Crude mafura oil had the highest initial total oxidation value which was 35.4. The olive oil blend had lower total oxidation values (ranging from 15.0 on day 0 to 61.8 on day 14) than extra-virgin olive oil ranging from 33.9 on day 0 to 103 on day 14. Sunflower oil had intermediate total oxidation values ranging from 8.88 on day 0 to 239 on day 14.

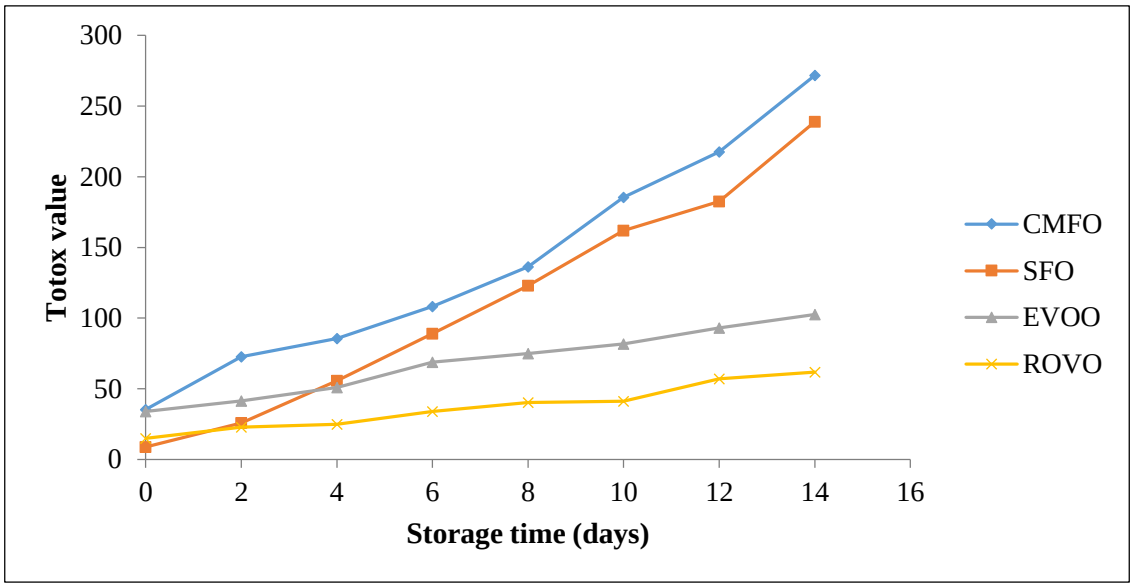


Figure 12 - Effect of storage time at 65ºC for 14 days on total oxidation (totox) value of traditionally extracted crude mafura oil (CMFO), sunflower oil (SFO), extra-virgin olive oil (EVOO) and olive oil blend (refined and virgin olive oils, ROVO).

2.1.6.4 Effect of refining on oxidative stability of mafura oil.

2.1.6.4.1 Peroxide value

Over the 14 day storage period, the peroxide values for traditionally extracted crude and refined mafura oils showed a similar trend of increasing from the beginning to the end of the storage period, as can be seen in Figure 13. Peroxide value of crude mafura oil increased from 5.50 mEq/kg oil on day 0 to 116 mEq/kg oil on day 14, while peroxide value for refined mafura oil increased from 2.83 mEq/kg oil on day 0 to 124 mEq/kg oil on day 14. On days 10, 12 and 14, refined mafura oil had slightly higher peroxide values than crude mafura oil.

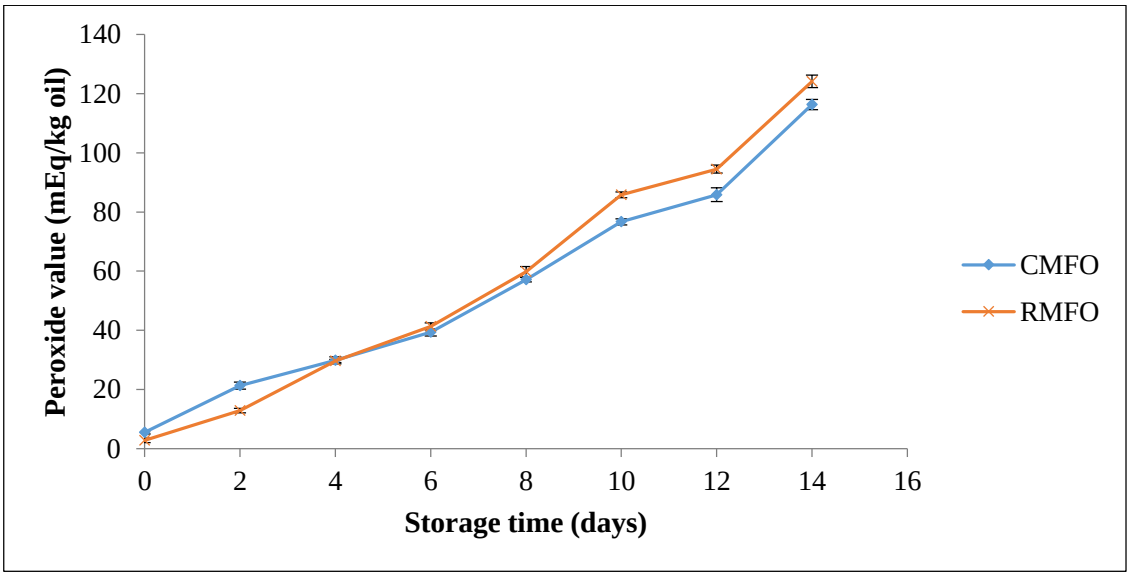


Figure 13 - Effect of storage time at 65°C for 14 days on peroxide value of traditionally extracted crude mafura oil (CMFO) and refined mafura oil (RMFO). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.4.2 Anisidine value

Over the 14 day storage period, anisidine values of traditionally extracted crude mafura oil were slightly higher than the anisidine values of refined mafura oil (Figure 14). Anisidine value of crude mafura oil increased gradually from 19.1 on day 0 to 36.5 on day 12 and then sharply to 60.5 on day 14. For refined mafura oil, anisidine value increased gradually from 14.0 on day 0 to 31.3 on day 12 followed by a sharp increase to 54.0 on day 14.

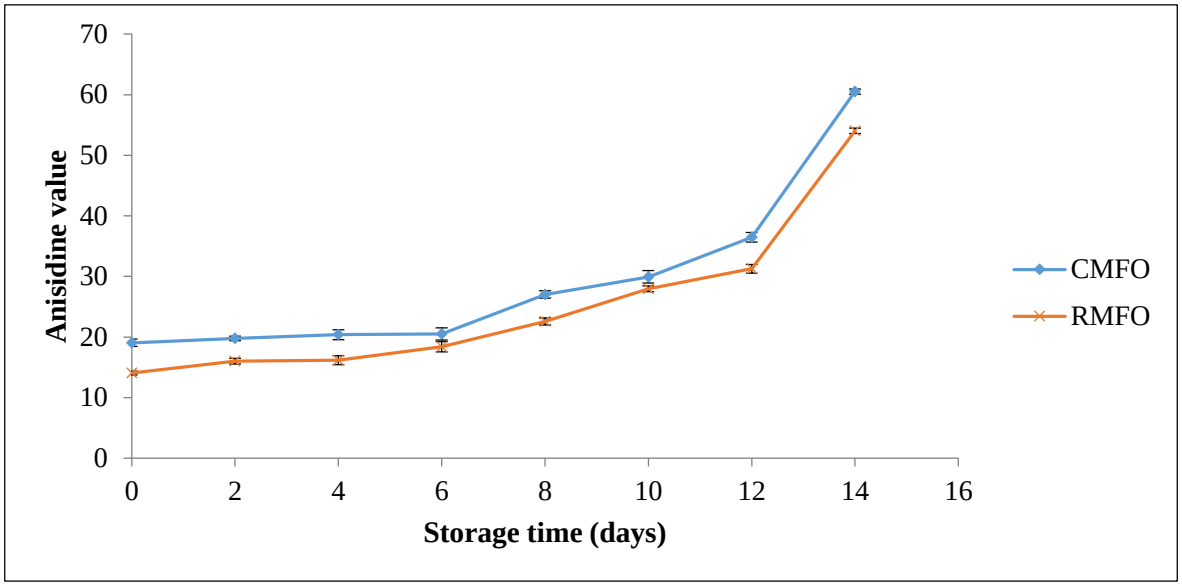


Figure 14 - Effect of storage time at 65°C for 14 days on anisidine value of traditionally extracted crude mafura oil (CMFO) and refined mafura oil (RMFO). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.4.3 Total oxidation value

The total oxidation values of traditionally extracted crude and refined mafura oils are presented in Figure 15. The total oxidation values of crude mafura oil ranged from 30.1 on day 0 to 293 on day 14. For refined mafura oil, total oxidation values ranged from 19.7 on day 0 to 302 on day 14.

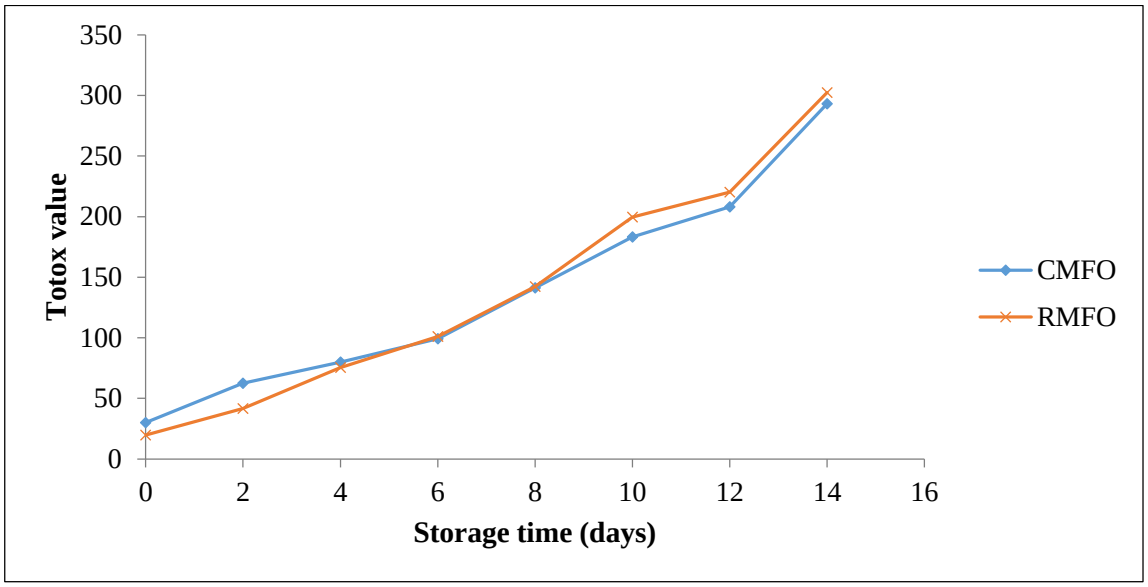


Figure 15 - Effect of storage time at 65ºC for 14 days on total oxidation (totox) value of traditionally extracted crude mafura oil (CMFO) and refined mafura oil (RMFO).

2.1.6.5 Effect of solvent extraction on oxidative stability of crude mafura oil.

2.1.6.5.1 Peroxide value

The peroxide values of the traditionally extracted crude mafura oil, hexane-extracted mafura oil and ethanol-extracted mafura oil increased progressively from the beginning to the end of the storage period (Figure 16). The rate of increase in peroxide value for crude mafura oil was lower than that for hexane-extracted and ethanol-extracted mafura oil samples. The peroxide value of crude mafura oil ranged from 5.83 mEq/Kg oil on day 0 to 113 mEq/Kg oil on day 14. For hexane-extracted mafura oil peroxide value ranged from 13.2 mEq/Kg oil on day 0 to 164 mEq/Kg oil on day 14, while, for ethanol-extracted mafura oil, peroxide value ranged from 14.8 mEq/Kg oil on day 0 to 145 mEq/Kg oil on day 14.

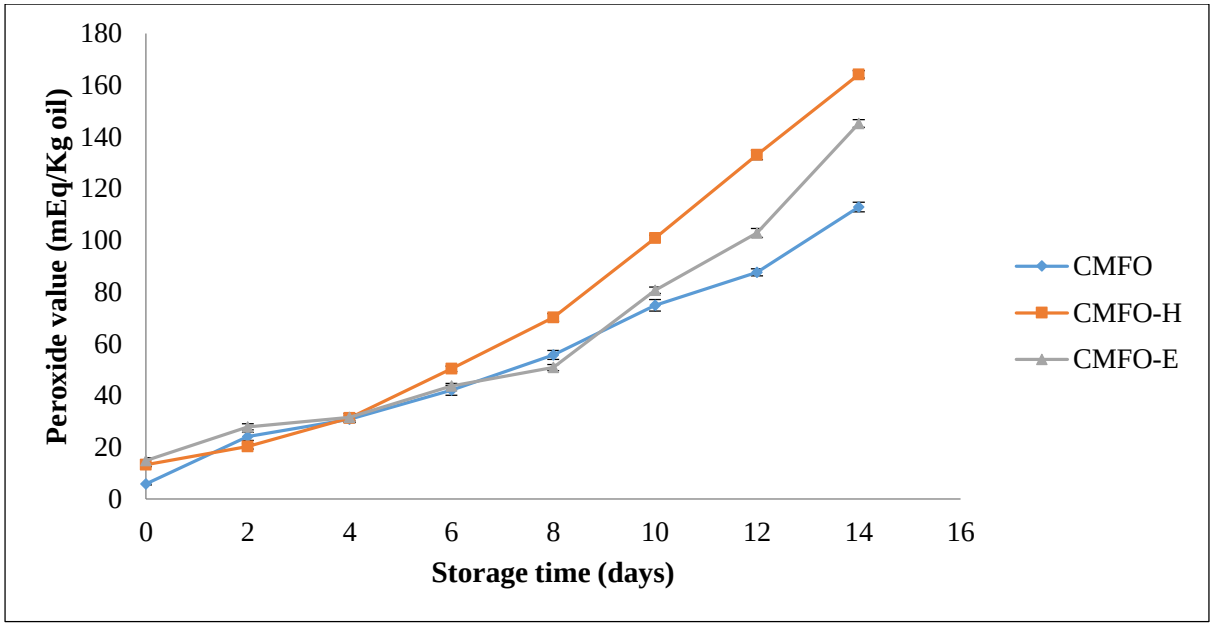


Figure 16 - Effect of storage time at 65ºC for 14 days on peroxide value of traditionally extracted crude mafura oil (CMFO), hexane-extracted mafura oil (CMFO-H) and ethanol-extracted mafura oil (CMFO-E). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.5.2 Anisidine value

Over the 14 day storage period, anisidine values of hexane-extracted mafura oil and ethanol-extracted mafura oil were much lower than the anisidine values of traditionally extracted crude mafura oil (Figure 17). The anisidine values of crude mafura oil were similar from day 0 (23.7) to day 8 (24.9) and increased sharply to 49.0 on day 14. The rates of increase in anisidine values for hexane-extracted and ethanol-extracted mafura oils were essentially similar although the ethanol-extracted oil had slightly higher anisidine values. Hexane-extracted mafura oil had anisidine values ranging from 1.41 on day 0 to 30.1 on day 14 while anisidine values of ethanol-extracted mafural oil ranged from 6.65 on day 0 to 35.5 on day 14.

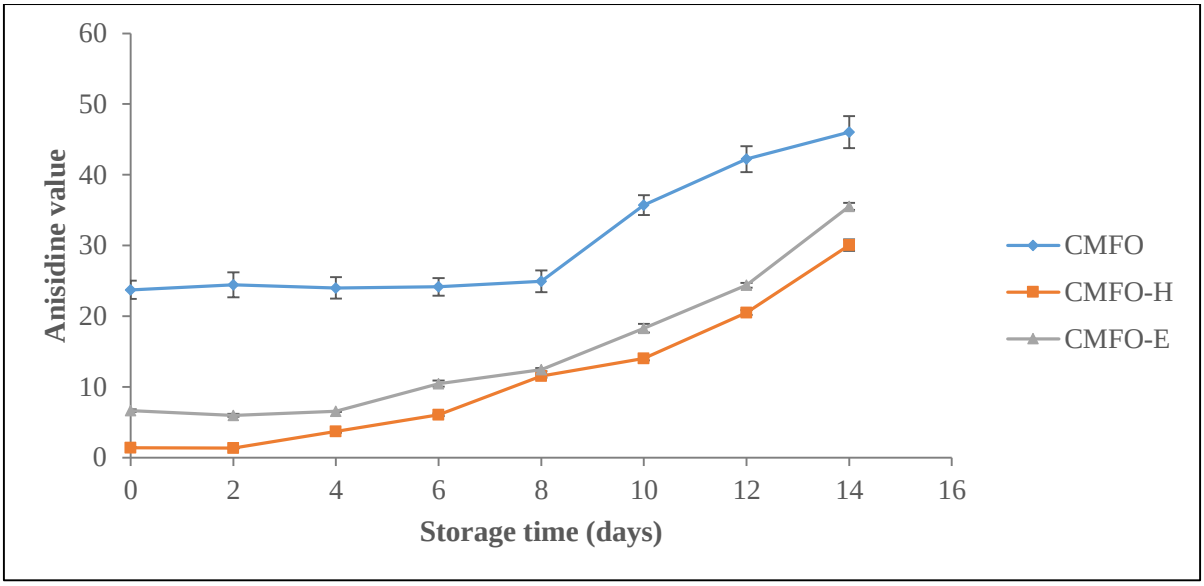


Figure 17 - Effect of storage time at 65ºC for 14 days on anisidine value of traditionally extracted crude mafura oil (CMFO), hexane-extracted mafura oil (CMFO-H) and ethanol-extracted mafura oil (CMFO-E). Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.1.6.5.3 Total oxidation value

Figure 18 shows the total oxidation values of the traditionally extracted crude mafura oil, hexane-extracted mafura oil and ethanol-extracted mafura oil. Hexane-extracted mafura oil had the lowest total oxidation value on day 0 (27.7) whereas ethanol-extracted mafura oil had the highest total oxidation value (36.3). From day 6, hexane-extracted mafura oil had higher total oxidation value (ranging from 107 on day 6 to 358 on day 14) than ethanol-extracted mafura oil which was 97.8 on day 6 and 326 on day 14. The total oxidation of traditionally extracted crude mafura oil was higher than the total oxidation value of hexane-extracted mafura oil from day 0 to day 6 and lower from day 8 to day 14 ranging from 136 on day 8 to 272 on day 14.

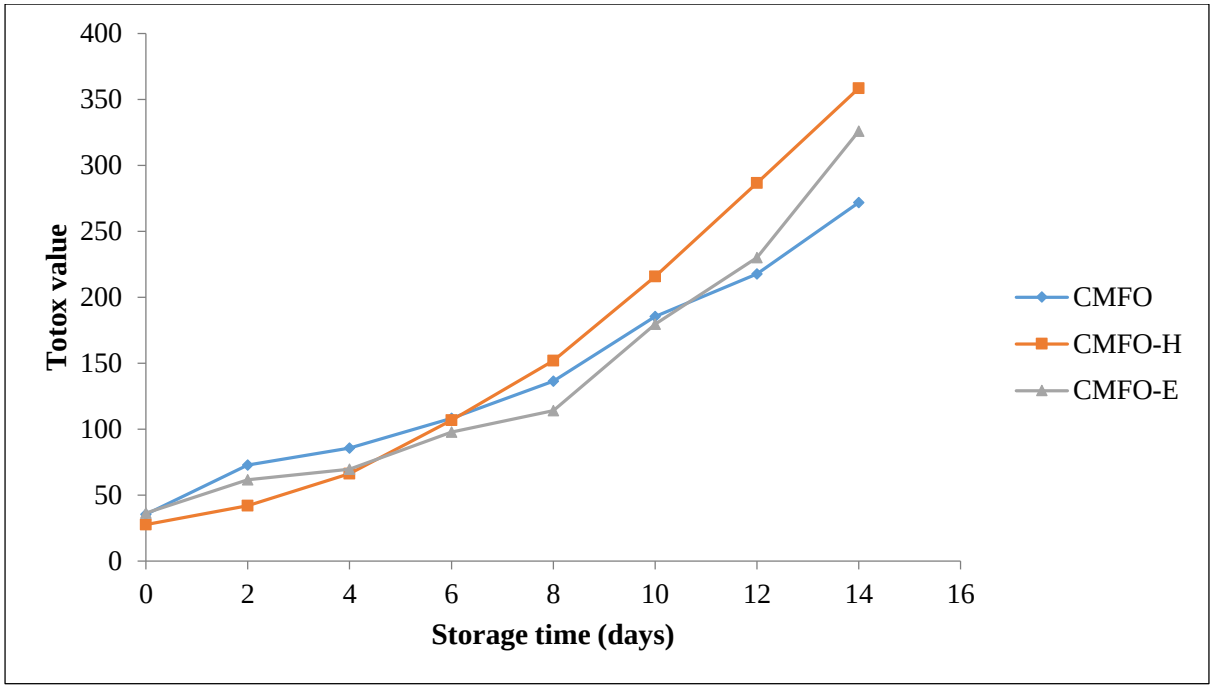


Figure 18 - Effect of storage time at 65ºC for 14 days on total oxidation value (totox) of traditionally extracted crude mafura oil (CMFO), hexane-extracted mafura oil (CMFO-H) and ethanol-extracted mafura oil (CMFO-E).

2.1.7 Discussion

2.1.7.1 Acid, iodine, saponification values and fatty acid composition of traditionally extracted mafura oils (crude and refined), sunflower oil and olive oils (extra-virgin and blend).

The higher acid value of the traditionally extracted crude mafura oil compared to sunflower and olive oil samples (Table 2) may be due to the extensive boiling of the pulp mixed with water and its exposure to light during the mafura oil extraction process. Fatty acids may be released via hydrolysis of ester bonds in lipids in the presence of moisture, exposure to light and heat (Pearson, 1976, Narayan et al., 1988, O'Brien, 2004, Vidrih et al., 2010). In addition, long storage of mafura seeds (6 to 12 months at -20ºC in the dark) before oil extraction may have contributed to increased acidity. Abdellah et al. (2012) found higher acid values in oil samples extracted from sunflower seeds stored for 12 months at room temperature (35ºC) than the oil extracted from newly harvested sunflower seeds. Oil from stored seeds exhibited increased acidity due to enzymatic hydrolysis of glycerides (Abdellah et al., 2012).

Ghasemnezhad and Honermeier (2009) also found that the content of free fatty acids increased during storage of high oleic sunflower seeds.

The acceptable limit of acid value for vegetable oils according to Codex standards is given as 0.6 mg KOH/g oil (Dimberu and Belete (2011). The acid value of crude mafura oil found in this study was higher than this threshold limit, whereas those of the sunflower and olive oil samples were either lower than or close to the acceptable limit. This is an indication of the relatively poorer quality of the traditionally extracted mafura oil due to a greater occurrence of hydrolytic rancidity. In this regard, a positive result was the observation that refining reduced the acid value of mafura oil to close to the acceptable limit (0.79 mg NaOH/g oil). Free fatty acids are removed during neutralization in the oil refining process and this observation is in agreement with Vidrih et al. (2010) who reported lower acid values of refined oils compared to unrefined.

Oils with high acid value as observed with the traditionally extracted crude mafura oil, which signifies high levels of free fatty acids, tend to have poor oxidative stability. The free fatty acids (especially polyunsaturated) are highly susceptible to oxidation thus reducing the oil oxidative quality and shelf-life (Frega et al., 1999, Vidrih et al., 2010).

The acid value of traditionally extracted crude mafura oil was much lower than the acid value reported by Adinew (2014) (8.13 mg KOH/g oil) also for mafura oil. These differences may be due to the different raw materials used for oil extraction, while traditionally extracted crude mafura oil was extracted from the pulp, mafura oil analysed by Adinew (2014) was extracted from the kernels.

The observed low, intermediate and high iodine values respectively of mafura oils, olive oils and sunflower oil samples were expected and this can be related to their fatty acid composition. Oils with low iodine value (e.g. lower than 100 g I_2/100 g oil) contain a greater proportion of saturated or monounsaturated fatty acids (Adinew, 2014) while oils with high iodine value tend to have significantly higher levels of unsaturation. The observed trends in iodine values of the oil samples are, therefore, in agreement with their fatty acid composition (Tables 3 and 4). Mafura oil with low iodine value of 77.1 g I_2/100 g oil (Table 2) was richest in saturated fatty acids (Table 4). On the other hand, the olive oil samples with intermediate iodine values of 88.8 – 93.8 g I_2/100 g oil (Table 2) had higher proportion of

monounsaturated fatty acids (Table 4) while the sunflower oil with high iodine value of 101 g I_2/100 g oil (Table 2) had higher proportion of polyunsaturated fatty acids (Table 4).

The saponification value of traditionally extracted crude mafura oil obtained in this study was much higher than the saponification value reported by Adinew (2014) and Daniel and McCrae (1908), (180.09 mg KOH/g oil and 202.5 mg KOH/g oil, respectively). Higher saponification value in traditionally extracted crude mafura oil may mean that the oil may potentially be used in soap making and cosmetics. The saponification value of the refined mafura oil was not significantly different than the crude mafura oil. This goes in line with the report of Ojeh (1981) in which refining did not affect the saponification value of cashew kernel oil. The saponification value was not directly affected by the refining process (Ojeh, 1981).

The observed iodine and saponification values suggest that sunflower oil and the olive oils should be less oxidatively stable than the mafura oils. Both iodine and saponification values are useful in predicting the degree of unsaturation and the type of fatty acids respectively in an oil. The free fatty acid content is also useful in explaining changes in oxidative stability of an oil during storage. The higher the amounts of free fatty acids present in an oil, the higher its susceptibility to oxidative degradation because free fatty acids (especially when unsaturated) are susceptible to oxidation (Frega et al., 1999, Vidrih et al., 2010).

The current study shows that traditionally extracted crude mafura oil is a relatively saturated oil with a high proportion of saturated fatty acids, in particular, palmitic acid. This is in agreement with previous findings of Khumalo et al. (2002) which reported that *Trichilia emetica* oil obtained by hexane extraction of decorticated sun-dried seeds had higher level of palmitic acid (52.7 g/100 g oil) than the other fatty acids. However, Engelter and Wehmeyer (1970) have reported that mafura oil had higher level of oleic acid (48.5 g/100 g oil) than palmitic acid (38.3 g/100 g oil).

2.1.7.2 Oxidative stability of traditionally extracted crude mafura oil, sunflower oil and olive oils (extra-virgin and blend).

According to Rossell (1986) and Matthäus (2010), refined vegetable oils of acceptable quality should have peroxide values of up to 10 mEq O_2/kg oil while Abramovic and Abram (2005) indicate an upper peroxide value limit of 20 mEq/kg oil for unrefined oils. In this regard, the initial peroxide values of all the oil samples analysed (less than 20 mEq O_2/kg oil) (Figure 10) were within acceptable limits.

According to Rossell (1986) the anisidine value of oils which have not undergone extensive oxidative deterioration should be less than about 10 and may be regarded as being of acceptable oxidative quality. Therefore, crude mafura oil with initial anisidine value of 23.7 (Figure 11) was of poor initial oxidative quality and an indication that the oil had already undergone extensive oxidative deterioration with the production of high levels of secondary oxidation products even before the beginning of the storage period. On the other hand, sunflower oil, extra-virgin olive oil and olive oil blend had anisidine values which were within the acceptable limits (2.95, 3.61 and 5.02, respectively) (Figure 11) and, therefore, had good initial oil oxidative quality.

Higher peroxide values of traditionally extracted crude mafura oil and sunflower oil as compared to the olive oil samples over the storage period indicates higher formation of primary oxidation products. High levels of primary oxidation products in traditionally extracted crude mafura oil were not expected due to its content of high levels of palmitic acid, a saturated fatty acid which would be resistant to oxidation. However, mafura oil did contain appreciable levels of linoleic acid (27 g/100 g oil) (Table 3) and furthermore, its high acid value (Table 2) suggests high levels of free fatty acids. An appreciable proportion of these free fatty acids could have been the polyunsaturated linoleic acid which could undergo oxidation to produce the observed high peroxide values of mafura oil during storage. On the other hand, the observed high levels of hydroperoxides in sunflower oil was not surprising since the oil is rich in polyunsaturated fatty acids, which are more prone to oxidation, especially at elevated temperature (Saga et al., 2011). Low peroxide values of olive oils (extra-virgin and blend) may be explained by their low acid value and high levels of oleic acid which would be less prone to oxidation compared to linoleic acid. Overall, the rate of

formation of primary oxidation products was higher for crude mafura oil and sunflower oil than the olive oil samples.

The observed higher anisidine values of traditionally extracted crude mafura oil compared to the other oils over the storage period (Figure 11) indicates high concentration of secondary oxidation products. As mentioned earlier, the anisidine value of traditionally extracted crude mafura oil at the start of the storage period was much higher than the acceptable limit which indicates that it had already undergone significant oxidation with the production of high levels of secondary oxidation products and could be considered rancid. Although both crude mafura oil and sunflower oil had similar peroxide values for most of the storage period (Figure 10), sunflower oil had lower anisidine values than crude mafura oil during storage. This suggests that high formation of hydroperoxides may not always necessarily lead to a high generation of secondary oxidation products. For the sunflower oil, the observed gradual increase in anisidine values after day 8 compared to the olive oil samples whose anisidine values remained relatively constant (Figure 11) is of importance. This may be related to the higher levels of polyunsaturated fatty acids in the sunflower oil which would make it more prone to secondary oxidation. Overall, these results indicate that the traditionally extracted crude mafura oil was the least oxidatively stable followed by sunflower oil with the olive oil samples showing highest oxidative stability. This is reflected in the total oxidation value which showed the traditionally extracted mafura oil having highest totox value over the storage period followed by sunflower oil with the olive oil samples having much lower totox values (Figure 12). The rate of increase of totox values for the mafura oil and sunflower oil samples over the storage period were much greater than for the olive oil samples (Figure 12).

The observed low oxidative stability of the mafura oil as shown by the high levels of peroxide and anisidine value could be due to high levels of free fatty acids which may lead to high formation of primary and secondary oxidation products in spite of the mafura oil being rich in saturated fatty acids. More importantly, these may be related to how the mafura oil was processed. The extensive heating treatment and the use of water when the pulp is boiled during mafura oil extraction may contribute to increasing the levels of free fatty acids and the concentration of oxidation products, thus lowering its oxidative stability. Furthermore, the exposure of the mafura seeds to the sun during drying (3 weeks) as well as the long storage period (6 to 12 months) of the seeds before oil extraction may potentially contribute to decreasing oxidative stability of the oil. Hilali et al. (2005) reported that argan oil from seeds

kept unprotected from sun light and in prolonged storage (6 months at room temperature, between 18 – 30^{o}C) before oil extraction exhibited high peroxide values.

2.1.7.5 Effect of refining on oxidative stability of traditionally extracted mafura oil.

Due to the low oxidative stability of crude mafura oil, a refining procedure was employed in an attempt to remove substances that may act as pro-oxidants and reduce its oxidative stability. According to the maximum limits reported by Rossell (1986), Abramovic and Abram (2005) and Matthäus (2010), the peroxide value of the non-oxidised crude (5.50 mEq/kg oil) and refined (2.83 mEq/kg oil) mafura oils (Figure 14) were within the acceptable limits.

Although some differences were observed in peroxide and anisidine values of crude and refined mafura oils over the 14 day storage period (Figures 13 and 14), these differences seemed to be marginal and not likely to be of real practical significance. This is reflected in the calculated total oxidation values (Figure 15) of the crude and refined mafura oils which were very similar for both oils over the storage period. These results indicate that refining did not improve the oxidative stability of the mafura oil although the acid value reduced significantly. Furthermore, the anisidine value of the refined oil (Figure 14) was also already above the acceptable limits at the beginning of storage, a possible indication of its inherent poor initial oxidative quality.

2.1.7.6 Effect of method of extraction on oxidative stability of mafura oil.

All the mafura oil samples analysed had acceptable initial peroxide values, but from day 2 onwards, their peroxide values were in excess of the acceptable limit of 20 mEq O_2/kg oil (Figure 16) meaning that there was high formation of primary oxidation products. On the other hand, the anisidine values of the solvent-extracted crude mafura oils were much lower than the anisidine values of the traditionally extracted crude mafura oil over the storage period (Figure 17). In addition, the anisidine values of the solvent-extracted mafura oils were less than or equal to about 10 for the first six days of storage meaning that they were within the acceptable limits for oxidative stability compared to the traditionally extracted mafura oil. These results, suggest that extraction using solvents improved the oxidative stability of mafura oil compared to the traditional extraction process, although this was not very obvious

from the calculated total oxidation value (Figure 18). It must be borne in mind that the totox value is a derived value from the peroxide value and anisidine values. In this instance, the totox value does not clearly show that the solvent-extracted mafura oils were more oxidatively stable than the traditionally extracted oil due to the trends observed in peroxide values of the three oils. The anisidine value gives the strongest indication that the solvent-extracted mafura oils were more oxidatively stable (lower production of secondary oxidation products) than the traditionally extracted mafura oil.

The observed differences in anisidine value may be probably because the different extraction methods extracted different fractions of lipids from the pulp. Hexane probably extracts more non-polar lipid substances such as tocopherols which may act as antioxidants thus enhancing the oxidative stability and lowering the anisidine value. On the other hand ethanol may probably extract more polar oxidised material resulting in higher anisidine value.

2.1.8 Conclusions

Traditionally extracted crude mafura oil (CMFO) is rich in saturated fatty acids especially palmitic acid. However, mafura oil is less oxidatively stable than sunflower oil which is a well-known polyunsaturated plant oil and olive oil which is a monounsaturated oil. The low oxidative stability of traditionally extracted mafura oil may be related to the extensive heating of the mafura fruit pulp in an aqueous medium during preparation of the oil. Refining of the traditionally extracted crude mafura oil does not improve its oxidative stability in spite of reducing its acidity. Solvent extraction of mafura oil improves the oxidative stability significantly compared to traditional extraction. Overall, it can be said that the extraction method has more effect on the oxidative stability of the mafura oil than its fatty acid profile. The adoption of strategies such as reduction in the boiling time during mafura oil processing or the use of antioxidants during storage could improve the oxidative stability of the oil and this could be of economic benefit to local mafura oil processors in rural communities in Mozambique.

2.2 Effect of a crude phenolic extract from sorghum bran on oxidative stability of mafura oil.

2.2.1 Abstract

Mafura oil as extracted traditionally, is consumed in its crude form by rural communities in the south of Mozambique. Earlier results in this research have shown that the traditional method of extraction produces mafura oil that is highly susceptible to oxidative deterioration. A potential strategy which could be easily adopted by rural mafura oil processors in Mozambique is the stabilization of the oil against oxidative deterioration during storage by using easily available natural antioxidants. In this study, the effect of a crude phenolic extract from sorghum bran on the oxidative stability of mafura oil during storage was investigated. The condensed tannin sorghum crude phenolic extract in its freeze dried form had higher total phenolic content and antioxidant activity than the non-freeze dried extract from sorghum bran and whole grain. The freeze-dried crude phenolic extract was rich in (-)-epicatechin and (+)-catechin. Crude phenolic extract at a concentration of 1000 ppm did not improve significantly the oxidative stability of crude mafura oil. When incorporated into the crude mafura oil at a concentration of 2000 ppm and stored at 65^{o}C over a 14-day period, the crude phenolic extract exhibited as good antioxidant activity as tertiary-butylhydroquinone in reducing the formation of secondary oxidation products. This study shows that condensed tannin sorghum bran is a good source of natural antioxidants which could be incorporated into mafura oil in the form of extracts to potentially improve the oxidative stability of the oil.

Key words: Mafura oil, sorghum, natural antioxidants, total phenols, antioxidant activity, oxidative stability.

2.2.2 Introduction

Oxidation is one of the major causes of deterioration of food lipids (Quiles et al., 2002, Lutterodt et al., 2011, Pardauil et al., 2011). Edible oils undergo oxidative deterioration through the formation of free radicals during storage and processing (Zhang et al., 2015). The rate and the extent of oxidation depend on the composition of the oil as well as the conditions in which the oil is stored (Kołakowska and Bartosz, 2013). Fatty acid composition is an important factor that determines the susceptibility of the oils to oxidation. Oils rich in polyunsaturated fatty acids are more prone to oxidation due to the double bonds where the oxidation takes place. On the contrary, oils that contain more saturated fatty acids are more stable to oxidation (Morelló et al., 2004, Ghasemnezhad and Honermeier, 2009). At high temperature (above 100^{o}C), the presence of oxygen and exposure to light during storage may accelerate the oxidation process in oils leading to the formation of oxidation products (Kołakowska and Bartosz, 2013). Therefore, in order to prevent oxidative deterioration, oils may be treated with antioxidants (Velioglu et al., 1998).

Antioxidants are compounds that are added to food lipids to increase oxidative stability and prolong their shelf life (Wanasundara and Shahidi, 1994, Lutterodt et al., 2011). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ) have been used to retard lipid oxidation (Matsukawa et al., 1997, Bera et al., 2006). However, some concerns regarding the safety of the use of synthetic antioxidants have been raised due to their possible carcinogenic effects. Therefore, there is a growing interest in the use of natural antioxidants from plants (Matsukawa et al., 1997, Wanasundara and Shahidi, 1998, Awika and Rooney, 2004, Martínez et al., 2013, Fan and Eskin, 2015) as an alternative way to retard lipid oxidation, which can be as effective as the use of synthetic antioxidants (Marinova and Yanishlieva, 1997, Abdalla and Roozen, 1999, Li et al., 2015). Natural antioxidants such as phenolic compounds may be found in fruits, seeds, nuts, leaves etc (Velioglu et al., 1998, Minussi et al., 2003, Zhang and Hamauzu, 2004, Roleira et al., 2015).

Sorghum (*Sorghum bicolor* (L.) Moench), is a drought-resistant cereal (FAO, 1995, Wu et al., 2012) that grows without addition of any fertiliser and is cultivated in semi-arid regions of Africa and Asia (FAO, 1995). It is an important food crop in Mozambique along with cassava, maize and rice (FAO, 2010). It is grown mainly in the North of the country where it

is one of the main staple food crops (FAO, 2010). The grain is normally decorticated to remove the bran and then ground into flour which is used in production of various foods (such as porridges and alcoholic beverages) for human consumption (FAO, 2010).

Sorghum contains various phenolic compounds such as phenolic acids, flavonoids and tannins (Awika and Rooney, 2004, Dykes and Rooney, 2006, Luthria and Liu, 2013). Phenolic compounds are secondary metabolites of plants which possess potent antioxidant activities and, therefore, may be used to prevent or delay oxidative degradation of food lipids. These compounds act as free radical scavengers by donating hydrogen atom to the lipid radicals formed in oils due to the presence of some initiators such as metals, atmospheric oxygen, etc (Dai and Mumper, 2010). Phenolic antioxidants produce stable free radicals (through resonance) or non-radical products thus preventing further oxidation from occurring (Kamal-Eldin, 2006, Shahidi and Zhong, 2010a). Sorghum may, therefore, be considered as a potential source of natural antioxidants.

Mafura (*Trichilia emetica* Vahl.) oil extracted from mafura seeds by rural processors in the South of Mozambique is rich in saturated fatty acids, especially palmitic acid. However, the oil tends to have poor oxidative stability during storage, probably due to the method of extraction. A potential strategy that can be used by rural mafura oil processors in Mozambique to stabilize the oil is to incorporate easily available natural antioxidants during storage. In this regard, phenolic extracts from sorghum bran could be potentially used as natural antioxidants to stabilise mafura oil against oxidation.

The aim of this study was to determine the effect of a crude phenolic extract from sorghum bran (as a natural antioxidant) on oxidative stability of mafura oil.

2.2.3 Materials

2.2.3.1 Mafura oil samples and sorghum grain.

Crude mafura oil was extracted using the traditional method of extraction, from mafura seeds obtained from Mozambique (Gaza province, Manjacaze district and Inhambane province, Zavala district) as described in section 2.1.3.3. The oil was placed in screw-capped 100 mL Schott glass bottles. A red condensed tannin type sorghum (NS 5511) was used.

2.2.3.2 Reagents

Acetic acid (glacial), acetone, chloroform, sodium carbonate, sodium chloride, di-sodium hydrogen orthophosphate anhydrous, sodium dihydrogen orthophosphate dehydrate, sodium thiosulphate pentahydrate, potassium iodide and starch (soluble) were obtained from Merck (Johannesburg, South Africa). Acetonitrile (LiChrosolv), p-anisidine, Folin-Ciocalteau reagent, hydrochloric acid 32% (LiChrosolv), methanol, potassium persulphate, trimethlypentane (isooctane) were obtained from Merck (Darmstadt, Germany). 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), caffeic acid, (+)-catechin hydrate, p-coumaric acid, (-)-epicatechin, ferulic acid, gallic acid, hesperetin, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), kaempferol, naringin, naringenin, quercetin dihydrate, rutin hydrate, sinapic acid, syringic acid, vanillic acid were obtained from Sigma-Aldrich. Tertiary-butylhydroquinone (TBHQ) was obtained from Merck (Germany).

2.2.3.3 Preparation of crude phenolic extract from red condensed tannin sorghum bran

Crude phenolic extract from sorghum bran was prepared using aqueous acetone according to Sikwese and Duodu (2007). 1000 g of the sorghum grain was dehulled for 6 min using an abrasive decortication device to obtain approximately 12% yield of bran which was sieved through a 710 µm sieve. Then 200 mL of 75% aqueous acetone was added to 40 g of sorghum bran in a 1000 mL beaker and stirred using a magnetic stirrer for 2 h. The mixture was then centrifuged (Rotanta 460 R centrifuge, Germany) at 3500 rpm for 10 min at 25^{o}C and decanted. The residue was re-extracted once with 200 mL solvent, centrifuged and decanted as mentioned above. The two supernatants were pooled and concentrated in a rotary evaporator (Buchi Rotavapor RE-120, Laboratoriums Technik AG, Switzerland**)** at 35^{o}C. The extracts obtained were frozen at -20^{o}C overnight and freeze-dried (Instruvac lyophilizer model 13 KL).

2.2.3.4 Treatment of crude mafura oil with freeze-dried crude phenolic extract from red condensed tannin sorghum bran and synthetic antioxidant tertiary-butylhydroquinone (TBHQ) in preparation for accelerated oxidative storage

The freeze-dried crude phenolic extracts from red condensed tannin sorghum bran were added to aliquots of 40 g of crude mafura oil at concentrations of 1000 ppm and 2000 ppm (mg extract per kg oil) in screw-capped 100 mL Schott glass bottles. The crude phenolic extracts were first dissolved in a very small volume of absolute methanol to facilitate incorporation into the oil. TBHQ was also dissolved in absolute methanol and added into another aliquot of 40 g crude mafura oil at a concentration of 200 ppm. The concentration of the TBHQ added is the legal limit according to the South African Foodstuffs, Cosmetics and Disinfectants Act 1972 (ACT 54 of 1972). Figure 19 shows the traditionally extracted crude mafura oil treated with antioxidants.

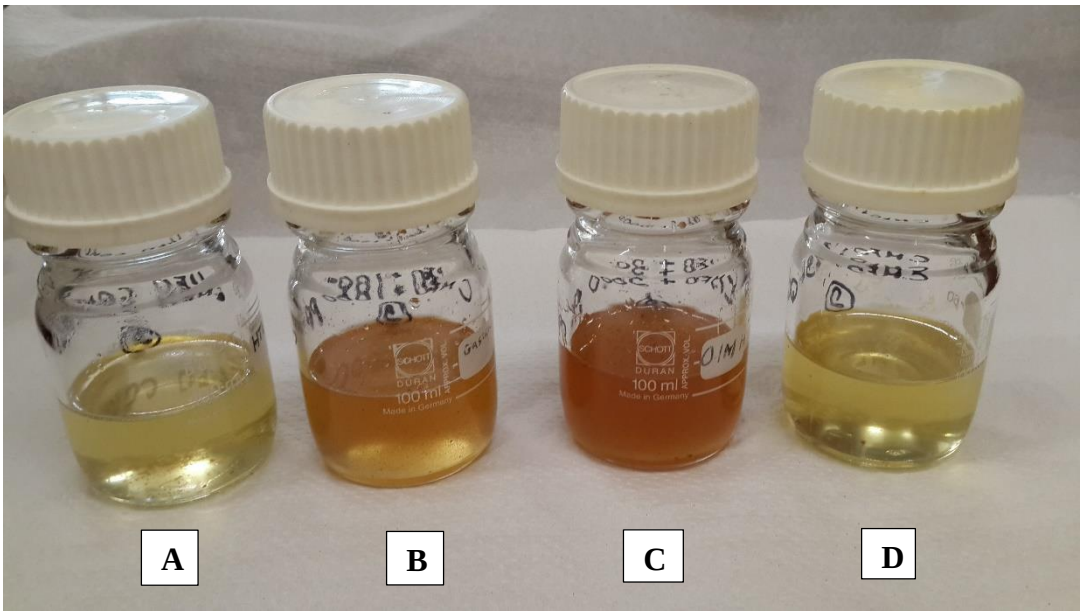


Figure 19 - Crude mafura oil (A) with no additive (control), crude mafura oil containing (B) 1000 ppm crude phenolic extracts, (C) 2000 ppm crude phenolic extracts and (D) 200 ppm TBHQ.

2.2.3.5 Sample extraction for total phenol content and antioxidant activity determination

Extracts from red condensed tannin sorghum (whole grain, bran and freeze-dried crude phenolic extract) were prepared according to Awika et al. (2003) with some modifications. 50 mg of each sample was added to 1 ml acidified methanol (1% conc. HCl in methanol) in 2 ml Eppendorf tubes and vortex mixed every 10 min for 2 h. Then the samples were centrifuged for 10 min at 3500 rpm (25^{o}C) in a desktop centrifuge (Labnet, force 712, South Africa) and decanted. Each sample residue was rinsed twice with 1 ml of the solvent, vortex mixed for 10 min, centrifuged and decanted as described above. The three supernatants were then combined and stored at – 20^{o}C in the dark prior to analysis.

2.2.4 Methods

2.2.4.1 Determination of oxidative stability

2.2.4.1.1 Accelerated oxidative storage

Crude mafura oil without additives, crude mafura oil treated with different concentrations of crude phenolic extract, and crude mafura oil treated with TBHQ were analysed for their oxidative stability through the measurement of their peroxide values, anisidine values and total oxidation values under accelerated oxidative conditions. The oil samples in screw-capped 100 mL Schott glass bottles were stored in a forced circulation oven (Labcon, type FSOE) at 65^{o}C for 14 days. Before storage, initial peroxide and anisidine values were determined on all the oil samples. A small amount of each oil sample was withdrawn and analysed every two days to assess the oxidative stability.

2.2.4.1.2 Determination of peroxide value, anisidine value and total oxidation value

The peroxide value, anisidine value and total oxidation value were determined as described earlier in section 2.1.4.5.2, 2.1.4.5.3 and 2.1.4.5.4, respectively.

2.2.4.2 Determination of phenolic composition of freeze-dried extract from red condensed tannin sorghum bran

Phenolic composition was determined by High Performance Liquid Chromatographic (HPLC) as described by Shelembe et al. (2014). The HPLC consisted of a Waters 1525 binary pump, Waters 2487 dual wavelength absorbance detector (Waters Associates, Milford, USA) and a Gemini C18 (250 x 4.6 mm i.d., 5 µm particle size) reverse phase column (Phenomenex, Torrance, CA, USA). The HPLC conditions were according to Kim et al., (2007). The mobile phase A was 0.1% acetic acid in water and mobile phase B was 0.1% acetic acid in acetonitrile. Solvents were delivered in linear gradient as follows: 8-10% B (2 min), 10-30% B (25 min), 30-90% B (23 min), 90-100% B (2 min), 100-8% B (2 min) and 8% B (7 min). Sample injection volume was 20 µl. Flow rate was maintained at 0.8 mL/min and total run time was 61 min. Phenolic compounds were detected at 280 nm and data acquired by Breeze system software (Waters Associates, Milford, USA).

Sample preparation for HPLC: 1 mg of freeze-dried crude phenolic extract from the condensed tannin sorghum bran was dissolved in 2 mL of 1% conc. HCl in methanol and filtered using 0.20 µm (PTFE) syringes filters (Millex – LG, Millipore, Japan) into 1.5 mL amber vials. Then 20 µl of the sample was injected into the HPLC column in duplicate.

Standards preparation: 17 phenolic compound standards were prepared at concentrations of 25, 50, 100, 150 and 200 ppm in acidified methanol. The phenolic compounds consisted of flavonoids ((+)-catechin hydrate, rutin hydrate, naringin, quercetin dihydrate, kaempferol, hesperetin, (-)-epicatechin and naringenin) and phenolic acids (gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acid, p-coumaric acid, sinapic acid, and ferulic acid). The standard solutions were filtered using 0.20 µm (PTFE) syringes filters (Millex – LG, Millipore, Japan) into 1.5 mL amber vials. Then 20 µl of each standard was injected into the HPLC system and chromatographed singly and as mixtures and a calibration curve peak area (vertical axis) and concentration (horizontal axis) for each standard was obtained.

Phenolic compounds in the extracts were identified by comparison of retention times with those of the standards. The concentration of each identified phenolic compound in the

extracts was calculated using their peak areas and the calibration curves and data was reported as µg/g of sample on dry weight basis.

2.2.4.3 Determination of total phenolic content

Total phenolic content (TPC) of whole grain sorghum, bran and freeze-dried crude phenolic extracts from red condensed tannin sorghum bran was determined using the Folin-Ciocalteu method as described by Ainsworth and Gillespie (2007). The Folin-Ciocalteu reagent reacts with polyphenols in an alkaline solution producing a blue chromophore consisting of a phosphomolybdic/phosphotungstic complex that can be detected spectrophotometrically (Blainski et al., 2013). In this assay, a 96-well microplate was used in which 18.2 µl of the sample (or standard) was mixed with 36.4 µl of Folin-Ciocalteau reagent (diluted 10% v/v with distilled water) and 145.4 µl of 700 mM Na_2CO_3 in each well. The microplate was shaken gently to mix and incubation was done for 2 h and absorbance read at 750 nm using a Thermo Scientific Multiskan microplate reader. (+)-Catechin was used as standard at concentrations of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml prepared in acidified methanol. The total phenolic content was expressed as milligrams of (+)-catechin equivalents per gram of sample on a dry basis.

2.2.4.4 Determination of antioxidant activity

Antioxidant activity of whole grain sorghum, bran and freeze-dried crude phenolic extracts from red condensed tannin sorghum bran was determined using the $ABTS^{\cdot+}$ radical scavenging assay according to Awika et al. (2003) with some modifications. This assay measures the relative ability of the antioxidant to scavenge $ABTS^{\cdot+}$ radicals compared to Trolox standard. $ABTS^{\cdot+}$ radical mother solution was prepared by mixing equal volumes of 8 mM ABTS and 3 mM $K_2S_2O_8$ (both prepared in distilled water) which was allowed to react for at least 12 h at room temperature in the dark. An $ABTS^{\cdot+}$ radical working solution was prepared by mixing the mother solution with 58 ml of 0.2 M pH 7.4 PBS (phosphate buffer saline). Trolox standards of concentration 0, 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 mM were prepared in PBS. In each well of a 96-well microplate, 10 µl of the standard (or sample) was mixed with 190 µl of $ABTS^{\cdot+}$ working solution and allowed to react for exactly 30 min at room temperature in the dark. Then, the absorbance was measured at 750 nm using a Thermo

Scientific Multiskan microplate reader. The antioxidant activity was expressed as millimolar of Trolox equivalent antioxidant capacity (TEAC) per gram of sample in dry basis.

2.2.5 Statistical analyses

All experiments were conducted in duplicates. Statistical analysis was done using ANOVA (Analysis of variance) performed using STATISTICA (Statsoft, Tulsa, OK, USA). Fisher´s Least Significance Difference test was used to compare mean values at a confidence level of 95%.

2.2.6 Results

2.2.6.1 Total phenolic content and antioxidant activity of whole grain sorghum, bran and freeze-dried crude phenolic extract from red condensed tannin sorghum bran.

Total phenolic content and antioxidant activity of whole grain sorghum, bran and freeze-dried crude phenolic extract (CPE) from red condensed tannin sorghum bran are presented in Table 5. The freeze-dried crude phenolic extract had significantly ($p<0.05$) higher total phenolic content (443.5 mg CE/g CPE) than sorghum bran (110.4 mg CE/g bran) and whole grain sorghum (20.45 mg CE/g whole grain). The freeze-dried crude phenolic extract also had higher antioxidant activity (4.12 mM TE/g CPE) than sorghum bran (0.62 mM TE/g bran) and whole grain sorghum had the lowest antioxidant activity (0.14 mM TE/g whole grain). Overall, the levels of total phenolic content and antioxidant activity decreased in the order CPE > bran > whole grain.

Table 5 - Total phenolic content and antioxidant activity of whole grain, bran and freeze-dried crude phenolic extract from red condensed tannin sorghum bran.

Samples	Total phenolics (mg CE/g)	Antioxidant activity (mM TE/g)
Whole grain	$20.45^{a} \pm 0.25$	$0.14^{a} \pm 0.01$
Bran	$110.4^{b} \pm 2.33$	$0.62^{b} \pm 0.05$
CPE	$443.5^{c} \pm 21.96$	$4.12^{c} \pm 0.15$

Results are means ± standard deviation.

Means within the same column followed by a different letter are significantly different ($p<0.05$).

CPE – Freeze-dried crude phenolic extract from sorghum bran. CE – (+)-Catechin equivalents. TE – Trolox equivalents.

2.2.6.2 Phenolic composition of freeze-dried crude phenolic extract from red condensed tannin sorghum bran.

Phenolic composition of the freeze-dried extract from red condensed tannin sorghum bran is shown in Table 6. The sorghum bran extract contained various phenolic acids and flavonoids. It contained high levels of (-)-epicatechin and (+)-catechin (17.7 µg/mg and 7.06 µg/mg respectively). (-)-Epicatechin was the most abundant phenolic compound in the sorghum bran extract. Levels of protocatechuic acid (0.87 µg/mg), 4-hydroxybenzoic acid (1.67 µg/mg), rutin hydrate (1.53 µg/mg), p-coumaric and sinapic acids (0.47 µg/mg), ferulic acid (0.46 µg/mg), quercetin (1.65 µg/mg) and hesperetin (0.47 µg) were significantly lower than (-)-epicatechin and (+)-catechin ($p<0.05$). Gallic acid (0.03 µg/mg), naringin (0.12 µg/mg) and naringenin (0.22 µg/mg) occurred in lowest quantities.

Table 6 - Phenolic composition of freeze-dried crude phenolic extract from red condensed tannin sorghum bran.

Phenolic compounds	µg /mg CPE
Gallic acid	$0.03^{a} \pm 0.01$
Protocatechuic acid	$0.87^{abc} \pm 0.01$
(+)-Catechin	$7.06^{d} \pm 0.69$
4-Hydroxybenzoic acid	$1.67^{b} \pm 0.12$
(-)-Epicatechin	$17.7^{e} \pm 2.02$
Rutin hydrate	$1.53^{bc} \pm 0.00$
p-Coumaric acid & Sinapic acid	$0.47^{abc} \pm 0.01$
Ferulic acid	$0.46^{abc} \pm 0.08$
Naringin	$0.12^{a} \pm 0.01$
Quercetin	$1.65^{b} \pm 0.40$
Naringenin	$0.22^{ac} \pm 0.08$
Hesperetin	$0.47^{abc} \pm 0.05$

Results are means ± standard deviation.

Means within the same column followed by a different letter are significantly different ($p<0.05$).

CPE – Freeze-dried crude phenolic extract from sorghum bran.

2.2.6.3 Effect of freeze-dried crude phenolic extract from red condensed tannin sorghum bran and tertiary-butylhydroquinone (TBHQ) on oxidative stability of traditionally extracted crude mafura oil.

2.2.6.3.1 Peroxide value

There was a progressive increase in peroxide values of traditionally extracted crude mafura oil with no added antioxidants (control) and crude mafura oil with phenolic extract over the 14 day storage period (Figure 20). However, there were differences in the rates of increase of peroxide value. The rate of increase in peroxide value for the control oil sample was higher (ranging from 9.33 mEq/Kg oil on day 0 to 177 mEq/Kg oil on day 14) than the samples containing phenolic extract. For mafura oil containing phenolic extract at a concentration of 1000 ppm, the peroxide value ranged from 10 mEq/Kg oil on day 0 to 157 mEq/Kg oil on

day 14 whereas for oil containing 2000 ppm phenolic extract, the peroxide value ranged from 10 mEq/Kg oil on day 0 to 111 mEq/Kg oil on day 14. Crude mafura oil with 200 ppm of synthetic antioxidant TBHQ had the lowest peroxide values ranging from 10 mEq/Kg oil on day 0 to 18.8 mEq/Kg oil on day 14.

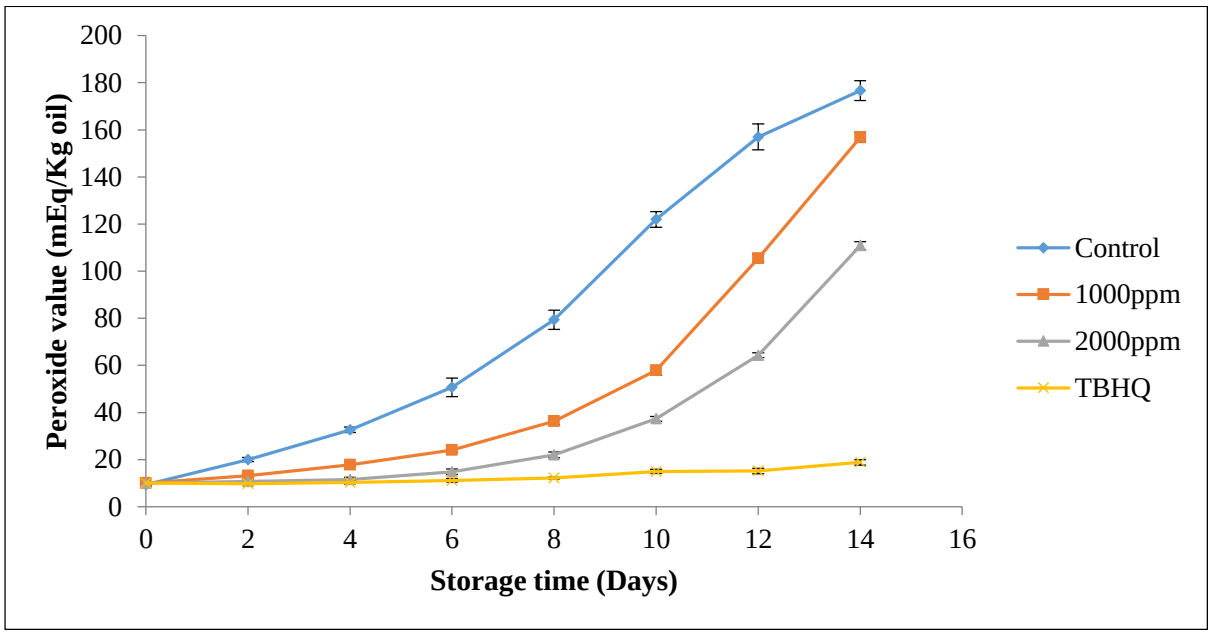


Figure 20 - Effect of freeze-dried crude phenolic extract from red condensed tannin sorghum bran (at concentration of 1000 ppm and 2000 ppm) and tertiary-butylhydroquinone (TBHQ) (at concentration of 200 ppm) on peroxide value of traditionally extracted crude mafura oil over 14-storage period at 65ºC. Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.2.6.3.2 Anisidine value

The anisidine values of the control oil sample (traditionally extracted crude mafura oil without antioxidant) were higher (ranging from 15.2 on day 0 to 56.7 on day 14) than the mafura oil samples containing 2000 ppm of phenolic extract (ranging from 11.3 on day 0 to 27. 1 on day 14) and 1000 ppm of phenolic extract (ranging from 14.8 on day 0 to 35.1 on day 14) and the oil containing 200 ppm of TBHQ (ranging from 10.6 on day 0 to 19.5 on day 14) (Figure 21). From day 10 to day 14, the control oil sample exhibited a sharp increase in anisidine value compared to the other oil samples.

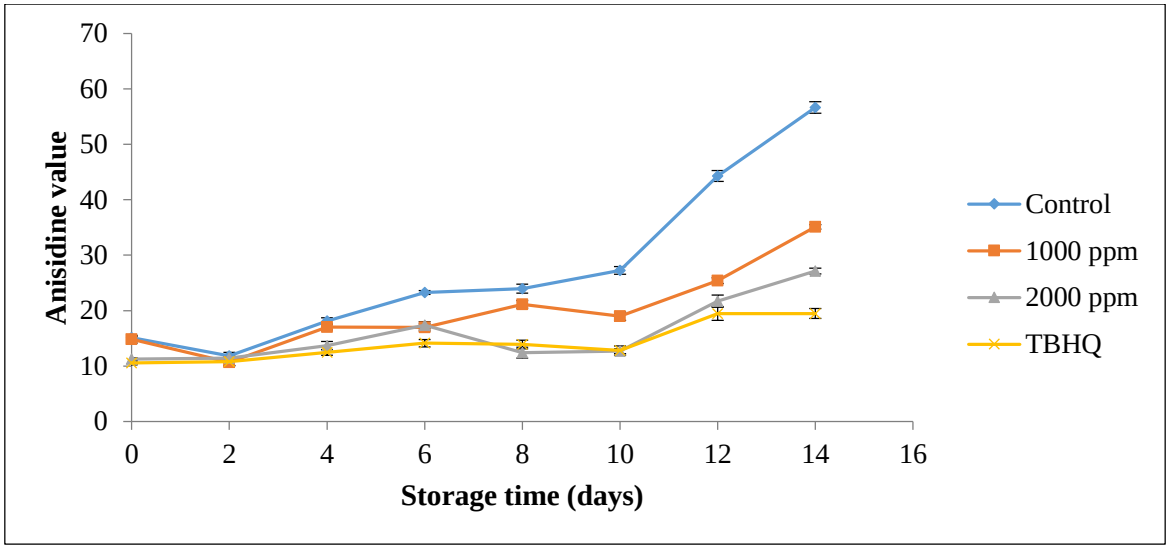


Figure 21 - Effect of freeze-dried crude phenolic extract from red condensed tannin sorghum bran (at concentration of 1000 ppm and 2000 ppm) and tertiary-butylhydroquinone (TBHQ) (at concentration of 200 ppm) on anisidine value of traditionally extracted crude mafura oil over 14-storage period at 65ºC. Each point on the graph is the average of 2 replicates conducted in different experiments. Bars represent standard deviation.

2.2.6.3.3 Total oxidation value

The total oxidation value of traditionally extracted crude mafura oil with no added antioxidants and mafura oil with natural and synthetic antioxidants are shown in Figure 22. Crude mafura oil containing 200 ppm of TBHQ had the lowest total oxidation values (ranging from 30.6 on day 0 to 57.2 on day 14). The control oil sample (mafura oil without antioxidant) had the highest total oxidation values (ranging from 33.8 on day 0 to 410 on day 14) over the whole storage period. The mafura oil sample containing 1000 ppm of phenolic extract had higher total oxidation values (34.8 on day 0 to 349 on day 14) than the oil sample containing 2000 ppm of phenolic extract (31.3 on day 0 to 249 on day 14).

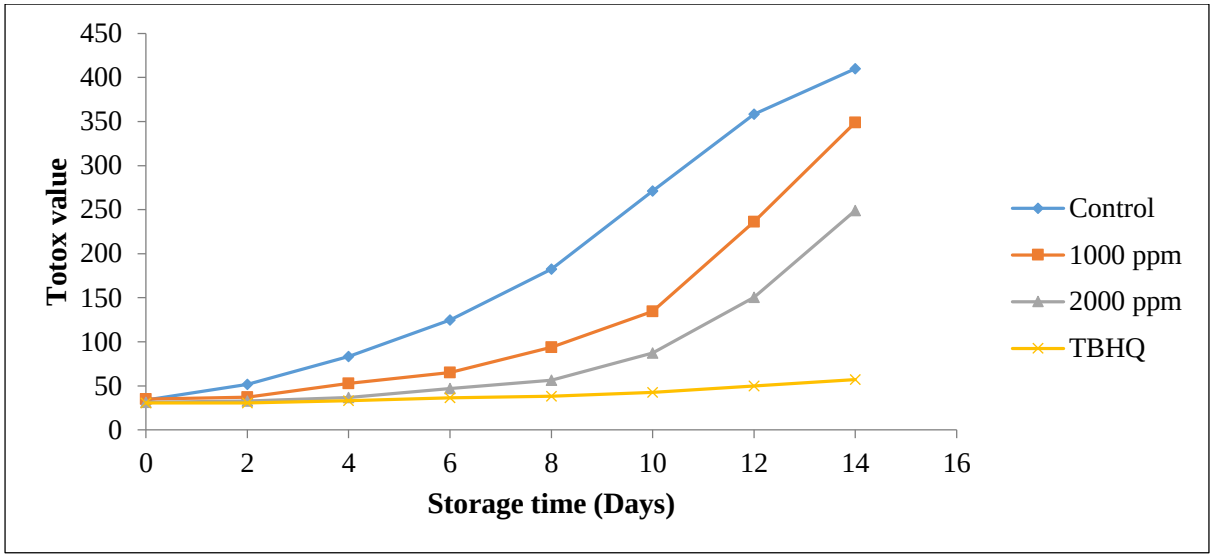


Figure 22 - Effect of freeze-dried crude phenolic extract from red condensed tannin sorghum bran (at concentration of 1000 ppm and 2000 ppm) and tertiary-butylhydroquinone (TBHQ) (at concentration of 200 ppm) on total oxidation (totox) value of traditionally extracted crude mafura oil over 14-storage period at 65^{o}C.

2.2.7 Discussion

2.2.7.1 Total phenolic content, antioxidant activity and phenolic composition of whole grain sorghum, bran and freeze-dried crude phenolic extract from red condensed tannin sorghum bran

The observed higher total phenolic content of sorghum bran than the whole grain (Table 5) is in agreement with the fact that phenolic compounds in sorghum are highly concentrated in the outer layers (pericarp and testa) of the grain (Kähkönen et al., 1999, Troszynska and Ciska, 2002, Awika and Rooney, 2004, O'Neil et al., 2010, Luthria, 2012, Luthria and Liu, 2013). The even higher levels of total phenolics in the freeze-dried crude phenolic extract from sorghum bran is a concentration effect. Freeze-drying is a dehydration process in which the majority of the water normally present in the food is removed under controlled conditions by sublimation (Ratti, 2001, Fellows, 2009, Potter and Hotchkiss, 2012). The removal of water would then concentrate the phenolics even further. The observed trend of higher total phenolic content of the freeze-dried extract compared to the whole grain is similar to that of Sikwese and Duodu (2007) who reported that freeze-dried crude phenolic extract from

sorghum bran of *Phatafuli* and *Shabalala* sorghum varieties had higher levels of total phenolics than their whole grains.

The trend in antioxidant activity was similar to that of total phenolics, which is an indication that phenolic compounds are most likely responsible for the antioxidant activity of the extracts due to their reducing power (Escarpa and Gonzalez, 2001, Cardoso et al., 2015). Similar results have been reported by Sikwese and Duodu (2007) and Awika et al. (2003).

The antioxidant activity exhibited by the condensed tannin sorghum bran may be explained by their content of phenolic acids and flavonoids (Table 6). Sorghum grain is an important source of phytochemicals such as phenolic acids, flavonoids and tannins which have been reported to have antioxidant activity (Waniska, 2000, Awika and Rooney, 2004, Dykes and Rooney, 2006). The freeze-dried crude phenolic extract analysed in the present study contained p-coumaric, sinapic, ferulic acids in higher amounts than the other phenolic acids. This is in agreement with previous findings which report that these phenolic acids were the most abundant in sorghum (Waniska et al., 1989). Shelembe et al. (2014), Awadelkareem et al. (2009) and Chung et al. (2011) have also reported the presence of various phenolic acids and flavonoids as those reported in this study in sorghum.

The observation that (-)-epicatechin and (+)-catechin occurred in highest quantities was expected as the sorghum type used was a tannin-containing sorghum. Sorghum condensed tannin (proanthocyanidin) is a polymer of (+)-catechin and (-)-epicatechin monomeric units (Figure 7D). Gupta and Haslam (1978), Schofield et al. (1998) and Gu et al. (2002) reported that proanthocyanidins in sorghum consisted of (+)-catechin as chain terminal units and (-)-epicatechin as chain extension units.

2.2.7.2 Oxidative stability of traditionally extracted crude mafura oil treated with antioxidants.

Traditionally extracted crude mafura oil with no added antioxidants had much higher peroxide values compared to the crude oil treated with either tertiary-butylhydroquinone or phenolic extract from sorghum bran (Figure 20). This is an indication of the formation of higher levels of primary oxidation products in mafura oil with no added antioxidants compared to the oil with TBHQ or phenolic extract.

The observation that over the storage period, oil samples treated with 1000 ppm of crude phenolic extract from condensed tannin sorghum bran had higher peroxide values compared to oil samples treated with 2000 ppm of crude phenolic extract suggests a dose-dependent effect of the extracts in reducing formation of primary oxidation products. Although tertiary-butylhydroquinone was clearly most effective and gave the lowest peroxide values, the crude phenolic extracts at the two concentrations (1000 and 2000 ppm) still showed appreciable ability to reduce the formation of hydroperoxides. These results are in agreement to those reported by Sikwese and Duodu (2007) in which crude phenolic extract from condensed tannin sorghum bran and tertiary-butylhydroquinone were able to reduce the levels of peroxide value of sunflower oil under accelerated oxidative conditions. Wanasundara and Shahidi (1998) also reported increased stability of marine oils stored at 65^{o}C for 84 h treated with extracts from green tea leaves (which contain up to 36% of polyphenols with (+)-catechins as the predominant group).

The control sample (crude mafura oil with no added antioxidants) and the oil treated with 1000 ppm of the crude phenolic extract had higher anisidine values than the oil samples treated with 2000 ppm of crude phenolic extract and 200 ppm of tertiary-butylhydroquinone. However, the rate of increase in anisidine values in the oil treated with 1000 ppm of crude phenolic extract was significantly lower than the crude oil with no added antioxidants (Figure 21). These results clearly indicate that the presence of antioxidant additives in the oil (either crude phenolic extract or TBHQ) reduced formation of secondary oxidation products and improved the oxidative stability of the oil. There is also an indication that the ability of the crude phenolic extract to reduce formation of secondary oxidation products seems to be dose-dependent. Similar results were obtained by Sikwese and Duodu (2007) who reported that crude phenolic extract from condensed tannin sorghum bran at 1000, 1500 and 2000 ppm and 200 ppm of tertiary-butylhydroquinone were able to inhibit formation of secondary oxidation products in sunflower oil.

Lower peroxide and anisidine values of all oil samples treated with the crude phenolic extract from condensed tannin sorghum bran than their control counterparts thus reflect the antioxidant activity of the crude phenolic extract. The antioxidant effect could be due to the ability of phenolic compounds in the extract to act as primary antioxidants and scavenge free radicals and, therefore, inhibiting the formation of oxidation products and degradation of the food lipids as mentioned earlier. Therefore, it can be said that phenolic extract from

condensed tannin sorghum bran may potentially be used to stabilize traditionally extracted mafura oil against oxidation during storage.

However, the addition of the phenolic extract to the mafura oil led to colour change of the oil samples which increased proportionally with the concentration of the extract (Figure 19). Changing of oil colour through the addition of the phenolic extract may be due to the presence of anthocyanins, tannins and other flavonoids (Table 6) which may impart colour to the oil. This may represent a constraint to their use as food additive as they can change the colour of the food (Bonilla et al., 1999) and, therefore, may have a negative impact on the use of condensed tannin sorghum bran as potential source of natural antioxidant if the colour is not acceptable to consumers. However, the impartation of the colour by the phenolic antioxidants may be reduced by their combination with other substances such as vitamin E and vitamin C which may exert synergistic antioxidant effects and lower the level of crude phenolic extract required to be incorporated into the oil (Liu et al., 2008).

2.2.8 Conclusions

Freeze dried crude phenolic extract from condensed tannin sorghum bran have higher total phenols and, therefore, higher antioxidant activity than whole grain and sorghum bran because phenolic compounds are more concentrated in the outer layers of the grain. The crude extract contains phenolic acids and high levels of (-)-epicatechin and (+)-catechin. Traditionally extracted crude mafura oil treated with 1000 ppm of the freeze-dried crude phenolic extract is less oxidatively stable than the oil treated with 2000 ppm which shows that the concentration of the extract influences the antioxidant activity. However, the crude phenolic extract at 2000 ppm is as effective as tertiary-butylhydroquinone in preventing the decomposition of the hydroperoxide into secondary oxidation products. Overall, freeze-dried crude phenolic extract from condensed tannin sorghum is able to increase the oxidative stability of the traditionally extracted crude mafura oil during storage under accelerated oxidative conditions. This may be explained by the presence of phenolic compounds (phenolic acids and flavonoids) which act as antioxidants through scavenging free radicals, thus preventing lipid oxidation. As a result, it can be said that addition of exogenous natural antioxidants could be a potential way of improving the oxidative stability of traditionally extracted mafura oil.

CHAPTER 3

3. GENERAL DISCUSSION

This chapter firstly presents a critical discussion of the methodology used in this research. Secondly it discusses the main findings regarding the oxidative stability of traditionally extracted crude mafura oil as compared to sunflower oil and olive oils (extra virgin and blend) and the effect of refining and solvent extraction on oxidative stability of mafura oil. Finally, the chapter presents some hypotheses in an attempt to explain the antioxidant activity of a crude sorghum phenolic extract used to stabilize the traditionally extracted mafura oil.

3.1 Discussion of methodology

One of the major ways of utilization of mafura seeds by rural communities in the south of Mozambique is for oil extraction. Due to their perishability, mafura seeds are sun-dried (for 2 to 3 weeks) as a means of preservation until required for oil extraction (Matakala et al., 2005). A major objective of this research was to study the oxidative stability of mafura oil which has been extracted in a way which mimics the method of extraction as conducted by the rural Mozambican communities as closely as possible. Therefore, mafura seeds were also sundried before oil extraction in this research.

Sun drying has associated disadvantages. Extended drying times in the sun may affect the quality of the product (Soysal and Öztekin, 2001, Pangavhane et al., 2002, Bankole et al., 2005). Some undesired chemical and physical reactions may occur such as colour losses or browning which is caused by oxidation of some phenolics (Karabulut et al., 2007), vitamin losses (Soysal and Öztekin, 2001) and the seeds may be contaminated by insects or fungal infestation as well as dust and other foreign materials (Basunia and Abe, 2001, Soysal and Öztekin, 2001, Bankole et al., 2005). Other disadvantages of sun drying include difficulty to control the drying process due to uncertainties in weather conditions and large area requirements (Basunia and Abe, 2001). However, sun drying presents some advantages such as low capital, simple equipment and low energy input (Karabulut et al., 2007). Perhaps, from the perspective of small scale processors in rural communities in Mozambique, the most important advantage of sun drying is the fact that it is cheap and inexpensive. If some measures are put in place to improve on hygiene and sanitation during sun drying (e.g. having

a means of covering and protecting the seeds from contamination), of the mafura seeds could be regarded as appropriate for these rural communities.

After sun drying, rural Mozambican processors store the seeds in sisal bags inside huts allowing smoke to reach the seeds for further preservation (Matakala et al., 2005). In this study, after sun drying the seeds were stored in paper bags at -20^{o}C until the oil extraction period. For oil extraction, the seeds were soaked in tap water for 2 days in order to facilitate pulp removal by manual pressing. The pulp was then mixed with water and filtered. The liquid was boiled for 3 h instead of 5 h commonly done by rural communities. The boiling time was reduced in an attempt to minimize or prevent oxidation which is caused by exposure to high temperature (Vlachos et al., 2006) for long periods of time. The crude oil was then stored at -20^{o}C for further analyses.

Some important factors which have an effect on susceptibility of an oil to oxidation include free fatty acid content, degree of unsaturation and fatty acid profile. Therefore in this study, acid value, iodine value, saponification value and fatty acid composition were determined in order to characterize the mafura oil as extensively as possible. Acid value is an important quality parameter of edible oils because it gives information about levels of free fatty acids produced from lipolysis (Pearson, 1976, Narayan et al., 1988, O'Brien, 2004, Vidrih et al., 2010). Acid value can be determined using methods such as near-infrared reflectance spectroscopy (NIRS) (Armenta et al., 2007, Rao et al., 2009). In this study, a more common titration method (section 2.1.4.1) was used. This method is labour-intensive, time-consuming, requires large amounts of organic solvents (Armenta et al., 2007, Rao et al., 2009) and it can be difficult to identify the end-point of the titration especially when using coloured solutions. However, this method was suitable for mafura oil since the oil is clear yellow (or a very light brown) and it was quite easy to identify the end-point of the titration.

The iodine value along with saponification value are useful for characterizing the type of fats and oil. The former measures the degree of unsaturation of fatty acids and the latter is useful on predicting the length of the fatty acid (Knothe, 2002, O'Brien, 2004). Therefore, their determination may be associated with fatty acid composition which gives information about the type of fatty acids present in the oil. Iodine value was determined by the Wijs method (mostly used as a standard method) which is regarded as an accurate method, except in the case of conjugated double bonds or when a double bond is near a carboxyl group (O'Brien,

2004). However, it is a time-consuming procedure (Kyriakidis and Katsiloulis, 2000) due to the fact that it is necessary to allow the reaction mixture to stand for 1 h in the dark for the addition reaction between iodine and the double bonds in the unsaturated fatty acids (section 2.1.4.2). Saponification value, determined by titrimetric method (which is based on the saponification of the oil with alcoholic KOH), is largely being replaced by fatty acid composition which may be determined by gas/liquid chromatography (GLC) (O'Brien, 2004) as was done in this study.

During processing and storage, edible oils undergo oxidation leading to rancidity (Vidrih et al., 2010). The extent of oxidation may be determined by oxidative stability tests. A number of methods are used for determination of oxidative stability (Shahidi and Wanasundara, 2008). In this study, the oxidative stability was determined through measurement of the peroxide and anisidine values under accelerated oxidative conditions. Peroxide value (which measures the concentration of primary oxidation products) was measured using an iodometric titration and anisidine value (concentration of secondary oxidation products) was measured spectrophotometrically at 350 nm. The iodometric titration method is a highly empirical method, which means that any variation in procedure may cause a variation of the results. Therefore, it must be conducted carefully controlling the temperature, the sample weight, the amount, type and grade of reagents as well as the time contact between the sample and reagents (O'Brien, 2004). Also the contact with air must be taken into account because hydroperoxides are formed when unsaturated fatty acids are in contact with oxygen, therefore, for the analysis flasks with glass stoppers are required. The anisidine value method is based on the formation of coloured products by the reaction of aldehydic compounds (principally 2-alkenlas and 2,4-dienals) present in the oil with p-anisidine solution (Labrinea et al., 2001). This experiment must be conducted in the dark in order to avoid exposure to actinic light (light that affects photographic films) because p-anisidine is light sensitive and tends to darken as a result of oxidation (AOCS, 1989). To avoid exposure of the solutions to light all containers were covered with aluminium foil and the experiment was conducted within the least possible time.

These two methods are very useful indicators of the oil quality and the two analyses should be conducted to give a full picture of the oxidation state of the oil. The peroxide value estimates the oxidation of the oil at the moment of the analyses whereas the anisidine value gives the whole history of the oxidation from the extraction period to the time of the

experiment (Shahidi and Wanasundara, 2008). The combination of peroxide and anisidine values gives the total oxidation value, which has practical application because it helps to estimate the oxidative deterioration of the food lipids (Shahidi and Wanasundara, 2008). The oxidative stability of mafura oil was determined under accelerated oxidative conditions (65^{o}C in the dark) in order to accelerate the development of rancidity because storage at room temperature would need longer test periods (Abou-Gharbia et al., 2000). The rate of decompositions of oils stored at elevated temperature is much higher than at lower temperatures (Frankel, 1993).

Refining of the crude mafura oil was conducted in an attempt to improve its oxidative stability by the removal of substances such as phospholipids, free fatty acids and other minor impurities. Crude oils may be refined either chemically or physically. In this study, the chemical refining process was applied to mafura oil. Mafura oil was refined in two steps namely degumming and neutralization. In the former, phosphatides are removed by the addition of water whereas in the latter, free fatty acids are removed through the saponification reaction. During degumming, the oil in contact with water (20%) at high temperature (75 ^{o}C) is agitated in order to allow the gums to coagulate and separate from the degummed oil. However, not all the water is absorbed in the gums and, therefore, it remains in the oil. After degumming the oil may be passed through a vacuum dryer to remove the excess water (James, 1958). In this study, however, neither degummed nor neutralized oil was further dried and therefore, it may affect the quality of the refined mafura oil lowering its oxidative stability and therefore, its shelf-life.

Mafura oil was also extracted using organic solvents (hexane and ethanol) and the oxidative stability was compared to the oil extracted traditionally. Although ethanol is a polar solvent and, therefore, gives lower oil yield than hexane (a non-polar substance), ethanol has also been used for oil extraction due to its ability to extract phenolic compounds (Hron Sr et al., 1982, Hron et al., 1994, Ferreira-Dias et al., 2003) which may contribute to increased oil oxidative stability. The solubility of triglycerides in ethanol is low but ethanol is able to extract oil because the gain of energy in the solute-solvent interaction is larger than the energy required for disruption of solute-solvent interaction (Johnson and Lusas, 1983). The solubility of the oil in hexane is high because of stronger solute-solvent interaction which compensates for energy losses. This is in accordance with the general principle of dissolution

which states that a non-polar solute is easily dissolved in a non-polar solvent (Johnson and Lusas, 1983).

Although the solvent-extracted oils were more oxidatively stable, the traditional method of extraction of mafura oil could be regarded as being more cost effective since only water is added and the solvent recovery step is eliminated (Cater et al., 1974, Rosenthal et al., 1996). Furthermore, it could also be regarded as being more environmentally friendly. However, the traditional method of oil extraction gave lower mafura oil yield because of its lower efficiency in oil extraction as compared to organic solvents. During the traditional mafura oil extraction process, the soaked seed pulp was boiled in excess water for a long period of time. On the other hand, during solvent extraction, the oil was extracted by agitating the mixture of the dried pulp and the solvent using a magnetic stirrer at room temperature. This is likely the most important characteristic of the two methods of extraction that determined the observed differences in oil oxidative stability.

Based on the knowledge that sorghum bran is rich in phenolic compounds (Awika and Rooney, 2004, Dykes and Rooney, 2006, Dykes et al., 2014) and that they have antioxidant capacity, a freeze-dried crude phenolic extract from condensed tannin sorghum bran was added to the traditionally extracted mafura oil in an attempt to stabilize it against oxidation. Sorghum grain was decorticated using a sorghum dehuller (section 2.2.3.3) to obtain the bran in which phenolics are concentrated (Awika and Rooney, 2004, O'Neil et al., 2010, Luthria, 2012, Luthria and Liu, 2013). It was of value to use sorghum bran as a source of antioxidants because it is a cheap, underutilized by-product of sorghum milling which would be readily accessible to rural communities. Total phenolic content and antioxidant activity of the condensed tannin sorghum whole grain, bran and freeze-dried crude phenolic extract were determined using Folin-Ciocalteu method and ABTS radical scavenging assay, respectively. Separation and identification of the phenolic composition of the freeze-dried crude phenolic extract from sorghum bran was determined using High Performance Liquid Chromatography (HPLC).

Phenolic compounds are extracted using several solvents such as methanol, ethanol and their aqueous mixtures (Krygier et al., 1982). For sorghum, phenolic compounds have been extracted using water, acidified water (Shelembe et al., 2014), aqueous acetone (Krygier et al., 1982, Awika et al., 2004, Awika and Rooney, 2004) and acidified methanol (Awika et al.,

2004, Awika and Rooney, 2004). In this study, crude phenolic extract from condensed tannin sorghum bran was prepared using 75% aqueous acetone (Sikwese and Duodu, 2007).

The Folin-Ciocalteu assay is a colorimetric method widely used for determination of total phenolic content (Kaluza et al., 1980, Awika et al., 2005). This method is based on the reducing power of aromatic hydroxyls which react with the Folin-Ciocalteu reagent producing coloured products that can be determined spectrophotometrically at 750 nm (Grigelmo-Miguel et al., 2009). One drawback of this method is that the Folin-Ciocalteu reagent is not specific and detects all non-phenolic reducing substances present in the extract such as L-ascorbic acid, reducing sugars (Prior et al., 2005) and extractable proteins (Andrés-Lacueva et al., 2009) leading to overestimation of the total phenolic content. Nevertheless, this method is simple, reproducible and convenient (Grigelmo-Miguel et al., 2009).

The ABTS radical scavenging assay (or Trolox equivalent antioxidant capacity, TEAC) is used to determine antioxidant capacity. This method is based on the ability of antioxidants to scavenge the 2,2´-azinobis (3-ethylbenzothiazoline-6 sulphonic acid) radicals ($ABTS^{\cdot+}$) as compared to that of a Trolox (water soluble vitamin E analogue) standard (Awika et al., 2003, Prior et al., 2005). One of the disadvantages of this method is that the $ABTS^{\cdot+}$ radical generation requires up to 12 h. The addition of the antioxidant before $ABTS^{\cdot+}$ formation may result in an overestimation of antioxidant capacity due to the possible interference of the antioxidant on radical scavenging or on radical formation. Therefore, the antioxidant (crude phenolic extract) is only added when the formation of radical is stable (Strljbe et al., 1997, Re et al., 1999, van den Berg et al., 1999) which occurs after 12h.

Tertiary butylhydroquinone (synthetic antioxidant) was added to mafura oil at the maximum allowable limit (200 ppm) and freeze-dried crude phenolic extract from sorghum bran (natural antioxidant) was added at two different concentrations (1000 ppm and 2000 ppm) in order to investigate the possibility of a dose response. Despite the superior efficacy of the TBHQ in stabilizing the mafura oil, the crude phenolic extract from condensed tannin sorghum bran exhibited good potential to be used to stabilize the oil in place of the synthetic TBHQ.

3.2 Discussion of main findings

Edible oils with high levels of unsaturation are the most susceptible to oxidation (Ramadan and Mörsel, 2004, Zambiazi et al., 2007, Moser, 2009, Mohdaly et al., 2011, Abdelazim et al., 2013). The oxidation reaction takes place between molecular oxygen and unsaturated fatty acids (Figure 23) (Shahidi and Wanasundara, 2008). In this study, however, traditionally extracted crude mafura oil with high levels of saturated fatty acids, was found to be highly susceptible to oxidative deterioration as compared to commercially available sunflower oil and olive oils (extra virgin and olive oil blend) which are rich in polyunsaturated fatty acids and monounsaturated fatty acids, respectively. These results indicate that the lower stability of mafura oil may not be related to the fatty acid composition.

Apart from fatty acid composition, the method of processing or extraction of the oil could influence its oxidative stability. This hypothesis was shown to be true in this research. The mode of preparation of the traditionally extracted mafura oil may have contributed to its decreased oxidative stability due to the extensive heating in excess water of the pulp during the extraction process. High temperatures in combination with moisture (O'Brien, 2004) and/or enzymes (Robards et al., 1988, Ramezanzadeh et al., 1999, Naz et al., 2004, Liauw et al., 2008) lead to hydrolysis of the ester bonds in fatty acids and formation of free fatty acids. The high acid value of crude and unrefined mafura oil demonstrated this. High contents of free fatty acids contribute to the formation of unstable primary oxidation products (hydroperoxides) (Martínez et al., 2013, Roman et al., 2013, Zhang et al., 2015). They can easily decompose into secondary oxidation products (carbonyl compounds) which are considered to be responsible for off-flavours and lowering of the shelf-life of edible oils (Guillén and Cabo, 2002). In this research, it seems the mafura oil was already in an advanced state of secondary oxidation even before the beginning of the storage period. The very high initial anisidine value of traditionally extracted mafura oil showed its poor initial oxidative quality and this anisidine value continued to increase further at high rates over the storage period.

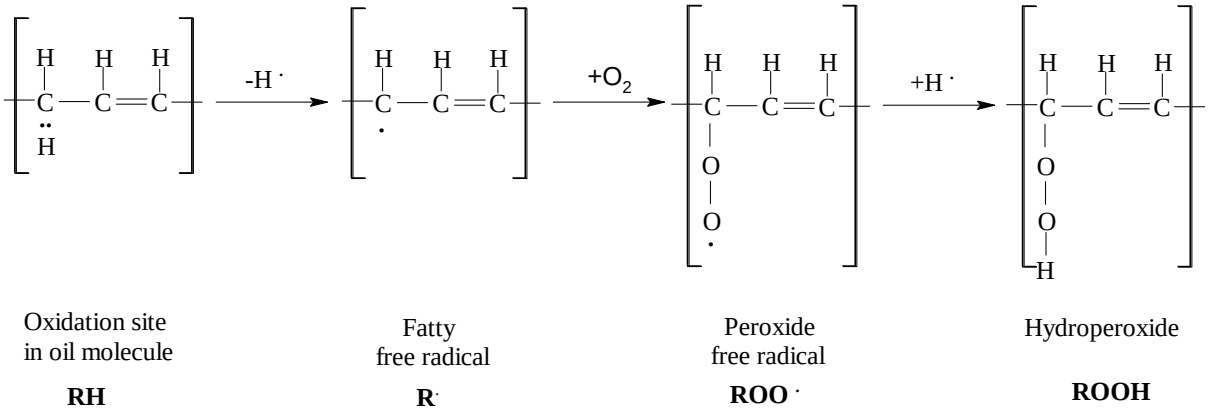


Figure 23 - Free radical oxidation of vegetable oil (Sherwin, 1978).

An important result from this research is the observation that the crude sorghum phenolic extract was able to stabilize the traditionally extracted mafura oil against oxidation by reducing formation of secondary oxidation products. The antioxidant capacity of the phenolic extract may be related to the presence of phenolic compounds which exert antioxidant properties. Phenolics act as primary antioxidants donating hydrogen atom or electrons to lipid radicals (Shahidi and Zhong, 2010b), thus terminating the oxidation at the initiation step (Sherwin, 1976).

(+)-Catechin (Figure 26), one of the major flavonoids identified in the crude phenolic extract may be used to illustrate the chemistry behind the antioxidant effect of the phenolic compounds. The (+)-catechin molecule could donate a hydrogen atom to the highly reactive lipid radical which is produced after the initiation step during the free radical chain reaction. This reaction produces a non-radical lipid derivative and a catechin radical which is stabilized through resonance (Figure 24) by delocalization of the unpaired electron around the aromatic ring of the (+)-catechin molecule (Kamal-Eldin, 2006, Shahidi and Zhong, 2010a). The resonance structures lack suitable sites for attack by molecular oxygen (Eskin and Przybylski, 2001) and have low energy, therefore they are stable and do not promote further reaction.

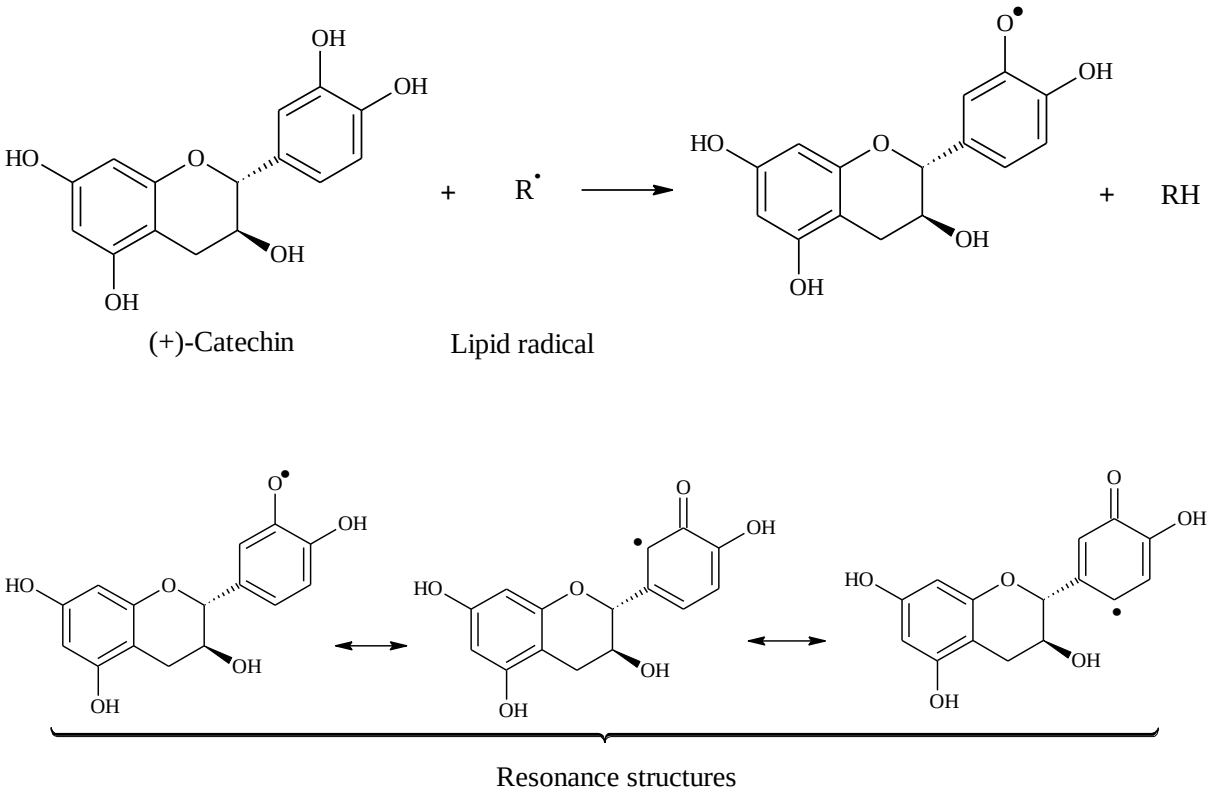
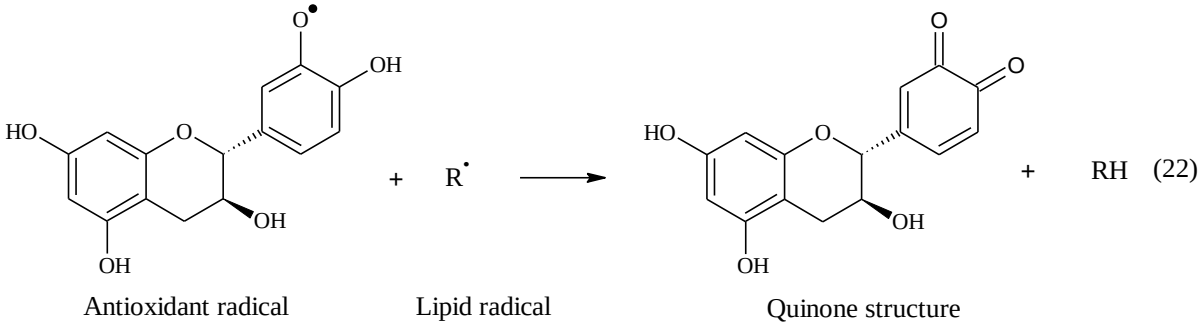


Figure 24 - Possible position for free radical scavenging by (+)-catechin and their resonance structures.

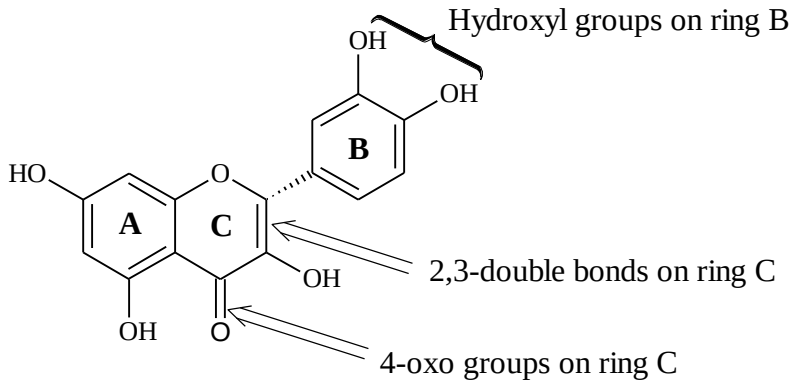
The antioxidant radical intermediate may also be stabilized by formation of a relatively stable and low energy quinone structure (Reaction 22) through reaction with a second radical (Pietta, 2000).



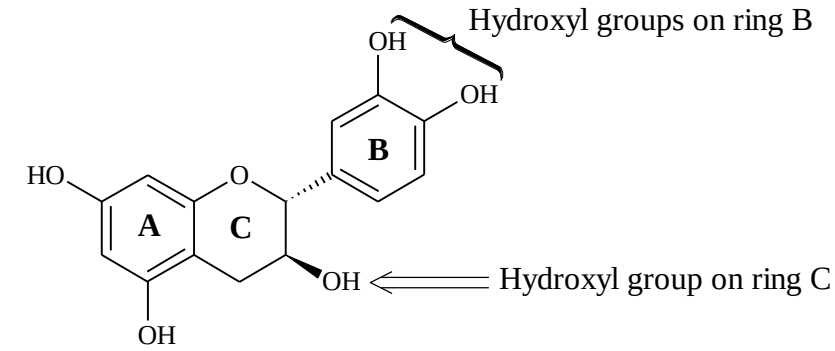
(22)

The antioxidant activity of the phenolic compounds identified in the crude phenolic extract from sorghum bran is related to their structure (Balasundram et al., 2006). For phenolic acids antioxidant activity depends on the numbers and position of the hydroxyl groups in relation to

the carboxyl functional group (Robards et al., 1999) whereas the antioxidant activity of the flavonoids depends on the structure and the nature of substitutions on rings B and C (Balasundram et al., 2006). The antioxidant activity in phenolic acids is enhanced with an increasing number of phenolic hydroxyl groups (Rice-Evans et al., 1996, Milić et al., 1998, Robards et al., 1999, van den Berg et al., 2000). For instance, gallic acid (Figure 7A), found in the crude phenolic extract used in this study, contains three hydroxyl groups substituted in the meta- and para-positions in relation to the carboxyl group and, therefore, exhibits high antioxidant activity (Milić et al., 1998). In the case of phenolic acids derived from hydroxycinnamic acids the presence of para hydroxyl group as in p-coumaric acid (also found in the extract), increases the reducing properties (Rice-Evans et al., 1996). According to Rice-Evans et al. (1996) and Fernandez et al. (2002), flavonoids that contain OH group attached to the 2,3-double bond and adjacent to the 4-carbonyl in the C ring as well as ortho-dihydroxylation in ring B exhibit maximum effectiveness of radical scavenging because they participate in the electron delocalization conferring high stability to the antioxidant radical formed. A similar arrangement is found in the structure of quercetin (Figure 25) which was found in the phenolic extract analysed in this study, and this could contribute to the antioxidant properties of the extract. The presence of the hydroxyl group in C-3 of ring C as well as the catechol group in ring B may increase the radical scavenging activity of (+)-catechin (Figure 25). Overall, the phenolic compounds found in the crude phenolic extract used in this study have structural characteristics that contribute to the antioxidant activity of the extract.



A Quercetin



(+)-Catechin

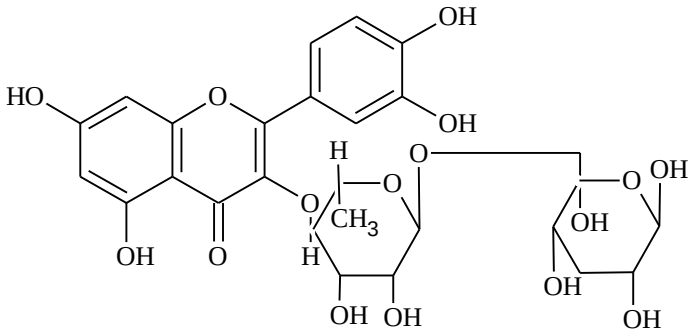
B

Figure 25 - Quercetin (A) and (+)-catechin (B) structures indicating important features for antioxidant activity.

It may be possible that the different phenolic compounds in the crude extract could work together to exert a synergistic antioxidant effect. According to Fernandez et al. (2002) flavonoids have synergistic effects with other antioxidants. Peyrat-Maillard et al. (2003), reported that a synergistic effect among phenolic compounds may be established when the less efficient antioxidant regenerates the more efficient one (Reaction 23).

$$A_2OH + A_1O\cdot \longrightarrow A_2O\cdot + A_1OH \qquad (23)$$

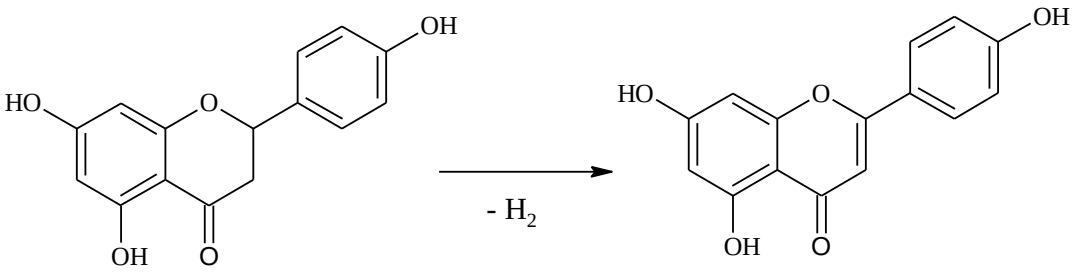
Becker et al. (2007) reported that the combination of quercetin (Figure 25 A) and rutin (Figure 26) (found in the crude phenolic extract analysed in this study) exhibited a synergistic effect in retarding lipid oxidation.



Rutin (flavan-3-ol rutinoside)

Figure 26 - Antioxidant rutin (flavan-3-ol rutinoside) (Becker et al., 2007).

In the cases in which a flavonoid lacks the double bond between C2 and C3 (e.g. naringenin), the 4-oxo group does not have much effect on antioxidant activity because it does not participate in delocalization of the unpaired electron (Rice-Evans et al., 1996). However, loss of hydrogen atoms and formation of a double bond may occur. This could be the case for naringenin (identified in the phenolic extract) whose antioxidant activity could be increased through the loss of 2 hydrogen atoms leading to formation of apigenin (Reaction 24). The presence of the double bond increases the planarity and stability of the antioxidant molecule which may be responsible for the higher antioxidant activity of apigenin (Fernandez et al., 2002).



(24)

Naringenin Apigenin

Despite the desirable antioxidative effect of the phenolic compounds present in the phenolic extract from sorghum bran, they may have undesirable effects such as off-colour and taste (Maxson and Rooney, 1972, Hahn et al., 1984 according to Sikwese, 2007). In this study, the phenolic extract from red condensed tannin sorghum bran imparted colour to mafura oil probably due to the presence of flavonoids (Table 6). This may represent a drawback in the use of red condensed tannin sorghum as a source of natural antioxidant because an antioxidant acceptable for food use must meet the following requirements: absence of sensory influence, effectiveness at low concentrations, compatible with substrate and must not affect the physical properties of food products (Eskin and Przybylski, 2001). The impartation of colour to the oil was more evident as the concentration of the phenolic extract increased. The possibility of using less concentrated extract in the presence of synergistic substances such ascorbic acid (vitamin C) could be considered. The synergistic effect appears to be due to the ability of the synergists to regenerate the antioxidant in the native form from their antioxidant free radicals (Liu et al., 2008). Depending on the consumers, the impartation of colour may not be perceived as negative impact because the oil will have increased shelf-life as well as better oxidative stability and, therefore, more appropriate for human consumption. Therefore, the slight change in colour of mafura oil may not necessarily mean that it will be rejected.

CHAPTER 4

4. CONCLUSIONS AND RECOMMENDATIONS

Mafura oil is rich in saturated fatty acids (mainly palmitic acid) which make it different from sunflower oil (rich in linoleic acid) and olive oil (rich in oleic acid). However, traditionally extracted crude mafura oil is highly susceptible to oxidative deterioration which is indicated by its higher peroxide and anisidine values as compared to sunflower oil and olive oil during storage under accelerated oxidative conditions.

Refining using a laboratory scale process of degumming and neutralization does not improve the oxidative stability of traditionally extracted mafura oil during storage under accelerated oxidative conditions. However, solvent-extracted mafura oil (using hexane or ethanol) has improved oxidative stability compared to traditionally extracted mafura oil during storage. This is an indication that the method of extraction rather than fatty acid composition is the main determinant of oxidative stability of traditionally extracted mafura oil during storage under accelerated oxidative conditions.

The extensive boiling treatment of the mafura fruit pulp in excess water (during preparation of traditionally extracted mafura oil) leads to increased hydrolysis and release of free fatty acids (high acid value). These free fatty acids then become prone to oxidation with production of high levels of primary oxidation products (high peroxide value) and secondary oxidation products (high anisidine value) in the oil during storage. The oxidative stability of traditionally extracted mafura oil may be improved by boiling the fruit pulp for a shorter period of time or drying the extracted oil under vacuum to remove the excess water after oil extraction. Also the oil could be extracted using mafura seeds that are as fresh as possible.

A crude phenolic extract prepared from red condensed tannin sorghum bran is rich in various phenolic acids (mainly protocatechuic acid and ferulic acid) and flavonoids (mainly catechin and epicatechin). Traditionally extracted crude mafura oil treated with the crude phenolic extract from red condensed tannin sorghum bran and tertiary-butylhydroquinone is stable to oxidative deterioration (as indicated by the low peroxide and anisidine values) under the accelerated oxidative conditions (65^{o}C) over 14-day storage period. The crude phenolic extract inhibits the formation of primary and secondary oxidation products in mafura oil due

to the antioxidant properties of the phenolic compounds which also appears to be dose-dependent. Although the crude phenolic extract at 2000 ppm is not as effective as tertiary-butylhydroquinone in inhibiting the formation of primary oxidation products, it is quite similar in preventing the formation of secondary oxidation products, which are responsible for rancidity.

However, the crude phenolic extract imparts colour to the mafura oil which could be perceived negatively by consumers. There is a need for a systematic sensory and market research study to establish the effect of the colour impartation to the oil by the crude phenolic extract on perception by consumers and processors of mafura oil in Mozambique. The colour effect could also be minimized by using the crude phenolic extract in combination with other antioxidants such as ascorbic acid which could lower the concentration of crude phenolic extract needed.

Overall, this study demonstrates that even though traditionally extracted crude mafura oil has poor oxidative quality due to the way it is extracted, there are opportunities for improving the stability by the use of readily available antioxidant additives from natural plant sources. This could be of relevance for rural mafura oil processors and consumers in Mozambique. Although, the rural processors may not be able to prepare phenolic extracts from sorghum bran due to lack of the means to do so (e.g. solvents and extraction equipment), it may be possible that the bran could be incorporated into the oil as is without extraction if ground finely.

There could also be opportunities for industrial scale production of mafura oil if alternative methods of extraction could be established which do not require the use of high temperature. Such methods should also take into account storage conditions for the mafura fruit due to their high perishability.

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