THE EFFECT OF EXTRACTION METHODS ON THE OXIDATIVE STABILITY OF MARULA AND MORINGA SEED OIL

by

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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Chemistry, School of Chemistry and Physics of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville campus, Durban, South Africa. National Research Foundation (NRF) financially supported the research.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION: PLAGIARISM

I, Anwuli Tracey Nwabuebo, declare that:

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(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

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ABSTRACT

Marula and moringa seeds are used as a source of food, and the seed oils are used in cosmetics, pharmaceuticals and in medicines. This is due to its high nutritive value and high content of unsaturated fatty acids, which however makes them highly susceptible to oxidation. Due to its high nutritive value, the demand for the seed oil often exceeds industrial supply. Therefore, a timeous and environmentally friendly extraction method, which produces an oil with better oxidative stability, is required. Hence, the reason for this investigation on the effect of screw press, aqueous extraction (37 and 60 °C) and solvent extraction (shaker and Soxhlet) method on the oxidative stability of marula and moringa seed oil.

The oxidative stability of the seed oils was determined by carrying out several tests, such as moisture content, acid value and peroxide value (PV). In addition, the anisidine value (AV), radical scavenging activity, conjugated diene (CD) and triene (CT) % were determined by UV-Vis spectroscopy. The fatty acid methyl ester content was determined using gas chromatography-mass spectrometry (GC-MS). The result for the oil yield showed there was a significant difference (P<0.05) in the different extraction methods. Soxhlet extracted marula and moringa seed oil had the highest oil yield of 53.99 % and 35.20 %, respectively. Aqueous extracted marula and moringa oil at 37 °C had the lowest oil yield of 18.67 % and 12.00 %, respectively. The fatty acid profile showed the presence of palmitic, stearic, oleic, linoleic and behenic acid in moringa seed oils with oleic acid being the most dominant in the seed oils with the different extraction methods. Soxhlet extracted marula and moringa seed oil had a fatty acid composition of 70.70 and 77.61 % respectively, while aqueous extracted marula and moringa seed oil at 37 °C was 72.36 and 79.94 % respectively. The oxidative stability test PV, a measure of the initial oxidation, and AV, a measure of secondary oxidation product, showed there was a significant difference (P<0.05) for the different extraction methods. Aqueous extracted marula and moringa seed oil at 37 °C had the highest radical scavenging ability compared to the other extraction techniques. The oxidative stability test carried out for 35 days at ambient and different accelerated storage conditions (45 and 65 °C) showed that aqueous extracted seed oil at 37 °C for both seed oils had lower values for the oxidative tests and lower values for the rate of change of PV and AV values thus suggesting a better stability oil. This could be as a result of the presence of the higher monounsaturated fatty

acid which aids stability, and lower polyunsaturated fatty acid present. A comparison of the stability tests for two seed oils showed that moringa seed oil had a better stability. This is probably due to the lower secondary oxidation products present in moringa oil as well as a higher quantity of monounsaturated fatty acid and lower quantity of polyunsaturated fatty acid present compared to that of marula. This study showed that aqueous extracted seed oils have a has a better resistance to degradation and oxidation reduction and shelf life in comparison with the other extraction methods. Also, moringa seed oil showed a better resistance to degradation as compared to marula seed oil

CONFERENCE PARTICIPATION

1. Anwuli T Nwabuebo and Brenda Moodley, **Effect of Extraction Methods on the Oxidative Stability of Marula and Moringa Seed Oil** (Poster presentation at the College of Agriculture, Engineering and Science Research Day, 29th November 2016, Howard Campus, UKZN).
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LIST OF ABBREVIATIONS

2,2-diphenyl-1-picrylhydrazyl	DPPH
American Oil Chemist Society	AOCS
Analysis of variance	ANOVA
Conjugated dienes	CD
Conjugated trienes	CT
Butylated hydroxyanisole	BHA
Electron transfer	ET
Fatty acid methyl esters	FAMEs
Fatty acids	FA
Ferric reducing antioxidant power	FRAP
Free radicals	A*
Gas chromatography coupled with a flame ionization detector	GC-FID
Gas chromatography mass spectrometry	GC-MS
Hydrogen atom transfer	HAT
Hydroperoxide	ROOH
Malonaldehyde	MA
Monounsaturated FAs	MUFAs
National Institute of Standards and Technology	NIST
Oxygen radical absorbance capacity	ORAC
p-anisidine value	*p*-AV or AV
Polyunsaturated FAs	PUFAs

Statistical Package	SPSS 24.0
Supercritical fluid extraction	SFE
Thiobarbituric acid	TBA
Total radical trapping antioxidant parameter	TRAP
Trolox equivalent antioxidant capacity	TEAC

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1.0 CHAPTER 1: INTRODUCTION

1.1 General overview

Plants store energy in the form of starch and storage lipids (Lüttge, 2012), mostly in seeds (seed oil) or pulp (fruit oil such as olive, avocado, palm) (Bora et al., 2001). In most plants lipids are stored in the form of triglycerides, jojoba being an exception where lipids are stored as wax (Murphy, 1990). Vegetable oil can be defined specifically as plant oils that are in a liquid state, but can generally be in any state (liquid, solid or gaseous) at a given temperature. Oils in a solid state at room temperature are called vegetable fat. In early days oils extracted from plants were used as a source of food, medicine, energy and cosmetic application (O'brien, 2008; Zimba et al., 2005). Oil is used in the treatment of ailments like muscle spasms, varicose veins and wounds, hair dandruff and in the production of lubricants and soap (Chivandi et al., 2008; Van Wyk et al., 2002). Examples of seed oils include sunflower oil, canola oil, sesame oil, grape seed oil, macadamia oil, marula and moringa oils and many others.

This work focused on moringa and marula seed oils, where oils were extracted from seeds of their respective trees. They are high oleic acid oils reported with high oxidative stability and used as carrier oils in cosmetic formulations. High oxidative stability is an important parameter for the cosmetics industry. Currently cold pressed oils are preferred as this extraction method is known to preserve oxidative stability. However, aqueous extraction is slowly gaining popularity due to the mild conditions required for extraction. Therefore, the aim of this study was to compare the effect of different extraction methods on the oxidative stabilities of moringa and marula seed oils.

Moringa olifera is part of the family *Moringaceae* having 14 species belonging to the genus *Moringa* (Morton, 1991). It is commonly known as the horse-radish or “drumstick” tree and indigenous to the western and sub-Himalayan tract including India, Pakistan, Asia Minor, Africa and Arabia (Mughal et al., 1999; Somali et al., 1984). It is now widely used in the tropical and sub-tropical areas (Anwar et al., 2007; Morton, 1991). Moringa is a deciduous tree which grows fairly quickly reaching a height of 5 to 10 m and

sometimes 15 m high (Morton, 1991; Somali et al., 1984). It is mostly found growing on pastureland or in river basins and grows well on hill sides (Yadav et al., 2016). The moringa pod changes colour to brown when it is matured and has about 10 to 50 seeds inside the pod, which is about 50 cm long (Figure 1.1). The matured dry seeds have a round shape and the outer part of the kernel is covered with a dark brown shell with three papery wings (Abdulkarim et al., 2005; Vlahov et al., 2002) (Figure 1.2).

Figure 1.1: Photograph showing moringa tree and pods (Oommen, 2015)

Figure 1.2: Photograph showing the (a) moringa kernels with the papery wings and (b) seeds

Due to the great importance of moringa tree in the food and medicinal industries, some specific non-governmental organizations such as Trees for Life, Church World Service

and Educational Concerns for Hunger Organization have recommended moringa as a natural nutrient (Fahey, 2005; Makkar et al., 1999; Manzoor et al., 2007; Sreelatha et al., 2009). The moringa tree is an all-purpose tree with the green pods and fresh dried leaves used as a vegetable which are rich in free leucine. The young leaves are edible and are commonly prepared and eaten like spinach or used to make soups and salads and are a good source of Vitamins A, B_1, B_2, B_3, B_6, B_7, C, D, E and K as well as minerals such as calcium, copper, iron, potassium, magnesium, manganese and zinc (Foidl et al., 2001). Moringa leaves are known to be very nutritious. For example, the content of vitamin C present in moringa was higher than that in orange, the vitamin A present was higher in moringa than in carrot, the calcium present was higher in moringa than that in milk, and potassium in moringa was higher than that in banana. Moringa has twice the amount of protein than in yogurt and a higher micro nutrient in dry leaves (Mahatab et al., 1987; Manzoor et al., 2007). In another study, it was reported that some parts of the tree showed antitumor, antipyretic, antiepileptic, anti-inflammatory, and antiulcer effects and are used in native medicines (Morimitsu et al., 2000; Siddhuraju et al., 2003; Singh et al., 1999).

The seed contains yellow edible oil that is used for medicinal purposes (Anwar et al., 2003; Foidl et al., 2001). The constituent of the fatty acid present in moringa seed oil is about 13 % saturated fatty acids, 82 % unsaturated fatty acids and a higher quantity of oleic acid (70 %) (Foidl et al., 2001; Rahman et al., 2009; Tsaknis et al., 1998). Oleic acid rich seed oils could be a substitute for hydrogenated vegetable oil because of their high stability and numerous health benefits (Rahman et al., 2009; Tsaknis et al., 1999). Moringa oil is also used as a lubricant for machinery because of its resistance to rancidity (Ferrao et al., 1970; Ramachandran et al., 1980). In addition the oil is used for cooking, used in making odours stable in the perfume industry and can absorb and retain volatile substances.

The cosmetic use of moringa oil dates back to the Egyptians, where it was reported that the addition of terebinth (frankincense), wax and fresh moringa oil was used to remove wrinkles (Kleiman et al., 2006). Moringa seed oil is presently used as a carrier oil in cosmetic preparations. It is also reported as a good oil for massaging and for aromatherapy applications, and also in body and hair care as a moisturizer and skin conditioner (Aney et al., 2009). Other uses include soap making and use in cosmetic preparations such as lip balm and creams (Aney et al., 2009). Armand-Stussi et al. (2003) reported that moringa

butter, a semisolid fraction of moringa oil, is used in skin products for babies and sensitive skin because it helps to soothe and soften the skin.

Marula (*Sclerocarya birrea*) belonging to the family *Anacardiaceae* subspecies *caffra*, is commonly found in the savannah regions in Africa and its geographical distribution runs from inland of West (Gambia and Nigeria), Central (Cameroon) and East (Ethiopia and Sudan) Africa and the lowlands of Southern Africa (South Africa) (Viljoen et al., 2008). The tree grows up to 10–15 m high with a grey cracked-like bark, strong, thick branches and light coloured leaves (Figure 1.3). The leaves are compound, pinnate and the flowers are greenish in colour. The fruits are yellow, and look like olives with white fruit pulp surrounded by a thick skin (Figure 1.4).

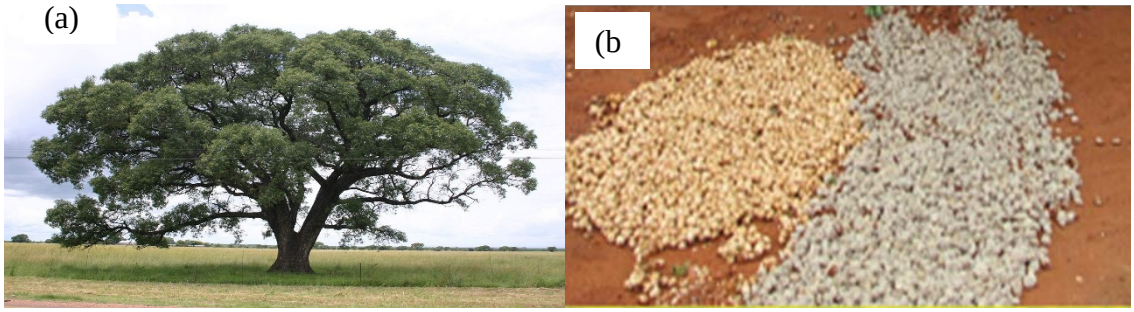


Figure 1.3: Photograph showing (a) Marula tree and (b) kernel

Figure 1.4: Photograph showing Marula fruit

The marula tree is an important source of food, and also has commercial, cultural and medicinal importance in Africa. The marula fruit has a strong aroma and is a fleshy fruit which is widely consumed due to its high nutritive value (Ojewole et al., 2010). The gum obtained from the tree is rich in tannins, and used for making a substitute for ink (Ojewole, 2003). Its African medicinal uses include bark extract for diarrhea and stomach pain, and the root extract is used for treatment of sore eyes (Eloff, 2001; Maroyi, 2011; Mathabe et

al., 2006). The leaves and roots are used for the treatment of fungal infections and snake bite (Dimo et al., 2007; Hamza et al., 2006). Marula wood is used by African villagers for making household dishes, mortars for crushing mealie, musical instruments, toys and decorative items (Breyer-Brandwijk, 1962). It was reported in literature that the bark yields 3.5–20.5 % tannin, 10.7 % tanning matter and traces of alkaloids (Breyer-Brandwijk, 1962).

Marula fruit contains a high vitamin C content which is triple that of oranges and has the same amount as that in guava (Baba et al., 2014). The fruit contains soluble phenolics, K, Na, Ca, Mg, Fe, Zn, Mn (Hillman et al., 2008), crude oils, carbohydrates, crude proteins, fibre, saponins (Ogbobe, 1992), minerals (Smith et al., 1996), and ascorbic acid (Eromosele et al., 1991). The main phenolic compounds in the fruit are byproducts of hydrolizable tannins, catechins, and hydroxycinnamic acid (Borochov-Neori et al., 2008). Literature has shown the presence of esters and hydrocarbons such as heptadecene (16.1 %), benzyl 4-methylpentanoate (8.8 %), benzyl butyrate (6.7 %), (Z)-13 octadecenal (6.2 %), cyclo-pentadecane (5.7 %), (Z)-3-decen-1-ol (8.4 %), 6-dodecen-1-ol (3.8 %), 11-hexadecanal (4.4 %), β-caryophyllene (91.3 %), α-humulene (8.3 %), and germacrene D (0.1 %) (Viljoen et al., 2008). It was also reported to contain caffeic acid, vanillic acid, *p*-hydroxybenzaldehyde, ferulic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid (Ndhlala et al., 2007). In several parts of Southern Africa the whole fruit is used for brewing beer and distilling spirits.

The kernels are rich in oil, which has a clear, pale, yellowish-brown colour with a pleasant smell. It is known to have a high nutritional value, high oxidative stability, and antioxidant activity, free radical scavenging properties and moisturizing properties. It is also used for cooking, preserving meat and for treatment of leather. Mono-unsaturated fatty acids and natural antioxidants are abundant in marula oil and the fatty acid composition of the oils makes the oil stable (Burger et al., 1987; Eromosele et al., 2003; Glew et al., 2004). It has been reported that the oil has an abundance of glutamic acid and arginine and also contains 64 % oleic acid, myristic, stearic and amino acids (Eloff, 2001). Due to the afformentioned properties it is used in industries for cosmetic preparation.

Both moringa (indigenous to India) and marula (indigenous to Africa) oils are high oleic acid oils. They are both reported to have high oxidative stability and were therefore

selected to investigate the effect of different extraction methods on their oxidative stability.

Traditionally oils have been extracted using solvents or by mechanical means (pressure). In East Africa, shea butter is still extracted by stirring ground seeds in boiling water and scooping of the lower density oil. Of the many oil extraction techniques available, hexane extraction is currently the most widely used commercially. However safety, environmental and worker health concerns associated with hexane is prompting the development of alternative more environmentally friendly methods (Latif, Diosady, et al., 2008).

Aqueous extraction, which is a type of cold pressing, is normally used for fruit oil (olive and avocado) extraction is more recently being applied to nuts and seed oil extraction, notwithstanding the lower yields. Marula and moringa are mainly pressed by mechanical means and currently there are no reports on the extraction of marula and moringa seed oils using aqueous extraction at 37 and 60 °C.

Cold pressing is gaining popularity due to the global drive towards natural extraction methods. Cold pressing implies that the temperature during extraction is kept below 60 °C where the low temperature is said to retain the physicochemical properties and stability of the seed oil.

1.2 Statement of problem

At present the common commercial extraction methods used are solvent and mechanical oil extraction (Febrianto et al., 2012). The mechanical pressing (screw press) has its advantages and disadvantages. One of its advantages is that it is a green method, which does not require the use of a solvent for extracting oil from the seed, but its disadvantage is that it has a lower oil yield with large amounts of residual oil left in the cake (about 7 % or 8 %). The advantage of solvent extraction is that it virtually extracts all the oil present in the seed leaving about 0.5 % in the cake. Its disadvantage is that the use of a solvent makes it not environmentally friendly and flammability of the solvent contributes to safety issues during the extraction process (Luthria et al., 2004; Anderson, 2016). A

better method of extraction, which will not affect the physicochemical properties of the seed oil, is required. This has led to this study of moringa and marula seeds, which are both naturally high in oleic acid with reported high antioxidant activities, and are evaluated for the impact of different extraction methods on oxidative stability, a key oil quality parameter. Aqueous extraction may be a suitable alternate method for the extraction of seed oils, since it uses milder extraction and processing conditions as well as it is a green method that may produce oils that are more stable and have a longer shelf life

1.3 Hypothesis

Aqueous extraction results in marula and moringa seed oils with better physicochemical properties, greater stability and of higher quality than hexane extracted and mechanically pressed oils.

1.4 Aim and objectives

Aim

To examine whether the method used to extract oil impacts oil quality, and specifically, the fatty acid profile, acid value, primary and secondary oxidation, antioxidant activity and shelf life of two high oleic acid oils namely, moringa and marula seed oils.

Objectives

1) To extract oil from moringa and marula seed using five methods, namely: hexane extraction (Soxhlet method at 69 °C), hexane extraction at ambient temperature (shaker method), aqueous extraction at 37 °C, aqueous extraction at 60 °C and mechanical pressing (screw press method).

2) To determine the quality parameters of the extracted oils (as per 1) specifically, the acid value (or, percentage free fatty acid), peroxide value, anisidine value and conjugated diene and triene values of the seed oils and analyze the results.

3) To determine the fatty acid composition and compare the profiles of the seed oils extracted with the different extraction methods (as per 1)

4) To determine the impact of the extraction method on the antioxidant activity of the different seed oils, if any.

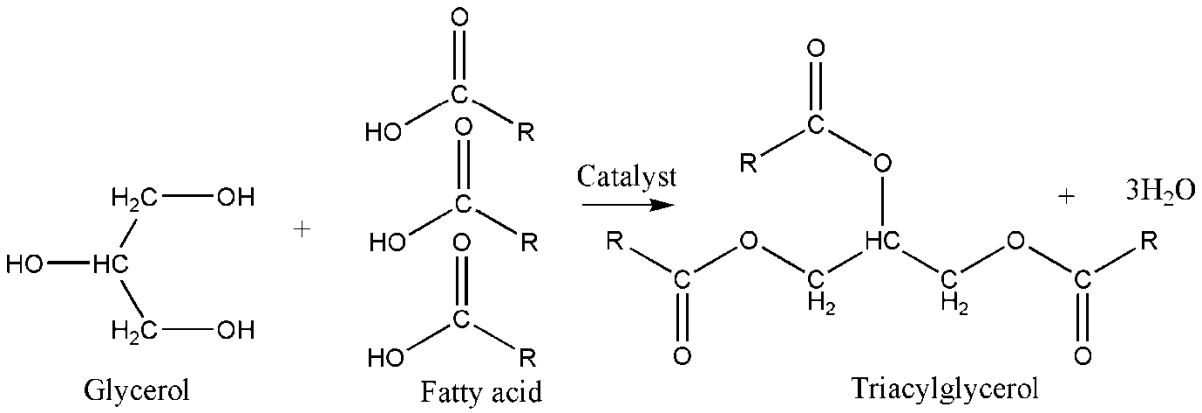
5) To conduct an accelerated shelf life study on the oils of (1) and compare the results.

6) To critically analyse the results obtained in 2-5 and determine whether the hypothesis is supported or not.

2.0 CHAPTER 2: LITERATURE REVIEW

2.1 Oils and Fats

Oils and fats are generally referred to as lipids; they are insoluble in water and soluble in organic solvents. The difference between oils and fats is that fats are solid at room temperature and oils are liquid at room temperature, with temperature ranging between 21 and 25 °C. Lipids are categorized depending on their acyl residue or their polarity (Belitz et al., 2009). Triacylglycerols are the generally used edible lipids and are made up of a glycerol backbone and three fatty acids (Scheme 2.1).



Scheme 2.1: Reaction of glycerol with 3 fatty acids to form triacylglycerol

Fats and oils are made up of triglycerides and other components like free fatty acids, tocopherol, sterols, trace metals, esters, carotenoids, ketones, phospholipids and other compounds. The origin of the oil determines its constituents (Belitz et al., 2009). In recent years, the request for edible oil has increased with the increase in population. Food industries are mindful of the type of oil they use because of health issues, and due to this, there has been an increase in the demand for oleic rich oil because of their high stability (Ahamd et al., 2015). Moringa and marula seed oils have been found to naturally have high oleic acid content (Abdulkarim et al., 2005; Eloff, 2001).

2.2 Extraction of oil

The process of obtaining this oil from seed or plant matrices is called extraction. There are many methods for the extraction of oil from plant matrices with the most widely used being chemical (solvent) and mechanical extraction (Halim et al., 2012; Mubarak et al., 2015). Cold pressing is now gaining more popularity as it is being associated with a more natural product.

2.2.1 Cold pressing

The term cold pressing does not mean that the temperature is fixed. Codex Alimentarius Commission et al. (2001) stated that cold pressed oil is obtained without the application of heat. However, heat is always generated in the process of pressing when there is friction between two surfaces (Parry et al., 2005). The temperature during the extraction of oil using a cold pressing technique depends on the structure of the seed and the oily fruits (Van Hoed et al., 2011) and should not be above 50 °C. However there are some exceptions, for example olive oil extraction using cold press should not exceed 30 °C (Van Hoed et al., 2011), pumpkin seed oil should not go above 60 °C during cold oil pressing (Goranovic, 2009), whereas cold pressing should be between the temperature of 40 to 60 °C for berry seed oil (Van Hoed et al., 2009).

2.2.1.1 Mechanical pressing

Mechanical extraction uses pressure to press the oil, with a screw press or hydraulic press. Mechanical extraction of oil is carried out by exerting pressure on the seed to break the cells and force out the oils from the seed. The main methods of extraction of oil through mechanical process are the screw or expeller press and the hydraulic press. In the screw press, a feeder and horizontal screw is used to apply pressure on the seed material. The barrel around the screw is used to let out the oil from the barrel. In a hydraulic press the material is loaded in a cage to which pressure is applied, the oil is pressed out and the cake is removed manually. A hydraulic press can be operated manually, whereas a screw press requires much larger horsepower and cannot be operated manually.

Mechanical pressing is the easiest method of extracting oil from a seed matrix. Unlike hexane extraction, where seed preparation is very important, in the mechanical pressing method, the plant matrix is cleaned (stones, sand, dirt, and spoiled seeds are removed by using a sifter or mesh and by hand picking) and then fed into the press with enough pressure generated to cause oil to flow out from the material (Mandal et al., 2013).

2.2.2 Solvent extraction

The most commonly used chemical techniques for extracting oil from plant matrices include organic solvent extraction, supercritical fluid extraction, accelerated solvent extraction, microwave assisted solvent extraction, ultrasonic assisted solvent extraction and aqueous extraction (Halim et al., 2011; Khoo et al., 2011).

Organic solvents such as *n*-hexane, benzene, toluene, diethyl ether, chloroform, ethanol, isopropane, methanol, acetone and ethyl acetate are used in organic solvent extraction (Dunnuck, 1991). Most of these solvents are toxic and inflammable, not environmentally friendly and may have adverse effects on human health (Bhattacharjee et al., 2007; Dunnuck, 1991). Of all the solvents mentioned, hexane (non-polar) has been used extensively throughout the world as a solvent for extracting vegetable oils because it does not cause much irritation on the skin and has less severe toxicity compared to the aforementioned solvents (Balasubramanian et al., 2011). Even though chemical extraction is popular, its main disadvantage is that it is inflammable (Iqbal et al., 2013).

Solvent extraction techniques are based on the principle of “like dissolves like”. An ideal solvent requires high levels of specificity towards lipids especially acylglycerols, and the solvent must be volatile enough to ensure low energy distillation to separate the lipid from the solvent. In the extraction of oil from plant matrices non-polar solvents such as hexane, benzene, toluene, chloroform and polar solvents such as methanol, acetone, ethyl acetate, diethyl ether and ethanol can be used (Mubarak et al., 2015).

The extraction efficiency is greatly affected by a number of parameters such as drying time of the plant material, particle size of the plant material, solvent type, amount of solute to solvent ratio, extraction temperature and extraction time (Daroch et al., 2013). Therefore, a good experimental

design is required to prepare the plant matrix within the range of process parameters to optimize process conditions (Chen et al., 2011). The percentage yield of oil can be improved by using solvents such as *n*-hexane, chloroform, methanol and/or a mixture of two or more solvents (Reshad et al., 2015). The commonly used and standard solvent for solvent extraction in industry is hexane because it is more efficient and reliable compared to other solvents, it uses a reduced amount of horse power, and it leaves a minute amount of oil 0.5 % in the cake (Reverchon et al., 2006).

2.2.2.1 Aqueous extraction

The extraction of seed oil from a plant matrix using an aqueous solution is referred to as an aqueous extraction process. This method is more environmentally friendly and safer to operate in comparison with organic solvent (hexane) extraction methods. The limitation of this method is its low efficiency of oil extraction (Aremu et al., 2015). However, industry prefers this method because of the safety; it is non-toxic and environmentally friendly. Much effort have been made for the past three decades in optimizing aqueous processing as an alternative for extraction of oil from plant matrices. Seed oil from coconuts and rice, peanuts, sunflower seeds, soybeans and lupine have been extracted using the aqueous extraction method (Aguilera et al., 1983; Hanmoungjai et al., 2000). Hanmoungjai et al. (2000) reported the aqueous extraction method to be favorable at a temperature between 45 to 85 °C.

2.2.2.2 Supercritical CO_2 extraction

The supercritical fluid extraction (SFE) technique is a process of removing or dissolving the extractant from its plant matrix with the use of supercritical fluid (CO_2) (Mubarak et al., 2015). A fluid is in its supercritical state when its temperature and pressure is above its critical point, thereby dispersing through a solid like a gas and dissolving materials like liquid above its critical point. Supercritical CO_2 is a widely used solvent for supercritical fluid extractions due to its intermediate critical pressure (7.4 MPa) and reasonably low critical temperature (31.1 °C) (Wang et al., 2006).

Supercritical fluids can be used as an extraction solvent to produce solvent free oil because the solvent pressure and temperature can be varied (Santana et al., 2012). Due to this, supercritical fluid extraction has been a valuable technique for the extraction of oils, fats and other natural

products from seeds (Akanda et al., 2012). Extracts from this method are usually contaminant free and in their original chemical form (Rozzi et al., 2002). In addition, the oil is extracted from the seed in a shorter time; CO_2 is inert, nontoxic, nonflammable and environmentally acceptable. The disadvantage of supercritical fluid extraction is that it is expensive to maintain.

The supercritical fluid extraction (SFE) technique has grown rapidly since its introduction and it is now the preferred method for industrial food processing (Akanda et al., 2012). It is regarded as a green processing technique and is an alternative to organic solvent-based extraction techniques (Herrero et al., 2010).

2.2.2.3 Ultrasound assisted solvent extraction of oil

Ultrasound assisted extraction is also known as sonication-assisted or ultrasound-assisted solvent extraction. This method is used to separate the extractant from the plant matrix with the aid of energy formed from the ultrasonic field that disrupts the cell walls of the plant. This then enables the solvent to enter the plant cells to release the seed oils (Vinatoru, 2001).

Elastic deformation in piezoelectric materials, which occurs due to the application of a high electric field frequency (50/60 Hz), is the basis of the principle behind ultrasonic waves. The distortion of the piezoelectric transducer is changed to a mechanical vibration, that is improved before being communicated to a resonating probe or sonotrode that is in connection with the processing medium (Raichel, 2006). Furthermore, ultrasonic technology in solvent extraction has been widely used in recent times, for the extraction of oil, pigment, proteins and flavonoids from plant matrices (Li et al., 2004). It is a fast technique and requires a shorter time. Its drawback is that it is costly.

2.2.2.4 Microwave assisted extraction of oil

Microwave extraction is an extraction technique that separates the extractant from the plant matrix with the help of microwave energy which is produced by the perpendicular oscillation between the electric and magnetic field which produces electromagnetic radiation with frequencies ranging from 0.3 to 300 GHz (Camel, 2001; De Monte et al., 2014). Microwave heating generally operates at a frequency of 2.45 GHz to avoid interferences with communication, domestic and industrial applications. Microwave–assisted extraction is widely used in the extraction of valuable

components from medicinal herbs and plants (Bayramoglu et al., 2008; Mahesar et al., 2008). The type of solvent used for this extraction method has an effect on the extraction yield of the oil (Rostagno et al., 2007). The main advantages of the microwave-assisted extraction method are its short extraction time, high oil yield, and it is energy saving. It does have some drawbacks in that it is expensive and less environmentally friendly (Pan et al., 2003; Priego-Capote et al., 2005; Shu et al., 2003).

Table 2.1 shows the oil yield obtained for extraction of moringa and cranberry seed using various extraction methods.

Table 2.1: Oil yield (%) of different extraction methods from different seed sources

Extraction methods	Oil source	Oil yield % (w/w)	Reference
Hexane	Moringa seed	38.40; 41.47	(Anwar et al., 2007; Ogbunugafor et al., 2011)
Screw press	Moringa seed	25.00; 39.10	(Mohamed, 2015; Palafox et al., 2012)
Supercritical CO_2	Moringa seed	21.00; 35.00	(Mohd-Setapar et al., 2013; Palafox et al., 2012)
Aqueous enzyme	Moringa seed	15.41	(Anwar et al., 2007)
Ultrasonication	Cranberry seed	24.21-32.35	(Thyagarajan, 2012)
Microwave	Cranberry seed	15.73-24.15	(Thyagarajan, 2012)

2.3 Rancidity of oil

Rancidity is the word used to signify an unpleasant flavor or taste in food, which is as a result of hydrolysis or oxidation. There are three types of rancidity: oxidative rancidity, hydrolytic rancidity and microbial rancidity.

Hydrolytic rancidity occurs when triglycerides are hydrolyzed to form free fatty acids in the presence of a catalyst. Oxidative rancidity results from absorption of oxygen from the environment (autoxidation). Microbial rancidity occurs when microorganism, such as bacteria use their enzymes (lipases) to break apart the chemical structure of the oil. Oil oxidation generates among others, aldehydes and ketones that cause an unpleasant odour or taste.

Oxidation in oil is very important in terms of quality, nutritional value, toxicity and taste, and odour of edible oil.

2.3.1 Oil oxidation

Autoxidation is any oxidation that occurs in open air or in the presence of oxygen (and sometimes UV radiation) and forms hydroperoxide. Hydroperoxide is formed from oxidation or changes of an unsaturated fatty acid or one of its intermediary oxidation products, in the presence of oxygen.

Many factors affect autoxidation of fatty acids; these include oil composition, the degree of unsaturation, the presence and activity of antioxidants, partial pressure of oxygen, temperature, light exposure, and moisture content of fat/oil (Belitz et al., 2009). In addition, the position of the double bond in the triacylglyceride molecule also affects the rate of autoxidation. Fast oxidation occurs on triacylglycerides containing unsaturated bonds in position 1- or 3- compared to triacylglycerides having an unsaturated bond in position 2 (Belitz et al., 2009).

2.3.2 Steps involved in autoxidation of oil

The quality and the stability of oil is affected by oxidation, which occurs in 3 stages: initiation, propagation and termination stage.

At the initiation stage, unsaturated oil (AH) loses it hydrogen radical in the presence of an initiator (I) to produce a few very reactive fatty acid molecules that have unpaired electrons called free radicals (A*). Initiators are singlet-oxygen molecules ($^1O_2$) which are highly reactive with high energy but they have a very short life.

In the propagation reaction stage the oil free radicals (A*) react with oxygen to produce peroxy radicals (AOO*) which are also very reactive. The AOO* react with another AH to produce AOOH

(hydroperoxide) and another A*. A* can then go around and repeat the reaction, thereby leading to a chain reaction.

The termination step occurs when two radicals (AOO* and A*) are joined to form a non-reactive unit. In a situation where oxygen is in excess, peroxy radicals will join to make the termination product (Frankel, 2012a; Upritchard, 2004). The initiation, propagation and termination steps are summarized in equations 1 to 5:

Initiation stage

$$I + AH \rightarrow A^* + IH \quad (1)$$

Propagation stage

$$A^* + O_2 \rightarrow AOO^* \quad (2)$$

$$AOO^* + AH \rightarrow AOOH + A^* \quad (3)$$

Termination stage

$$AOO^* + A^* \rightarrow AOOA \quad (4)$$

$$A^* + A^* \rightarrow AA \quad (5)$$

The primary oxidation product that occurs in oil is the formation of hydroperoxide from the main oxidation process that occurs during the initiation and propagation stages. The maximum level of hydroperoxide increases rapidly in highly unsaturated oils because the hydroperoxide decomposes easily. When the hydroperoxide gets to its maximum level it further decomposes into secondary oxidation products (Frankel, 2012a; Upritchard, 2004).

Oxidation in oil can be avoided by the addition of antioxidants, metal inactivators or by capping the container of oil with nitrogen to protect the oil. When unsaturated fatty acids are exposed to light they are susceptible to photooxidation, when exposed to lipoxygenases they are susceptible to enzymatic oxidation, and when exposed to autoxidation, direct reaction of molecular oxygen with organic compounds takes place (Frankel, 2012a; Upritchard, 2004).

2.3.3 Oxidation products

Hydroperoxides are oxidation products of polyunsaturated fatty acids. This oxidation product is referred to as a primary oxidation product because they are detected at the early stage, due to the removal of hydrogen from polyunsaturated fatty acids (Akoh et al., 2008; Belitz et al., 2009). Further decomposition of these hydroperoxides leads to the formation of secondary oxidation products (aldehydes, ketones, alcohols, acids, and lactones) which leads to the unfavorable taste, flavor and texture of the oil (Kamal-Eldin, 2003). The oxidative status of oil can be determined by quantitatively measuring the primary and secondary oxidation products of the oil as carried out in this study.

Peroxide value and conjugated diene are the commonly used methods for determining the primary oxidation state while *p*-anisidine value is the most widely used quality parameter for measuring secondary oxidation products of edible oils (Shahidi et al., 2005).

2.4. Methods to measure oxidation products in oil

2.4.1 Peroxide value

The preferred method of quantifying primary oxidation in the oil and food industry is by measuring the peroxide value (Shahidi et al., 2005; Yildiz et al., 2003). This method gives a quantitative measure of hydroperoxide present in the initial stage of oil oxidation. The peroxide concentration reveals the extent of oxidative deterioration of the oil.

The quantification of peroxides in oil is determined *via* HI or Fe^{2+}, based on the principle of reduction of the hydroperoxide group in the oil.

The ferric thiocyanate method is developed based on the ability of hydroperoxide to oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) in an acidic medium which are complexed by either thiocyanate or xylenol orange to form red-violet or blue-purple chromophores, respectively (Eymard et al., 2003; Jiang et al., 1991). The increased sensitivity is as a result of the lower

sensitivity of ferrous ions to spontaneously oxidize in air, as compared to the high susceptibility of iodide solutions to oxidize. The ferric ions form chromophores which when complexed to thiocyanate can be measured by spectrophotometry with absorption at wavelengths of 500-510 nm (Belitz et al., 2009; Eymard et al., 2003).

Determination of the peroxide value using the iodometric method is based on the reaction of saturated potassium iodide (KI) solution with the hydroperoxide (ROOH) in the oil. From this reaction iodine (I_2) is liberated which, when titrated against a standardized solution of sodium thiosulfate with starch as an end point indicator, gives a quantitative measurement of the hydroperoxide present (Shahidi et al., 2005). The peroxide value is reported as milliequivalent of oxygen per kilogram of sample (meq O_2/kg). The reaction steps are shown in equation 6 and 7:

$$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+ \quad (6)$$

$$I_2 + 2NaS_2O_3 \rightarrow Na_2S_4O_6 + 2NaI \quad (7)$$

Even though the iodometric method is the preferred method of peroxide value determination, it has several limitations, which include the absorption of iodine across the double bonds, oxidation of iodide by dissolved oxygen, difficulty in determining the endpoint, and its sensitivity is lower than the ferric thiocyanate. It is further labour intensive, consumes a lot of sample and generates organic solvent waste (Dobarganes et al., 2002; Ruíz et al., 2001).

2.4.2 Conjugated dienes and trienes

Polyunsaturated fatty acids form hydroperoxides during oxidation. However, if there is a shift or rearrangement in the double bond during the formation of hydroperoxides, these then form conjugated dienes, which absorb at 234 nm. When there is a further shift in three or more double bonds conjugated trienes form, which absorb at 268 nm. (Shahidi et al., 2005). Conjugated dienes and trienes are oxidation products and their formation in fats and oils is revealed by an increase in UV absorption. The detection of conjugated dienes and trienes using the ultraviolet method is fast and requires little sample and no chemical reagents. It is also simple and does not depend on chemical or colour reactions. This method has a low specificity and sensitivity in comparison with

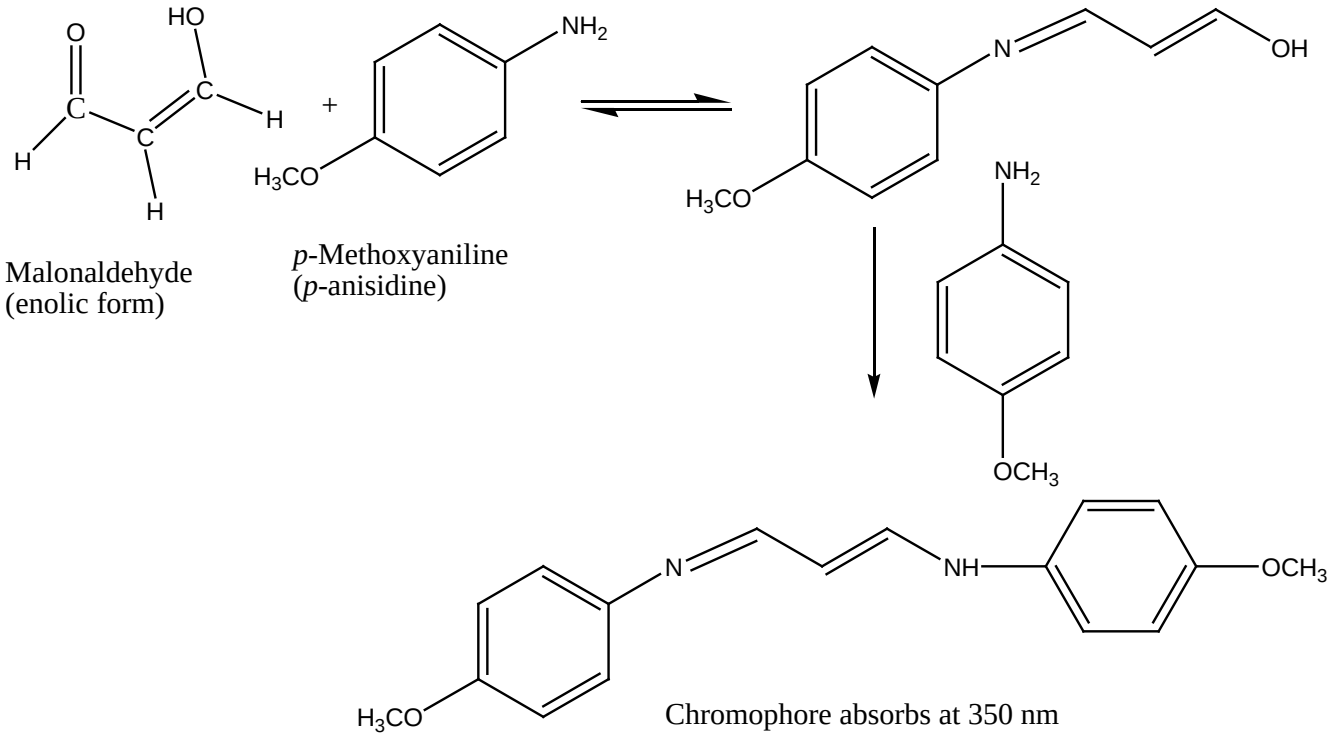
the peroxide value for the detection of hydroperoxide in oil (Frankel, 2012b; Shahidi et al., 2005). Conjugated dienes are generally measured using ultraviolet measurements and hydroperoxides are measured using the iodometric titration which determines the peroxide value (Shahidi et al., 2005). The limitation of the conjugated diene measurement is that it is dependent on the type of fatty acid present.

2.4.3 Measurement of secondary oxidation products

The decomposition of the primary oxidation product (hydroperoxide) gives rise to the formation of secondary oxidation products, which could be volatile or non-volatile. These secondary oxidation products include carbonyl containing compounds, hydrocarbons, volatile organic acids, alcohols, epoxy compounds and many others. Several methods exist for measuring the secondary oxidation product such as thiobarbituric acid (TBA) test, *p*-anisidine value (*p*-AV), carbonyl test, totox value, conjugated triene value and many others. The commonly and widely used method is the *p*-anisidine test

2.4.4 p-anisidine value

The anisidine test measures the content of aldehydes (2-alkenals and 2,4-alkadienals) produced during the decomposition of hydroperoxide (Frankel, 2012b). The anisidine value is defined as the absorbance of a solution after reacting 1 g fat in 100 mL of isooctane solvent and *p*- anisidine reagent (0.25 % anisidine in glacial acetic acid). As shown in scheme 2.2, the reaction of *p*-anisidine with an aldehyde in acidic conditions yields a yellowish product which absorbs at 350 nm (Shahidi et al., 2005). The *p*-anisidine value is a good and reliable method for measuring the amount of secondary oxidation products in fats and oils (List et al., 1974). The anisidine test on oils that have been newly pressed can be used to determine their stability. This test can also be used to determine hidden oxidation based on the presence of high molecular weight decomposition compounds produced during vegetable oil extraction before removal of the odour of the oil (Frankel, 2012b). The disadvantage of the anisidine test is that the reagent used must be carbonyl free to prevent the interference with the carbonyl present in the sample. Also, the reaction requires a water free reagent because in the presence of water the reaction will be incomplete (White, 1995).



Scheme 2.2: Proposed reaction between *p*-anisidine reagent and malonaldehyde (Shahidi et al., 2002)

2.4.5 Thiobarbituric acid (TBA) value

Thiobarbituric acid is an old method used for determining the lipid oxidation state. The thiobarbituric acid (TBA) value is defined as the extent of lipid oxidation and is expressed as milligram of malonaldehyde (MA) equivalents per kilogram of sample or as micromoles MA equivalent per gram of sample. The product of oxidation of polyunsaturated fatty acids is the malonaldehyde which reacts with the thiobarbituric acid reagent to form a pink complex which absorbs at 530-532 nm (Frankel, 2012b; Shahidi et al., 2005). Because the reaction consists of a large number of secondary oxidation products, the reacting secondary products are referred to as TBA-reactive substances or TBARS. The test is standardized by using malonaldehyde generated from 1, 1, 3, 3-tetraethoxypropane by acid hydrolysis (Frankel, 2012b). Again, the TBA test specifically detects malonaldehyde, which is one particular oxidation product, and whether this is representative of the oxidation status of the oil depends on the oil.

The thiobarbituric acid reactive substances assay is a well-known assay but suffers from lack of sensitivity and specificity. The state of the reaction such as high temperatures, changes in pH, long heating times, and presence of antioxidants and metal ions are known to significantly affect the colour development which may affect the spectrophotometric analysis (Antolovich et al., 2002). Nevertheless the main limitation is as a result of several compounds reacting with the TBA reagent and therefore results in an overall higher intensity of the colour complex (de las Heras et al., 2003). This test is usually carried out in the assessment of meat but in the case of oils, the *p*-anisidine value test is preferred to the thiobarbituric acid reactive substances assay due to the aforementioned limitations (Shahidi et al., 2005). For this reason, the *p*-anisidine value test was used in this study.

2.4.6 Oxidative stability

Oxidative stability is the ability of an organic compound to resist oxidation, and further degradation, which results in rancidity, and loss of quality. There are many methods that can be used to determine the oxidative stability of lipids, and a number of different tests have been designed to accelerate the normal oxidation process. The accelerated oxidation method allows one to understand how the lipid may deteriorate once exposed to oxidation. The oxidative stability of oil is often determined by subjecting it to an accelerated process using high temperatures or bubbling with oxygen for the purpose of research. For a better understanding of the oxidative stability of oils, measuring the primary oxidation product and secondary oxidation products is important to get an indication of its oxidative stability. These products of oxidation are quantified using the peroxide value and the *p*-anisidine tests as described in sections 2.4.1 and 2.4.4.

2.5 Fatty acid composition

The major constituents of oil from all vegetable, animal and marine (fish or algal) are fatty acids (FA). Fatty acids are made up of carbon as their backbone in the form of an alkyl chain, and with a carboxylic acid group and methyl group attached at each end as shown in scheme 2.3. Physical and functional properties of FAs are affected by the number of carbon atoms present including the

carbon of the carboxylic group, and the location and number of double bonds present in the straight chain (Belitz et al., 2009; Gurr et al., 2008).

$$R\text{-}(CH_2)_n\text{-}COOH$$

Scheme 2.3: General formula of a fatty acid

FAs are essential in human and animal diets, they are a source of glucose and metabolic energy in the body, and they also play a significant role in the formation and maintenance of cellular membranes (Belitz et al., 2009). Monounsaturated FAs (MUFAs) are FAs that contain one double bond in their alkyl chain, while, the ones with more than one double bond are called polyunsaturated FAs (PUFAs). The structural integrity of cellular membranes is maintained by PUFAs (Gurr et al., 2008), and they have also been reported to have health-promoting functions against various diseases e.g. cardiovascular, heart, diabetes, cancer, rheumatoid arthritis and inflammatory bowel disease (Abete et al., 2009; Caygill et al., 1996; Zurier et al., 1996). Table 2.2 provides a list of the commonly found fatty acids in plant seed oil.

Table 2.2: List of fatty acids found in plant seed oil (Stobart et al., 2003)

Systematic Name	Common Name	Structure (C: D)
Dodecanoic acid:	Lauric acid	12:0
Tetradecanoic acid	Myristic acid	14:0
Hexadecanoic acid	Palmitic acid	16:0
Hexadec-*cis*-9-monoenoic acid	Palmitoleic acid	$16:1^{\Delta 9}$
Octadecanoic acid	Stearic acid	18:0
Octadeca-9-*cis*-monoenoic acid	Oleic acid	18:1
Octadeca-*cis*-9,12-dienoic acid	Linoleic acid	$18:1^{\Delta 9,12}$
Octadeca-*cis*-9,12,15-trienoic acid	α-Linolenic acid	$18:3^{\Delta 9,12,15}$
Octadeca-*cis*-6,9,12-trienoic acid	γ-Linolenic acid	$18:3^{\Delta 6,9,12}$
Octadeca-*cis*-6,9,12,15-tetraenoic acid	Stearidonic acid	$18:4^{\Delta 6,9,12,15}$
Eicosanoic acid	Arachidic acid	20:0
Eicosa-*cis*-9-monoenoic acid	Gadoleic acid	$20:1^{\Delta 9}$
Docosanoic acid	Behenic acid	22:0
Docsa-*cis*-9-monoenoic acid	Erucic acid	22:1

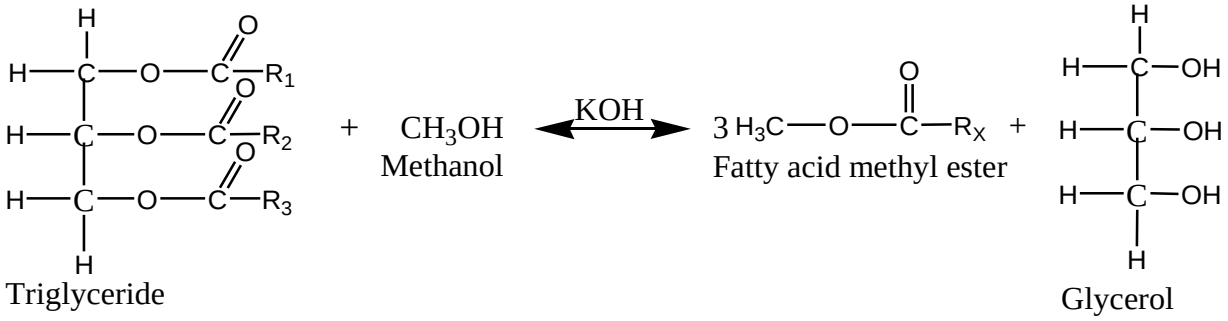
Where C is the number of carbon atoms in the fatty acid; D is the number of double bonds in the fatty acid while Δ^x (delta-x) indicates the position of the double bond on the x^{th} carbon-carbon bond counting from the carboxylic acid end.

Several analytical techniques have been used for determining fatty acids, namely enzymatic, spectrophotometric, high performance liquid chromatography, gas chromatography coupled with a flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). GC-MS is the preferred method because it has a high sensitivity and resolution.

The gas chromatography mass-spectrometry technique comprises of a gas chromatograph coupled with a mass spectrometer. The sample to be analyzed is injected into the GC inlet where it is volatilized and enters the column with the help of an inert gas usually (helium) which is the mobile phase. The mobile phase carries the sample through a packed or capillary column that separates the components of the sample based on the different boiling points of the analytes in the sample which inturn affects the strength of interaction with the stationary phase. The mobile phase helps the volatalized analyte make its way out of the column to the detector (mass spectrometer). The mass spectrometer ionizes the chemical compound to generate charged molecular ions and fragments, and measures their mass to charge ratio. The ion then travels through the mass analyser that separates them based on their mass to charge ratio. The detector amplifies the signal and counts the number of ions associated with their specific mass to charge ratio. The information is then processed *via* the computer software and a mass spectrum is created.

Fatty acids are polar and non-volatile. Therefore, in order for the fatty acid to be volatile and be separated by GC, derivatization must be carried out. Methylation is the general method of converting non-volatile fatty acids to volatile fatty acid methyl esters (FAMEs) (scheme 2.4.). Methylation of fatty acid is performed with BF_3 methanol or methanolic potassium hydroxide as a derivatizing agent.

In gas chromatography (GC) we inject the sample, which may be a gas or a liquid, into an gaseous mobile phase (often called the carrier gas). The mobile phase carries the sample through a packed or capillary column that separates the sample's components based on their ability to partition between the mobile phase and the stationary phase



Scheme 2.4: Reaction of triglyceride and KOH methanol to form fatty acid methyl ester and glycerol

2.6 Antioxidants

Antioxidants are substances that protect cells from oxidative stress and the effects of free radicals. Oxidative stress is an imbalance between free radicals produced in the body and the ability of the body to fight against the negative effect of the free radicals by neutralizing with the help of an antioxidant. Thus antioxidants play an important role by repairing the damages caused by free radicals (McCall et al., 1999; Wittenstein et al., 2002).

Antioxidants play an important role in delaying or preventing autoxidation. There are two classes of antioxidants, primary or chain-breaking antioxidants and secondary or preventative antioxidants (Madhavi et al., 1995). The primary antioxidants have the ability to scavenge free radicals and inhibit initiation, propagation and the β- scission reaction. Secondary or preventative antioxidants are compounds that delay the rate of oxidation by reacting with prooxidants or oxidation intermediates (Antolovich et al., 2002). Many methods have been developed for assessing the activity of antioxidants. These methods are based on hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays are the major methods that measure HAT while Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and diphenyl-l-picrylhydrazyl (DPPH) assay represent ET-based methods. The DPPH assay is the most commonly used method to test the activity of antioxidants in plant materials.

2.6.1 2, 2-Diphenyl-l-picrylhydrazyl (DPPH) assay (Radical Scavenging ability)

DPPH is used to determine the antioxidant activity (Oyaizu, 1986) of a substance or compound. This method is based on reducing tolueneic DPPH solution in the presence of hydrogen donating antioxidant (present in the oil) resulting in the formation of the non-radical form of DPPH-H. It is a stable chromogen radical with a deep purple colour. As shown in equation 8 and 9, DPPH radicals are reduced in the presence of antioxidants (AH) or reaction with radical species (R^*), and the reduction reaction results in loss of colour of the solution. This loss in colour can be measured spectrophotometrically at 517 nm, which reveals the reducing ability of antioxidants (AH).

$$DPPH^* + AH \rightarrow DPPH - H + A \quad (8)$$

$$DPPH^* + R^* \rightarrow DPPH \rightarrow R \quad (9)$$

3.0 CHAPTER 3: MATERIALS AND METHOD

3.1 Materials and reagents

Moringa seeds were harvested in March 2016 in Bushbuckridge, South Africa while marula seeds were harvested from January to March 2016 in Majeje, South Africa. The moringa seeds were stored in the refrigerator at 4 ℃ and the marula seeds were stored in a dark dry room at ambient temperature because the quantity of the marula seeds was too large to store in the refrigerator. Both seeds were extracted within a week of the seeds arriving at the laboratory. The moringa seeds were carefully crushed using a pestle and mortar to de-hull the seeds. Thereafter, the seeds were manually cleaned by separation from the hulls while the marula kernels were crushed at the Everpix factory site. Screw press extraction was carried out at Everpix, Pietermaritzburg, South Africa. The other extraction processes were carried out in the laboratory at UKZN, Westville Campus. The extracted oils were stored in glass amber bottles that had been flushed with nitrogen and then capped and stored in the refrigerator at 4 ℃ until analysis (Adejumo et al., 2013). All the reagent and solvents used were HPLC grade and purchased from Merck and Capital Labs in South Africa.

3.2 Determination of moisture content

The moisture content of the seed was determined gravimetrically using the oven method. A 4.9993 ± 0.0001 g sample of the ground seed was weighed in a petri dish and dried in the oven at 105 °C for 24 hours. After drying, the seeds were cooled in a desiccator for 30 minutes, and were thereafter weighed until a constant value was obtained. This process was repeated in triplicate each time on fresh samples to get the average value of moisture content of the seeds (Adejumo et al., 2013). The percentage moisture content of the seed was calculated as follows:

$$\% M = \left[\frac{m_1 - m_2}{m_1 - m_0}\right] \times 100\ \% \qquad (10)$$

Where m_0 = mass of the empty weighing container in grams

m_1 = mass of the weighing container and sample before drying in grams

m_2 = mass of weighing container and sample after drying in grams

The moisture content was determined in triplicate, each time with a fresh sample of seed.

The moisture content of the oils were determined using the same method described above for moisture content of seeds.

3.3 Sample preparation

3.3.1 Oil extraction

The seeds were extracted using various extraction methods: Soxhlet extraction with hexane at 69 °C, hexane extraction at ambient temperature using a shaker, aqueous extraction at 37 and 60 °C, and screw press extraction. The aqueous extraction temperature of 37 °C, was chosen because oil would not be extracted at ambient temperature alone. The aqueous extraction temperature of 60 °C, was chosen because any higher temperatures would result in the seed oil becoming denatured. The temperature of 69 °C, for hexane Soxhlet extraction is the standard temperature used in literature.

3.3.2 Soxhlet extraction with hexane

The seed samples were blended using an electroline domestic blender. The blended seed (approximately 50 g that was the average of triplicate weighings) was placed in a thimble, which was positioned in the center of the Soxhlet apparatus and connected to the round bottom flask containing 300 mL of hexane and anti-bumping stones to prevent it from violent boiling. The set up was placed on a heating mantle for 8 hours at 69 °C (Figure 3.1). After the extraction process was completed the extract was quantitatively transferred to a Heidolph rotary evaporator (model number 517-61000-00-0) at 45 °C to remove the solvent. The final oil extract was purged with nitrogen for 40 minutes to remove residual solvent and was kept in the refrigerator until analysis

was carried out (Adejumo et al., 2013). The extraction process was carried out in triplicate each time on fresh samples (Gandure et al., 2011).

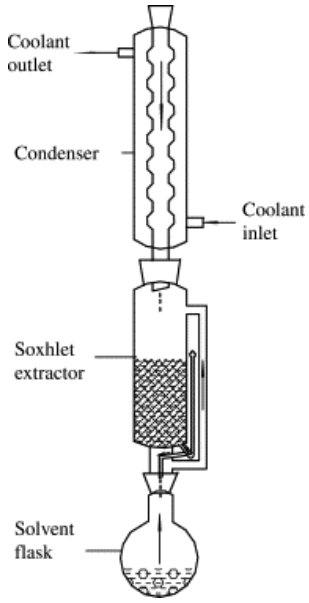


Figure 3.1: Diagram of a Soxhlet extraction setup (Wang et al., 2006)

3.3.3 Hexane extraction at ambient temperature (shaker method)

A 120.00 g mass of the blended seed sample was weighed into an amber bottle, 300 mL of hexane was added and the mixture placed on a shaker for 8 hours (Figure 3.2). After the extraction process, the heterogeneous mixture was centrifuged (Rotofix 32 A) at 6000 rpm for 30 minutes in order to separate the solid residue from the solvent. The decanted solvent was transferred to a Heidolph rotary evaporator (model number 517-61000-00-0) to evaporate the solvent at a temperature of 45 °C. The final oil extract was weighed and stored in a tightly sealed amber bottle purged with nitrogen and kept in the refrigerator until analysis was carried out (de Oliveira et al., 2014). The extraction process was carried out in triplicate each time on fresh samples.

Figure 3.2: Photograph of the shaker extraction method setup

3.3.4 Aqueous extraction

The seed sample (150.00 g) was blended using an electroline domestic blender with 35 mL of deionized water heated up to 37 °C and 60 °C for each extraction. Thereafter, the samples were placed in a glass beaker in a water bath at their respective temperatures and continuously stirred manually for 1 hour The sample was then centrifuged at 6000 rpm for 30 minutes and the supernatant was collected using a pasteur pipette. The extracted oil was weighed and stored in a tightly sealed amber bottle purged with nitrogen and kept in the refrigerator until analysis was carried out. The extraction process was carried out in triplicate each time on fresh samples.

3.3.5 Screw press extraction

The seed (14.50 kg) was sorted by hand, cracked using a cracking machine and then fed into the screw press where the oil was pressed. Sufficient pressure was generated for the oil to flow out of the seed oil and the oil was then filtered to remove any debris present. After the filtration process the oil was packed in an amber bottle, infused with nitrogen gas and tightly sealed and stored in the refrigerator until analysis was carried out. Figure 3.3 shows a schematic diagram of the screw press extraction. The extraction process was carried out once.

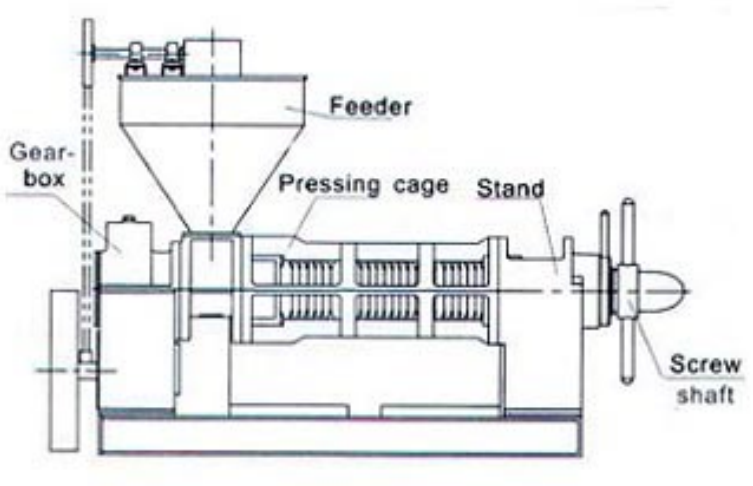


Figure 3.3: Schematic diagram of a screw press (Kmec, 2008)

3.3.6 Determination of percentage oil yield and efficiency

The oil yield was determined after extraction of the seed oil. For the Soxhlet and shaker extractions with hexane, the extracted oil was placed in a round-bottom flask and the solvent removed using a rotary evaporator with the water bath temperature set at 45 °C, the mass of the oil was weighed, and the oil yield was calculated using the equation:

$$Oil\ yield\ = \frac{mo}{ms} \times 100\ \% \qquad (11)$$

Where *mo* = weight of oil recovered after extraction

ms = weight of seed samples

For the aqueous extracted oil, the oil and water extract was centrifuged at 3000 rpm for 30 minutes and the oil layer (top) was carefully removed using a Pasteur pipette, and weighed. The oil yield was determined as described in the above equation.

The extraction efficiency of the seed oil was calculated based on the result obtained in this study for the Soxhlet extracted seed oil, because Soxhlet extraction is the standard analytical method for determination of fat content. Thus, it is considered to be 100 % of the fat content. The extraction efficiencies of the other extraction methods were calculated in relation to the Soxhlet extraction using the equation:

$$Extraction\ efficiency = \frac{my}{mm} \times 100\ \% \quad (12)$$

where my is the percentage oil yield for the other extraction methods, and mm is the percentage oil yield for Soxhlet extraction which is considered to have a 100 % extraction efficiency because it is the standard analytical method. A sample calculation of the extraction efficiency is provided in Appendix A

3.3.7 Determination of acid value and percentage free fatty acid

Acid value is an essential parameter used to determine the quality of seed oil (Gharby et al., 2012) and it reports the extent of hydrolytic degradation that has occurred in the seed. Hydrolytic degradation is due to the water content that comes out with the oil during extraction thereby reacting with triglycerides to form free fatty acids and glycerol (Soetaredjo et al., 2008). Further degradation could be accelerated by the presence of a high moisture content in the seed.

To standardize an approximately 0.1 M potassium hydroxide, 0.1000 M hydrochloric acid was prepared by pipetting 0.95 mL of 32 % concentrated hydrochloric acid into a 100 mL volumetric flask and made up to mark with deionized water. A 20 mL aliquot of 0.1000 M HCl was pipetted into a 250 mL Erlenmeyer flask and 3 drops of phenolphthalein indicator was added. The solution was then titrated against the potassium hydroxide solution until there was a colour change from colourless to light pink. The standardization was carried out in triplicate and since the mole ratio of KOH: HCl was 1:1, the molarity was calculated using the equation:

$$M(KOH) = \frac{V(HCl) \times M(HCl)}{V(KOH)} \quad (13)$$

where M= molarity

V = volume

The acid value was determined following an adaptation of the AOCS Official Method Cd 3d-63, 1998a. The oil (2.00 g) was weighed and dissolved in 20 mL of ethanol. The solution was titrated against the standardized 0.1 M KOH using phenolphthalein as the indicator. The acid value (AV) was calculated using the equation:

$$AV = \frac{56.1 \times V \times M}{m} \quad (14)$$

where 56.1 is the equivalent weight of KOH

V = volume, in mL, of KOH

M = molarity of KOH

m = the mass, in grams

3.3.8 Determination of the peroxide value

The peroxide value was determined following the AOCS Official Method Cd 8-53, 1998c. For the standardization of approximately 0.01 N sodium thiosulfate solution, 1.2405 g of sodium thiosulfate pentahydrate was dissolved in 125 mL of deionized water and made up to mark in a 500 mL volumetric flask. A 0.01 N potassium dichromate solution was prepared by firstly drying it at 120 °C for 4 hours and dissolving approximately 0.2485 g of the dried dichromate in 125 mL of deionized water and making it up to the mark in a 500 mL volumetric flask with deionized water. A 1 mL aliquot of concentrated sulfuric acid was added to 80 mL of deionized water in an Erlenmeyer flask. To this solution, 10 mL of 0.01 N potassium dichromate solution and 0.9996 g potassium iodide was added and the reaction was allowed to stand for 5 minutes. The solution was titrated with the sodium thiosulfate solution until the yellow colour was almost discharged. Starch indicator (5 mL) was added to the solution, which turned the solution blue, and it was titrated against sodium thiosulfate solution until the blue colour disappeared. The following equation was used to calculate the concentration of sodium thiosulfate:

$$N = \frac{Millilitres\ of\ K_2Cr_2O_7 \times 0.1000\ N}{Millilitres\ of\ Na_2S_2O_{3\ consumed}} \quad (15)$$

where N = Normality

For the sample analysis, 2.00 g of oil was weighed into a conical flask and 30 mL of solvent mixture of glacial acetic acid: chloroform in the ratio of 3:2 (v:v) was added to the oil samples. To commence the reaction, saturated KI solution (1 mL) was added and allowed to stand for 1 minute, then 30 mL of water (H_2O) was added and the liberated iodine was titrated against 0.0100 N sodium thiosulfate solution with vigorous shaking until the yellow colour was almost discharged.

About 5 mL of 1 % (w:v) starch indicator was added which turned the solution blue black. The titration continued with vigorous shaking until the end point was reached indicated by the disappearance of colour. This process was repeated in triplicate and a blank test was carried out under the same conditions to determine if any of the titrating agents contained peroxides. The following equation was used to determine the peroxide value, which is expressed as millequivalent per kilogram of oil (meq/kg of oil).

$$Peroxide\ value = \frac{(S-B)\times N\times 1000}{Sample\ weight\ (g)} \quad (16)$$

where S = sample titre value in L

B = blank titre value in L

N = Normality of $Na_2S_2O_3$ in meq/L

1000 = conversion factor

A sample calculation is provided in Appendix B.

3.3.9 Determination of p-anisidine value

The *p*-anisidine value was determined following the AOCS Official Method Cd 18-90, 1998b. A sample of the oil (1.00 g) was measured into a 25 mL volumetric flask and made up to the mark with isooctane. The absorbance of the solution was measured at 350 nm with isooctane as the blank. The oil solution (5 mL) was pipetted into a test tube and another 5 mL of isooctane into another test tube followed by the addition of 1 mL *p*-anisidine to each of the test tubes. The solutions were mixed in their individual test tubes and the absorbance of the solution, with the reference cuvette containing isooctane and anisidine solution as a blank was measured after 10 minutes. The *p*-anisidine value (*p*AV) was calculated using the following equation:

$$p-\mathrm{AV} = \frac{25\ mL(1.2\times A_2 - A_1)}{M} \quad (17)$$

where A_1 = absorbance of oil solution

A_2 = absorbance of *p*-anisidine oil solution

M = mass of sample

25 mL = volume of isooctane used to dissolve the sample

1.2 = correction factor (= 6 mL/5 mL) for dilution of the 5 mL sample solution with 1 mL of anisidine reagent dissolved in acetic acid

Instrumental conditions

The sample was analyzed using a UV-3600 Shimadzu UV-Vis spectrophotometer using a quartz cuvette with a path length of 1 cm. The base line was corrected with isooctane. The absorbance was measured at 350 nm.

3.3.10 Determination of conjugated dienes and trienes

The conjugated diene and triene value was determined following the AOCS Official method Ti 1a-64 (AOCS & Firestone, 1994) at wavelength 232 and 270 nm, respectively. A sample of oil (0.98 g) was weighed into a 100 mL volumetric flask and 75 mL of isooctane was added to dissolve the sample completely which was then further made up to the mark with isooctane. The conjugated diene and triene percentage was calculated from the following equations:

$$Conjugated\ diene\ \% = 0.84\left[\left(\frac{A_1}{bc} - 0.07\right)\right] \quad (18)$$

$$Conjugated\ triene\ \% = 0.84\left[\left(\frac{A_2}{bc} - 0.07\right)\right] \quad (19)$$

where A_1 = absorbance of the sample at 232 nm for conjugated diene and A_2 = absorbance at 270 nm for conjugated triene

b = cell length (1 cm)

c = concentration of sample used for absorbance measurement in g/L

0.07 = absorptivity constant (ε) of the ester group

0.84 = is a factor that accounts for any extraneous absorption (Holman, 1957)

Instrumental conditions

The absorbance of the sample was measured using a UV-3600 Shimadzu UV-Vis spectrophotometer with a quartz cuvette having a path length of 1 cm. The absorbance of the sample was recorded at 232 nm and 270 nm.

3.3.11 Determination of fatty acid composition

Sample preparation

The fatty acid profile was determined as fatty acid methyl esters (FAMEs) by gas chromatography-mass spectrometry. The methyl esters were prepared using the method prescribed by the International Olive Oil Council, (2001). Fatty acids are polar compounds and are not volatile. In order for them to be analyzed on a gas chromatography instrument they have to be volatile and it is for this reason that derivatization was carried out before the GC-MS analysis. The most widely used method for converting non-volatile fatty acids into volatile fatty acid methyl esters is methylation, which is carried out with methanolic potassium hydroxide. A stock solution of 1000 mg/L was prepared by dissolving 10 mg of oil in 10 mL of hexane. The oil solution was further made up to a lower concentration (100 mg/L) using appropriate dilution. A 0.20 mL aliquot of methanolic potassium hydroxide (0.2000 M) was added to 2 mL of the oil solution. The mixture was shaken vigorously, allowed to stand and separate. An aliquot (1 mL) of the hexane phase (the upper layer containing the methyl ester) was transferred into a GC vial for analysis using the GC-MS (Haiyan et al., 2007).

Preparation of stock solutions

The lower working concentration of all oils was prepared using the dilution factor method and the following equation:

$$C_1V_1 = C_2V_2 \quad (20)$$

For example, from a stock solution of 1000 mg/L, a lower working concentration of 100 mg/L was prepared by transferring 1000 mg of the stock solution into a 10 mL volumetric flask and making it up to the mark with n-hexane.

$$1000\frac{mg}{L} \times V_1 = 100\frac{mg}{L} \times 0.01\ L$$

$$V_1 = 1 \times 10^{-3} L$$

A concentration of 100 mg/L was found to be appropriate for instrumentation analysis using GC-MS.

Instrumental conditions

The sample was analyzed using gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP-2010 SE Ultra Japan), with a DB-5MS capillary column of length 30 m, 0.25 µm internal diameter and 0.25 mm film thickness. The carrier gas was helium with a flow rate of 1.04 mL/min and a total flow of 5.1 mL/min, a linear velocity of 37.20 cm/sec at purge flow of 3.00 mL/min. The injection temperatures were set at 250 °C. The oven temperature was set at 60 °C and held for 1.00 min, ramped to 300 °C at a rate of 10 °C/min and held for a further 5.00 min. A 3 µL injection volume was used in a splitless injection mode. Ion source temperature was 230 °C, interface temperature 250 °C, ion source voltage was 70 eV, and total run time 30 min.

The result was expressed as area % using the following equation:

$$\%\ Analyte = \frac{A_a}{\sum A_1} \times 100 \qquad (21)$$

$where\ A_a = peak\ area\ of\ analyte$

$\sum A_1 = sum\ of\ all\ peak\ areas\ in\ the\ chromatogram$

3.3.12 Radical scavenging activity towards 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

The radical scavenging activity of each extracted seed oil was determined by monitoring the decrease in absorbance at 517 nm. A 3 mL freshly prepared 0.01 mM toluene solution of 2, 2-

diphenyl-1-picrylhydrazyl (DPPH) was added to 3 mL of a 5 mg/mL extracted oil sample. The sample was mixed thoroughly and left to stand for 30 minutes in the dark to allow the radical-antioxidant reaction to occur. After the reaction, the absorbance was measured at 517 nm against a blank. A synthetic antioxidant, butylated hydroxyanisole (BHA), was also prepared in the same manner as the oil sample and was used as a positive control or reference standard for this assay (Ahmad et al., 2014). Triplicate measurements were taken.

Instrumental conditions

The samples were analyzed using a Shimadzu UV-3600 spectrometer with a quartz cuvette with a path length of 1 cm. The base line was corrected with methanol. The absorbance was measured at 517 nm.

The radical scavenging activity was expressed by the inhibition percentage and calculated using the following equation:

$$\%\ Radical\ scavenging\ activity = \frac{A\ control - A\ sample}{A\ control} \times 100 \qquad (22)$$

A control = Absorbance of control

A sample = Absorbance of sample

3.3.13 Oxidative stability

The oxidative stability test was determined by transferring 25 mL of each of the seed oils extracted with the different extraction methods into different amber bottles, filled to the top with no head space, and capped with screw caps. The amber bottles containing the oil from different extraction methods were placed in the dark at ambient temperature and at the different accelerated temperatures in an incubator regulated at 45 and 65 °C respectively. The samples were stored at their different storage temperatures from day 0 to 35. A 2.00 g and 1.00 g sample of each oil was periodically collected and analyzed for peroxide value and anisidine value, respectively. This is described in section 3.3.8 and 3.3.9 (Andarwulan et al., 2014).

3.4 Statistical analysis

The data were expressed as mean ± standard deviation calculated from triplicate determinations, and the analysis of variance (ANOVA) was performed using Statistical Package for the Social Science 24.0 for windows (SPSS 24.0). A probability value at $P<0.05$ was considered a statistically significant difference. The correlation of data was assessed using the Pearson correlation test. Student-t-test, comparing two parameters, was used to compare results from the oxidative test for the two seed oils extracted using aqueous extraction at 37 °C.

4.0 CHAPTER 4: RESULTS AND DISCUSSION

4.1 Moisture content of seed and seed oil

The amount of water present in the seed has a negative effect on the seed because high water content allows the seed to start to grow moulds thereby affecting the storability of seeds, and quality of oil extracted from seeds, including its taste and odour (Torres et al., 2006). Moisture content primarily depends on the length of drying and the ambient storage conditions especially temperature and humidity. High moisture after harvesting, and temperature allows the seed to deteriorate fast. From the results (Table 4.1), the moisture content of marula and moringa seeds were 5.56 and 7.26 % (dry weight), respectively. The result was well below the maximum accepted moisture content of seeds (10 %) so fungal growth is not expected in the seed (Adejumo et al., 2013). Anwar et al. (2005) reported a moisture content of 5.90 to 7.00 % (dry weight) and yield of 33.23 to 40.90 % for moringa seed oil, which is similar to that reported in this study. Zharare et al. (2004) reported a moisture content value of 5.00 to 5.40 % for different species of marula seed. The difference in moisture content value of marula and moringa seed reported in this study as compared to literature could be as a result of geographical area or climatic conditions as well as the length of drying time and storage condition (Maskan, 2001). The result in Table 4.2, which was determined from the results of the analysis of variance test (ANOVA), showed that there was a significant difference in the moisture content of the seed oils (marula and moringa) extracted using the different extraction methods. The moisture content for aqueous extracted (37 and 60 °C) marula seed oil was 5.41 and 5.30 %, respectively while the solvent extracted (Soxhlet and shaker) extracted marula seed oil was 4.77 and 4.96 %, respectively. Moringa seed oil extracted using aqueous extraction (37 and 60 °C) had a moisture content of 6.94 and 6.65 %, respectively while the solvent extracted seed oil (Soxhlet and shaker) had a moisture content of 5.94 and 6.21 %, respectively. This result showed that aqueous extracted seed oil had the highest moisture content, which is expected because of water used for the extraction process. Also, it is expected that the aqueous extracted seed oil should have lower stability because of the amount of moisture present as high moisture in harvested seeds hastens the deterioration process. However, the reverse was the case, which could be as a result of the higher antioxidant present in the aqueous extracted seed

oil as shown in Table 4.19 for radical scavenging activity and the fatty acid profiles which are discussed in section 4.3. Overall, moringa seed oil had the higher moisture content compared to marula, which correlates with the moisture results of the seeds.

Table 4.1: Moisture content of marula and moringa seeds (n = 3)

	Marula	Moringa
Moisture content (% w/w)	5.56 ± 0.04	7.26 ± 0.02

Table 4.2: Moisture content of marula and moringa seed oil (n=3)

	Moisture content (% w/w)	
Extraction methods	Marula seed oil	Moringa seed oil
Hexane extraction (Soxhlet)	4.77 ± 0.02^{a}	5.94 ± 0.02^{f}
Hexane extraction (shaker)	4.96 ± 0.01^{b}	6.21 ± 0.02^{g}
Screw press	5.18 ± 0.01^{c}	6.40 ± 0.06^{h}
Aqueous extraction at 60 °C	5.30 ± 0.01^{d}	6.65 ± 0.05^{i}
Aqueous extraction at 37 °C	5.41 ± 0.05^{e}	6.94 ± 0.08^{j}

Values are mean ± SD of marula and moringa seed oils from different extraction methods analyzed in triplicate. Mean values in the same column followed by different superscript letters are significantly different ($P < 0.05$) for marula and moringa seed oil, respectively

4.2 Oil yield and extraction efficiency

Oil yield is the amount of oil that can be extracted from the seed. It is expressed as a percentage of the starting material (w/w) while extraction efficiency is the percentage of oil extracted in

relation to total amount of oil present. The ANOVA test was used to determine the significant differences ($P<0.05$) for the yields and extraction efficiencies for marula and moringa seed oil for the different extractions methods. The results showing the statistical differences between the extraction methods are shown in Tables 4.3 and 4.4.

Table 4.3: Oil yield and extraction efficiency of marula seed oil (n = 3)

Marula seed oil		
Extraction methods	Oil yield (%)	Extraction efficiency (%)
Hexane extraction (Soxhlet)	53.99 ± 0.01^{a}	100.00 ± 1.00^{f}
Hexane extraction (shaker)	42.74 ± 0.51^{b}	79.17 ± 1.07^{g}
Aqueous extraction at 60 °C	20.00 ± 0.01^{c}	37.05 ± 0.39^{h}
Aqueous extraction at 37 °C	18.67 ± 0.68^{d}	34.56 ± 0.36^{i}
Screw press	21.88^{e}	54.09^{j}

Values are mean ± SD of marula seed oils from different extraction methods analyzed in triplicate except screw press method. Mean values in different column followed by different superscript letters are significantly different ($p<0.05$) for the oil yield and extraction efficiency %, respectively.

Table 4.4: Oil yield and extraction efficiency of moringa seed oil (n = 3)

Moringa seed oil		
Extraction methods	Oil yield (%)	Extraction efficiency (%)
Hexane extraction (Soxhlet)	35.30 ± 1.14^{a}	100.00 ± 1.00^{f}
Hexane extraction (shaker)	27.19 ± 0.91^{b}	77.01 ± 0.55^{g}
Aqueous extraction at 60 °C	13.05 ± 0.35^{c}	36.98 ± 0.63^{h}
Aqueous extraction at 37 °C	12.00 ± 0.00^{d}	33.98 ± 0.32^{i}
Screw press	14.43^{e}	60.91^{j}

Values are mean ± SD of moringa seed oils from different extraction methods analyzed in triplicate except screw press method. Mean values in the same column followed by different superscript letters are significantly different ($p<0.05$) for the oil yield and extraction efficiency %, respectively.

Soxhlet extraction using hexane (b pt. 69 °C) gave the highest oil yield of 53.99 % and 35.30 % for marula and moringa, respectively; this being the total or 100 % fat content as hexane Soxhlet extraction is the standard method for extracting fat content. The shaker method (at ambient temperature) gave a lower oil yield for both marula and moringa of 42.74 % and 27.19 %, and extraction efficiency of 79.17 % and 77.01 %, respectively. The higher oil yield and extraction efficiency recorded with Soxhlet extraction was due to the temperature of extraction (69 ℃) which leads to cell walls breaking. This together with the organic solvent used and continuous recycling of the hot solvent through the Soxhlet extraction process promotes better extraction efficiency by penetrating into the cell wall of the seed which was also broken during the process of grinding, solubilizing all the oil present (Daroch et al., 2013; Li et al., 2016). The oil yield value and extraction efficiency obtained at 60 °C using water for extraction was also higher compared to that obtained at 37 °C and this could be attributed to the difference in temperature. In this extraction, the mechanical grinding led to cell walls breaking but in addition, the higher temperature also contributed to better extraction efficiency by also breaking the cell walls thus increasing the cell

surface area (Erickson, 1990). Temperature, ranging from 37 to 60 °C, applied in the extraction method helps to reduce moisture in the cells, and reduces the viscosity of the oil thereby resulting in easier flow of the oil during extraction. It thus speeds up the extraction process and gives a better oil yield (Sefah, 2006). The disadvantage of temperature is that when it is too high the oil will be oxidized and become rancid (Sefah, 2006). The results showed that moringa seed with high moisture content had lower extraction efficiency and marula with a lower moisture content had a higher extraction efficiency. This indicates that the lower the moisture content, the higher the extraction efficiency which was also found by Adejumo et al. (2013).

Researchers have reported fat content for solvent extraction (Soxhlet method with hexane) of marula oil within the range of 50.00 % to 65.00 % (Mariod et al., 2004; Zharare et al., 2004) which was in line with the results obtained for this study. For solvent extracted moringa seeds using a Soxhlet method with hexane, researchers reported oil yields between 33.23 % - 42.00 % (Tsaknis et al., 1999; Anwar et al., 2003; Anwar et al., 2003) with the value obtained in this study (Table 4.3 and 4.4) similar to the reported range. The variation in the results of the oil yield could be as a result of different plant varieties studied, and environmental and climatic conditions (Anwar et al., 2003). Sharma and Gupta (1982) also reported that the variation in oil yield could be because of genetic characteristics.

4.3 Determination of fatty acid profile

In order to determine the fatty acid composition of the seed oil using GC-MS, the fatty acid present in the oil must be volatile. However, fatty acids are generally not volatile and therefore the oil is derivatized prior to GC-MS analysis. The normal method for determining the fatty acid profile is by using the fatty acid methyl ester standards and comparing the retention time of the standard with that of the sample. Due to the unavailability and high cost of the appropriate column (capillary column CP-Sil 88 (60 m × 0.25 mm i.d, thickness of 0.25 µm) for analyzing fatty acid methyl esters, an inert cap 5 ms/sil column with dimensions of 30 mm × 0.25 nm i.d, × 0.25 µm film thickness was used instead supplied by Restek. The use of this column resulted in some standards not being eluted which made identification and quantification based on calibration graphs difficult. However, on analysis of the sample itself, all the analytes of interest eluted. Thus instead of basing

identification of the analytes in the sample on the retention time of standards, the identification was based on each analytes mass spectral fragmentation pattern. Once the analyte peaks were identified in the chromatogram, quantification of the analytes of interest were determined based on the percentage of peak area of the analyte *versus* the total peak areas of all analytes in the chromatogram. The gas chromatogram for marula oil is shown in Figure 4.1. The peak with retention time 16.825 was identified as palmitic acid based on its mass spectrum (Figure 4.2). The mass spectrum showed a molecular ion peak at *m/z* 270 which corresponded to the molecular mass of the methyl ester of palmitic acid having a formula of $C_{17}H_{34}O_2$. From the fragmentation pattern of the mass spectrum shown in Figure 4.2 the peak at *m/z* 239 corresponded to the loss of $–OCH_3$, which is seen, by the loss of 31 mass units. The peak at *m/z* 227 corresponded to the loss of a carbon, which is possibly the carbon from the methyl group at the opposite end of the palmitic acid methyl ester structure, with the hydrogens attached to the main structure to stabilize it. The peak at *m/z* 213 corresponded to the loss of a methylene group shown by the loss of 14 mass units. Likewise, the other peaks in the mass spectrum show the loss of $–CH_2$ groups, which confirmed the hydrocarbon like backbone of palmitic acid. Furthermore, a comparison of the mass spectrum obtained for palmitic acid methyl ester with that found for palmitic acid methyl ester in the NIST library of the GC-MS showed a good match (Figures 4.2 and 4.3). All the peaks on the gas chromatogram were identified based on similar analysis of their corresponding mass spectra.

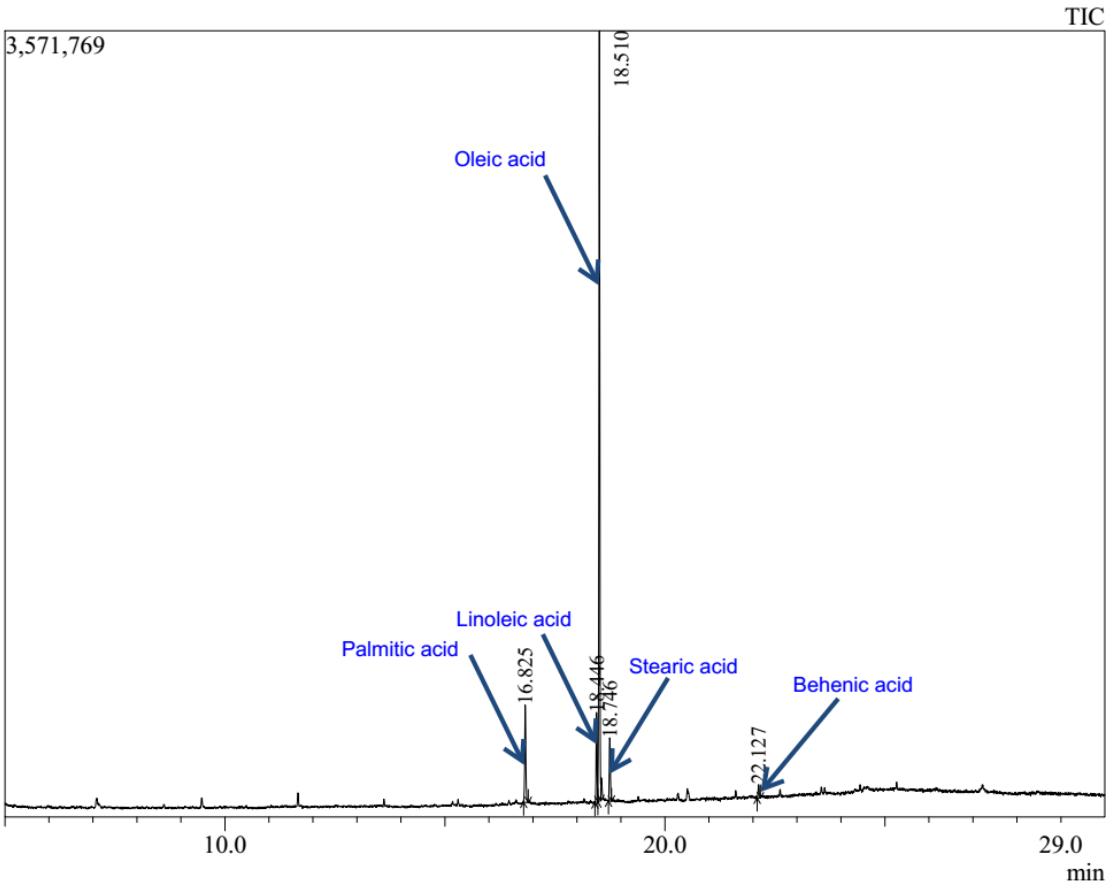


Figure 4. 1: GC-MS chromatogram of 5 fatty acids present in aqueous extracted (37 °C) marula seed oil

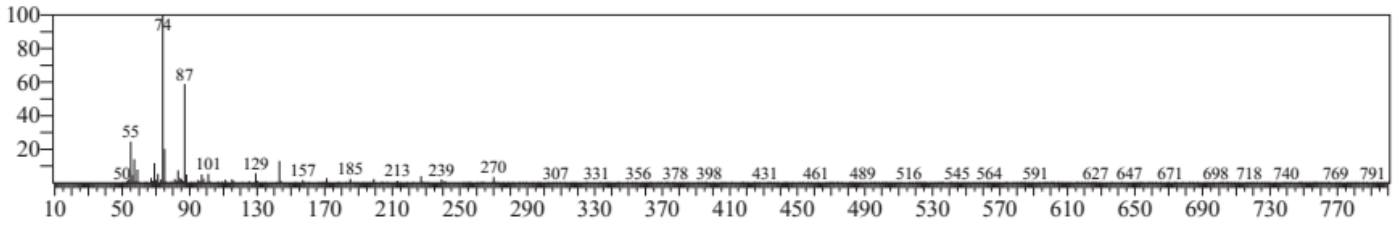


Figure 4.2: Actual mass spectrum of palmitic acid methyl ester in marula seed oil

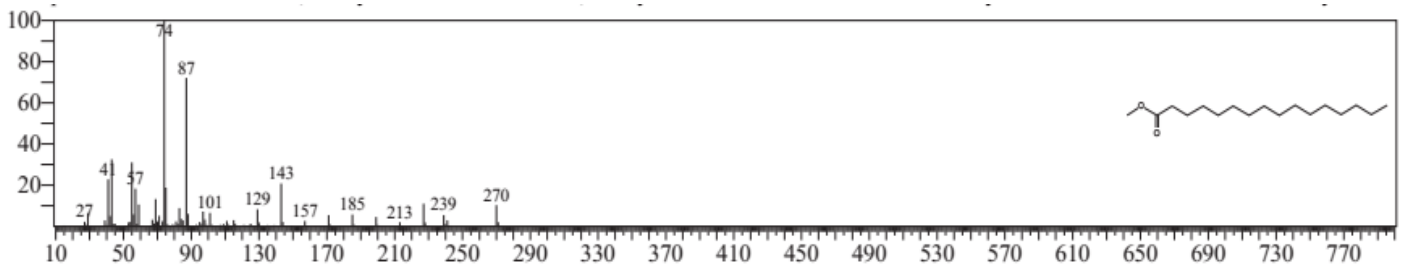


Figure 4.3: Library match for palmitic acid methyl ester from the NIST library

The results in Tables 4.5 and 4.6 show the presence of palmitic acid (hexadecanoic acid methyl ester), linoleic acid (9, 12- octadecadienoic acid methyl ester), oleic acid (9- octadecenoic acid methyl ester), and stearic acid (octadecanoic acid methyl ester) in both seed oils. Behenic acid (docosanoic acid methyl ester) was only detected in moringa seed oil and in shaker and aqueous extracted marula seed oil at 37 °C. Furthermore, according to the analysis of variance test (ANOVA) $P<0.05$ shows that there is a significant difference in the fatty acid composition for the different extraction methods for marula seed oil. There was also a significant difference in the fatty acid composition for the different extraction methods for moringa seed oil. The results in Tables 4.5 and 4.6 showed that the highest fatty acid concentration present in marula seed oil was oleic acid. Aqueous extracted marula seed oil at 37 and 60 °C had an oleic acid value of 72.36 and 72.01 %, respectively, screw press seed oil was 71.12 % and the values for solvent extracted oil were 70.70 and 70.19 % for the Soxhlet and shaker methods, respectively. Mariod et al. (2004) reported oleic acid as 67.20 % for solvent (Soxhlet method with hexane) extracted marula seed oil.

For moringa seed oil, oleic acid was the most dominant fatty acid. The percent composition of oleic acid in aqueous extracted moringa seed oil was 79.94 % and 79.67 % at 37 °C and 60 °C, respectively, solvent extracted oil (Soxhlet and shaker method) had 77.61 and 78.78 %, respectively, and screw press extracted oil was 79.59 %.

Latif and Anwar (2008) reported oleic acid as being the dominant fatty acid present in moringa seed oil. They reported that solvent extracted moringa seeds using a Soxhlet method with hexane had an oleic acid percentage of 67.30 % and aqueous extracted moringa seed oil (at boiling temperature) had an oleic acid percentage of 68.07 %. For both seed oil, oleic acid was dominant in aqueous extraction compared to the other extraction methods. For the polyunsaturated fatty, linoleic acid was found to be dominant in Soxhlet extraction as compared to the other extraction methods while aqueous extraction had the lowest and screw press was in between for both seed oil. Polyunsaturated fatty acids are highly susceptible to oxidation due to the presence of the double bonds. As the number of double bonds increase, the rate of oxidation also increases. The polyunsaturated values for all the extraction methods are similar and therefore oils extracted from all the methods are prone to oxidation. However, in ranking the extraction methods in order of being susceptible to oxidation, Soxhlet extraction is most susceptible to oxidation. Comparing the two seed oils, moringa is expected to be more stable compared to marula seed oil because it has a

higher oleic acid content, which is a monounsaturated fatty acid, which has relatively higher oxidative stability and lower quantity of polyunsaturated fatty acids (linoleic acid) which is easily susceptible to oxidation. Seed oils rich in oleic acid (monounsaturated fatty acid) are reported to have a high stability and can be used as replacement for hydrogenated oils (Rahman et al., 2009; Tsaknis et al., 1998).

The variation in the percentage of the fatty acid present in the different extraction methods could be as a result of the solubility and different partition coefficients of the different triglycerides in the process of extraction where some triglycerides are more soluble at specific temperatures and in specific solvents used during extraction. In addition, different triglycerides are present in different parts of the seed cell and the different extraction processes may cause specific parts of the seed cell to be exposed leading to extraction of those triglycerides present in that part of the cell (Hegel et al., 2011; Günerken et al., 2015). This is seen in Table 4.5 and 4.6 where the monounsaturated oleic acid has the highest percentage extraction from the aqueous extraction method at 37 °C and the lowest extraction from extraction methods using high temperature and solvent (Soxhlet and shaker). The saturated and polyunsaturated fatty acids were preferentially extracted using Soxhlet extraction which suggests these triglycerides require high temperature and a solvent for their extraction.

The fatty acid present in seed oils may also depend on the geographical area, processing method such as the extraction technique used for extracting the seed oil, and storage conditions. The variation in our results of the fatty acids with that of literature could be because of the harvesting time, environmental and climatic conditions, storage process and extraction techniques (Anwar et al., 2003; Mariod et al., 2010).

Table 4.5: Relative fatty acid composition of marula seed oil

Extraction methods					
Fatty acid (%)	Soxhlet	Shaker	Aqueous 60 °C	Aqueous 37 °C	Screw press
Palmitic acid	12.75 ± 0.12^{a}	11.62 ± 0.27^{b}	11.96 ± 0.27^{c}	10.85 ± 0.00^{d}	11.26 ± 0.45^{e}
Stearic acid	6.27 ± 0.10^{p}	7.41 ± 0.07^{r}	6.70 ± 0.12^{s}	6.84 ± 0.28^{t}	6.86 ± 0.20^{t}
Oleic acid	70.70 ± 0.42^{k}	70.19 ± 0.22^{l}	72.01 ± 0.41^{m}	72.36 ± 0.12^{n}	71.12 ± 0.11^{o}
Linoleic acid	9.32 ± 0.13^{f}	9.16 ± 0.08^{g}	8.98 ± 0.26^{h}	8.29 ± 0.25^{i}	9.03 ± 0.08^{h}
Behenic acid	Not detected	0.94 ± 0.00^{v}	Not detected	0.94 ± 0.00^{v}	Not detected
Total saturated	19.02 ± 0.16^{w}	19.97 ± 0.28^{x}	18.66 ± 0.30^{y}	18.63 ± 0.28^{y}	18.12 ± 0.49^{q}
Total polyunsaturated	9.32 ± 0.13^{f}	9.16 ± 0.08^{g}	8.98 ± 0.26^{h}	8.29 ± 0.25^{i}	9.03 ± 0.08^{h}
Total monounsaturated	70.70 ± 0.42^{k}	70.19 ± 0.22^{l}	72.01 ± 0.41^{m}	72.36 ± 0.12^{n}	71.12 ± 0.11^{o}

Values are mean ± SD of marula seed oils from different extraction methods analyzed in triplicate. Mean values in the same row followed by different superscript letter are significantly different ($P<0.05$) according to the analysis of variance test.

Table 4.6: Relative fatty acid composition of moringa seed oil

Extraction methods					
Fatty acid (%)	Soxhlet	Shaker	Aqueous 60 °C	Aqueous 37 °C	Screw press
Palmitic acid	8.05 ± 1.28^{a}	7.06 ± 0.33^{b}	7.55 ± 0.17^{c}	6.49 ± 0.25^{d}	6.83 ± 0.33^{e}
Stearic acid	7.39 ± 0.17^{p}	6.01 ± 0.33^{r}	5.92 ± 0.62^{s}	6.96 ± 0.08^{t}	6.37 ± 0.00^{u}
Oleic acid	77.61 ± 1.18^{k}	78.78 ± 0.77^{l}	79.67 ± 1.90^{o}	79.94 ± 0.48^{n}	79.59 ± 0.24^{o}
Linoleic acid	1.33 ± 0.00^{f}	1.03 ± 0.06^{g}	0.87 ± 0.04^{h}	0.67 ± 0.11^{i}	0.95 ± 0.15^{j}
Behenic acid	5.92 ± 0.13^{v}	6.82 ± 0.00^{w}	5.99 ± 0.02^{x}	6.62 ± 0.00^{y}	6.20 ± 0.00^{z}
Total saturated	21.36 ± 1.30^{m}	19.89 ± 0.47^{q}	$19.46 \pm 0.64^{\#}$	20.07 ± 0.26*	$19.40 \pm 0.33^{\#}$
Total polyunsaturated	1.33 ± 0.00^{f}	1.03 ± 0.06^{g}	0.87 ± 0.04^{h}	0.67 ± 0.11^{i}	0.95 ± 0.15^{j}
Total monounsaturated	77.61 ± 1.18^{k}	78.78 ± 0.77^{l}	79.67 ± 1.90^{o}	79.94 ± 0.48^{n}	79.59 ± 0.24^{o}

Values are mean ± SD, of moringa seed oils from different extraction methods analyzed in triplicate. Mean values in the same row followed by different superscript letters are significantly different (P<0.05) according to the analysis of variance test. Total saturated fatty acids are all significantly different

4.4 Acid value

Table 4.7, shows a significant difference (P<0.05) in the acid value of the seed oils extracted using Soxhlet, shaker, screw press and aqueous extraction methods. The statistical differences between the different extraction methods were determined from the analysis of variance test (ANOVA).

Table 4.7: Acid value of marula and moringa seed oil (n = 3)

Acid value (mg KOH/g oil)		
Extraction methods	Marula seed oil	Moringa seed oil
Hexane extraction (Soxhlet)	3.59 ± 0.03^{a}	3.44 ± 0.09^{f}
Hexane extraction (shaker)	3.44 ± 0.03^{b}	3.31 ± 0.06^{g}
Aqueous extraction at 37 °C	2.30 ± 0.00^{c}	2.04 ± 0.03^{h}
Aqueous extraction at 60 °C	2.45 ± 0.03^{d}	2.23 ± 0.03^{i}
Screw press	3.10 ± 0.06^{e}	2.81 ± 0.00^{j}

Values are mean ± SD of moringa seed oils from different extraction methods analyzed in triplicate. Mean values in the same column followed by different superscript letters are significantly different ($P < 0.05$) for marula and moringa seed oil, respectively

The results showed that aqueous extracted marula and moringa seed oil at 37 °C and 60 °C had the lowest acid value compared to screw press, shaker and Soxhlet extracted oil (Table 4.7) which to some extent indicates that aqueous extracted seed oil has a better quality. The aqueous extracted seed oil was expected to have a higher acid value, because it used water as the extracting solvent, which may contribute to the hydrolytic degradation or the extent to which the glycerides in the oil decomposes, by lipase action. The reverse was the case in this study, which showed that aqueous extraction had the lowest acid value. This could be due to the mild operating conditions that did not require the use of an organic solvent or high pressure. This suggests that water alone is not the only parameter that contributes to hydrolytic degradation. The high acid values for the Soxhlet extracted seed oils could be because of the organic solvent and temperature used in the extraction process. High temperature used in Soxhlet extraction may result in the free fatty acids being more soluble in the solvent and thus were easily transferred to the seed oil. In addition, the breakdown of free fatty acids in certain solvents may also contribute to more free fatty acids being extracted resulting in higher acid values. In addition, these two parameters (temperature and organic solvent) are known to negatively affect the natural antioxidants present, which reduces their ability to

scavenge the free fatty acids present thereby leading to a lower quality of oil (Bhatnagar et al., 2009). Storage of the seeds before extraction may also play a role in the acid value of the extracted seed oil. Poor storage conditions may lead to microbial activities, which may result in the production of free fatty acids thus leading to higher acid values. The seeds stored in the refrigerator may produce less free fatty acids and thus result in less free fatty acid being extracted in the extraction process as compared to seeds stored at ambient temperature. Also, the enzymatic activity whereby the lipases break down fatty acid from lipid molecules may contribute to the high or low acid values of the seed oils.

Lastly, moringa seeds had a higher moisture content than marula seeds (Table 4.1), which was also seen in the seed oils (Table 4.2). A higher water content in the oil is expected to lead to greater hydrolytic activity and a higher acid value. Moringa seed oil had higher moisture content and was expected to have a higher acid value compared to marula seed but the reverse was the case whereby moringa seed oil had a lower acid value even though it had high moisture content. This reversal in acid value for marula and moringa could be because moringa seed oil had a higher radical scavenging ability compared to marula seed oil as reported further in this study (section 4.7), which means that moringa has a higher natural antioxidant content that can scavenge the free fatty acid and lead to a low acid value. The lower acid values for aqueous extracted marula and moringa seed oil shows that aqueous extraction resulted in a better oil quality.

Danlami et al. (2014) reported an acid value of 3.60 mg KOH/g for marula seed oil using solvent (hexane) extraction using the Soxhlet method, which compared well with that reported in this study. There are no reports in literature on the acid value of aqueous extracted seed oil at 37 and 60 °C for both marula and moringa seed oil. Therefore, this study presents the first report on the acid value of aqueous extracted seed oil at 37 and 60 °C from marula and moringa seeds.

4.5 Measurement of oxidation products

The oxidative state of the seed oil was determined using the peroxide value, conjugated diene and triene values at 232 nm and 270 nm, and the *p*-anisidine value.

4.5.1 Peroxide value

The peroxide value is an indication of the initial stage of oxidation that takes place in the oil and is also a measure of the primary oxidation products (peroxides and hydroperoxide) in oil (McGinely, 1991). Oil is oxidized during processing and storage *via* autoxidation in which triplet oxygen and singlet oxygen react with the oil.

The higher the peroxide value the more susceptible the oil is to rancidity and having a shorter shelf life. Table 4.8 shows the results of the different oxidative parameters of the screw press, aqueous (37 and 60 °C) and solvent (Soxhlet and shaker) extraction methods. The results in Table 4.8, shows that there was a significant difference ($P<0.05$) in the peroxide value of the seed oils extracted with the different extraction methods. The statistical differences were determined according to the analysis of variance test (ANOVA).

Table 4.8: Peroxide value of marula seed oil (n = 3)

	Peroxide value (meq/kg of oil)	
Extraction methods	Marula seed oil	Moringa seed oil
Hexane extraction (Soxhlet)	0.83 ± 0.06^{a}	0.67 ± 0.06^{f}
Hexane extraction (shaker)	0.70 ± 0.06^{b}	0.50 ± 0.00^{g}
Aqueous extraction at 37 °C	0.15 ± 0.10^{c}	0.20 ± 0.00^{h}
Aqueous extraction at 60 °C	0.10 ± 0.06^{c}	0.10 ± 0.00^{i}
Screw press	0.30 ± 0.12^{e}	0.30 ± 0.00^{j}

Values are mean ± SD of marula seed oils from different extraction methods analyzed in triplicate. Mean values in the same column followed by different superscript letters are significantly different ($P < 0.05$)

The peroxide value results (Table 4.8) showed that Soxhlet extraction using hexane as the solvent produced the highest peroxide value and thus the least stable oil, which is probably due to the combination of the high temperature and use of a solvent in the extraction process. The high peroxide value for Soxhlet extracted oil could also be due to the low radical scavenging ability (section 4.7) observed which would result in a lower ability to scavenge the free radicals in the oil thus leading to a high oxidation product content and eventually a high peroxide value and a lower quality oil.

Aqueous extraction at 37 °C and 60 °C for both marula and moringa seed oil had a lower peroxide value. This could be as a result of the effect of the extraction process where a mild extraction technique with lower temperatures produced lower amounts of primary oxidation products, leading to a better quality of oil. Aqueous extracted oil (37 °C and 60 °C) had a higher scavenging ability, and would therefore be able to scavenge the free radicals in the oil resulting in a low peroxide value for this oil. In addition, aqueous extracted marula and moringa seed oil contained lower values of polyunsaturated fatty acid (linoleic acid) as compared to the other extraction methods

leading to the seed oil being less susceptibility to oxidation thereby producing lower amounts of primary oxidation product (peroxides).

Overall Moringa seed oil had a lower peroxide value compared to marula seed oil across all extraction methods suggesting that Moringa seed oil has lower oxidation products and therefore is more stable.

The peroxide values of the different extraction methods were within the maximum acceptable value of 15 meq/kg for oil as recommended by Codex Alimentarus Commission (Joint FAO/WHO (Codex Alimentarus Commission, 1999)).

Anwar et al. (2003) reported a peroxide value of 0.59 meq/kg of oil for solvent extraction (Soxhlet method) of moringa oil with hexane, which was in accordance with that reported in this study. Tsaknis et al. (1999) reported a peroxide value of 0.36 meq/kg of oil for screw pressed moringa seed oil, which was similar to that reported in this study. There were no available published results for comparison of the aqueous extraction at 37 and 60 °C.

4.5.2 Conjugated diene and triene value

Conjugated dienes and trienes are formed during lipid oxidation. An increase in the absorption at 232 nm is caused by the formation of conjugated dienes. Absorption at 270 nm increases when conjugated trienes are formed. The conjugated diene and triene values show the oxidative deterioration of the seed oils (Iqbal et al., 2013).

The results in Table 4.9 showed that there was a significant difference ($P<0.05$) between the different extraction methods. The statistical differences in the conjugated diene and triene values obtained were based on the analysis of variance test (ANOVA) for the different extraction methods.

Table 4.9: Conjugated diene and triene values of marula and moringa seed oil (n = 3)

	Marula seed oil		Moringa seed oil	
Extraction methods	Conjugated diene value	Conjugated triene value	Conjugated diene value	Conjugated triene value
Hexane extraction(Soxhlet)	1.16 ± 0.00^{a}	0.79 ± 0.01^{f}	1.08 ± 0.01^{l}	0.64 ± 0.01^{q}
Hexane extraction (shaker)	1.09 ± 0.00^{a}	0.67 ± 0.01^{g}	1.02 ± 0.01^{l}	0.57 ± 0.00^{r}
Aqueous extraction at 37 °C	0.66 ± 0.00^{c}	0.39 ± 0.01^{h}	0.65 ± 0.02^{n}	0.37 ± 0.01^{s}
Aqueous extraction at 60 °C	0.78 ± 0.01^{d}	0.48 ± 0.01^{i}	0.70 ± 0.01^{n}	0.42 ± 0.01^{ts}
Screw press	0.99 ± 0.02^{e}	0.54 ± 0.00^{j}	0.87 ± 0.00^{p}	0.47 ± 0.01^{t}

Values are mean ± SD of marula seed oils from different extraction methods analyzed in triplicate. Mean values in the same column followed by different superscript letters are significantly different ($P < 0.05$) for conjugated diene and triene values, respectively.

Conjugated dienes and trienes are products formed during oxidation as a result of the shift in the double bond. The shift in the double bond is initiated by parameters such as temperature and light, which are specific to different extraction techniques, and cause formation of oxidation products. Research has shown that increases in temperature of approximately 10 °C increments will cause the rate of reaction of oxygen with oil to double (Khan and Shahidi, 2002; Pan et al., 2004; Rossell, 1992; Berger, 1994).

Aqueous extraction method had the lowest conjugated diene and triene values. Low values of conjugated dienes and trienes obtained in aqueous extracted seed oils may be attributed to the mild extraction conditions used in this technique. Also, the shift in the position of the double bond in aqueous extraction method may not be as frequent as the solvent extraction method (Soxhlet and shaker method) method resulting in lower conjugated diene and triene values.

The presence of conjugated dienes and trienes in the seed oils, in this study, can be justified by the presence of the polyunsaturated fatty acid, which may easily undergo oxidation resulting in high conjugated diene and triene values. Comparing the two seed oils, marula seed oil had a higher

quantity of polyunsaturated fatty acid compared to moringa seed oil (Table 4.2) and is therefore expected to form oxidation products more easily thus resulting in higher conjugated diene and triene values, which have been observed.

Evaluation of the relationship between the oxidative stability parameters using the Pearson correlation test provides the direction and strength of two variables. The correlation test is between -1, which shows a negative strong correlation in the downward direction, and +1 a positive strong correlation in the upward direction. The closer the coefficients are to +1 and -1, the greater is the strength of the relationship between the variables. A strong positive correlation shows the variables are in a direct relationship with each other. The conjugated diene strongly correlated with the peroxide values of the respective oils, with a correlation coefficient of 0.931 and 0.946 for marula and moringa seed oil, respectively. This further confirms that an increase in peroxide value results in an increase in the conjugated diene.

Comparing the relationship between the conjugated diene and triene percentage for marula and moringa seed oil, a strong positive correlation coefficient of 0.981 and 0.961, respectively was shown suggesting that if there is a high quantity of dienes present in the oils, there is a corresponding high tendency for trienes to also form. This study is the first to report conjugated diene and triene values for aqueous extracted marula and moringa seed oil at 37 and 60 °C.

4.5.3 p–anisidine value

The anisidine value measures the secondary product of lipid oxidation, which is the content of aldehydes (2-alkenals and 2,4-alkadienals) produced during the decomposition of hydro-peroxides (Frankel, 2012b). The lower the anisidine value the more stable the oil. Tables 4.10 shows the results of the anisidine values for the different extraction methods; Soxhlet, shaker, screw press, and aqueous at 37 and 60 °C. The results in Table 4.10 showed that there was a significant difference ($P<0.05$) in the anisidine values of the oils produced by the different extraction methods. The statistical differences were determined based on the analysis of variance test (ANOVA).

Table 4.10: Anisidine value of marula and moringa seed oil (n = 3)

Anisidine values		
Extraction methods	Marula seed oil	Moringa seed oil
Hexane extraction(Soxhlet)	1.41 ± 0.03^{a}	1.37 ± 0.08^{f}
Hexane extraction (shaker)	1.39 ± 0.07^{a}	1.27 ± 0.05^{g}
Aqueous extraction at 60 °C	1.11 ± 0.06^{c}	1.04 ± 0.07^{h}
Aqueous extraction at 37 °C	1.00 ± 0.03^{d}	0.88 ± 0.04^{i}
Screw press	1.28 ± 0.05^{e}	1.21 ± 0.08^{g}

Values are mean ± SD of marula seed oils from different extraction methods analyzed in triplicate. Mean values in the same column followed by different superscript letters are significantly different ($P < 0.05$) for marula and moringa seed oil, respectively

The anisidine value for Soxhlet, shaker and screw press method for marula and moringa seed oil was higher compared to the other extraction methods while aqueous extraction at 37 and 60 °C was the lowest. According to Table 4.10, the result showed that more secondary oxidation products have formed in the Soxhlet extracted oil thereby resulting in higher anisidine values. High values in both the Soxhlet extracted seed oils, could be due to the temperature and solvent used in the extraction process. The high temperatures used and the presence of a solvent have encouraged the formation of secondary oxidation products resulting in high anisidine values. In addition, the Soxhlet extracted seed oil had a higher polyunsaturated fatty acid content (Table 4.5 and 4.6) and lower monounsaturated fatty acid as compared to other extraction techniques. The Soxhlet extracted seed oil will therefore tend to produce more primary oxidation products, which will further oxidise to secondary oxidation products resulting in higher anisidine values. Aqueous extracted marula and moringa seed oil had the lowest anisidine values, which are probably due to the low amounts of polyunsaturated fatty acid and high monounsaturated fatty acid present.

Overall, moringa seed oil had lower anisidine values for all extraction methods which indicates a lower presence of secondary oxidation products and should therefore be a more stable oil.

The results for the oxidative stability tests (peroxide value, conjugated diene, conjugated triene and anisidine value) as shown in Table 4.8 to 4.10 all showed a lower value for aqueous extracted seed oils at 37 °C and 60 °C for both marula and moringa seed oil compared to the screw press and solvent extraction (Soxhlet and shaker) method. Assessment of the relationship between the oxidative stability parameters statistically showed a strong positive correlation between all the tests. The correlation coefficient between the peroxide value and conjugated dienes formed during the formation of hydroperoxide for marula and moringa seed oil was 0.931 and 0.946, respectively indicating that peroxide value and conjugated dienes have a good correlation. This implies that as the peroxide value was increasing the conjugated diene value was also increasing Farhoosh and Pazhouhanmehr, (2008) showed conjugated dienes to be a good indicator of oxidative stability by showing a high correlation with peroxide value, as was also shown in this study. In addition, Gomez et al. (2004) also reported a high correlation coefficient of 0.995 for olive oil indicating that peroxide value and conjugated dienes are in good correlation in oxidation of olive oil. The correlation coefficient between the oxidation parameters (conjugated trienes and anisidine value) for marula and moringa seed oil was 0.966 and 0.952 respectively, which also showed a strong correlation meaning that as the anisidine value was increasing conjugated triene value was also increasing.

4.6 Determination of oxidative stability of the seed oils based accelerated shelf life studies

The objective of every manufacturer and retailer using seed oils is to verify the shelf life of the oil. Giese (2000) defines shelf life study as the period of time for a product to meet its acceptable expectations regarding its quality. In reality, carrying out shelf life tests take time due to the time required for an oil with high stability to deteriorate. For this reason, accelerated storage shelf life tests were designed to hasten or increase the rate of oxidation and shorten the time required to achieve a change in the quality of the oil, thereby enabling estimation of oxidation rates to the expected storage temperature (Labuza and Schmidl 1985). The shelf life test was carried out to determine the effect the different extraction methods have on stability of the extracted oil. Shelf life of oil is reliant on many factors such as temperature of storage, exposure to light, and the presence of antioxidants (McClements and Decker 2008).

Moringa and marula seed oils were extracted using the 5 selected methods and were subjected to accelerated shelf life studies. This involved storing the oil at ambient temperature in the dark, and at higher temperatures (45 and 65 °C) which were chosen to stimulate a fast degradation for a period of time in an incubator, and analysing weekly collected samples for peroxide value, an indicator of primary oxidation, and anisidine value, the indicator of choice for secondary oxidation.

Peroxide value measures the quantity of hydroperoxide present in the oil. It is also used to determine the initial stage of oxidation and is commonly used in both industry and the academic world (Shahidi et al., 2005; McClements and Decker 2008). Statistically, the results in Tables 4.11 and 4.12 showed that there was a significant difference ($P<0.05$) in the peroxide values and anisidine values for the shelf-life studies for the different extraction methods. The statistical differences were determined based on the analysis of variance test (ANOVA) at the different stages of incubation period, day 7 to day 35, and for the different extraction methods. The result for the peroxide value at day 0 showed no significant difference ($P>0.05$) at ambient temperature, 45 °C and 65 °C. The results showed an increase in the peroxide value after each week, which showed that the longer the oils were stored, the greater their tendency to be oxidized, which is to be expected. A faster increase in peroxide values and *p*-anisidine values at elevated temperatures than at ambient temperature was observed hence storage at higher temperature accelerates oxidation. This was observed by the increase in the steepness of the gradient of the straight line from storage at ambient temperature to storage at 65 ℃ for all extraction methods (Figures 4.4 to 4.23).

Tables 4.11 and 4.12 also showed that the aqueous extraction method (37 °C and 60 °C) at the different stages of incubation had lower peroxide values compared to screw press and solvent (Soxhlet and shaker) extraction methods which had the highest peroxide values. The lower peroxide value could be as a result of the higher antioxidant activity in the aqueous extracted seed oil (Table 4.19), which enables scavenging of the oxidation products leading to a lower peroxide value.

The *p*-anisidine value measures the secondary product of oxidation, which is as a result of decomposition of hydroperoxide (Frankel, 2012b). From Tables 4.11 and 4.12 the results showed that there was a significant difference ($P<0.05$) in the *p*-anisidine values for the shelf life study for the different extraction methods. The statistical differences were determined based on results obtained for the analysis of variance test (ANOVA) at the different stages of incubation from day

7 to day 35 and for the different extraction methods. The day 0 anisidine value at ambient temperature, 45 °C and 65 °C showed no significant difference (P>0.05). After each week, the results showed an increase in the *p*-anisidine value, which suggests that the longer the oils are kept the more the production of secondary oxidation products. The results showed that there was also a significant difference for the different extraction methods. Overall, aqueous extracted seed oil at 37 °C for marula and moringa had the lowest *p*-anisidine value, and Soxhlet extracted marula and moringa seed oil had the highest *p*-anisidine value. From the results, the seed oil extracted using the aqueous extraction method at 37 °C had a better stability throughout the shelf life study compared to the screw pressed and solvent extraction methods. The anisidine values of the seed oils stored at the accelerated degradation temperature for the different extraction methods statistically revealed that there was a significant difference (P<0.05) between samples stored at higher temperatures for the different extraction methods for both oils.

A student t-test was carried out comparing one parameter, peroxide value, between the two seed oils. Statistically, the results in Tables 4.13 and 4.14 comparing the two seed oils at ambient temperature for 35 days showed that there was no significant difference at day 0 for the peroxide value of the two seed oils. From day7 to 35 onward there was significant difference (P<0.05) in the oxidative stability test for marula and moringa seed oil. Tables 4.11 and 4.12 showed a higher peroxide value for marula seed oil compared to moringa seed oil for aqueous extracted oil. A comparison of the anisidine value at ambient temperature, for both seed oils that were aqueous extracted (Table 4.14), showed that marula seed oil had the higher anisidine value for seed oil extracted at 37 °C compared to moringa seed oil extracted at 37 °C, which implies that moringa seed oil has a better stability than marula seed oil. The lower quantity of polyunsaturated fatty acid and higher quantity of monounsaturated fatty acid in aqueous extracted moringa seed oil as shown in Table 4.6 could also be the reason why aqueous extracted moringa seed oil had a better stability. In addition, the antioxidant activity in the aqueous extracted seed oil as reported in this study (Table 4.19) could be a contributing factor towards improved stability of the oil as antioxidants helps to fight against free radicals that make an oil unstable. The high presence of polyunsaturated fatty acid (linoleic acid) in marula seed oil makes the seed oil more susceptible to oxidation because of the presence of the double bonds in the polyunsaturated fatty acid.

Table 4.11: Oxidative stability (shelf life) results of marula seed oil

	PR	Soxhlet Extraction			Shaker Extraction			Aq. Extraction 37 °C			Aq. Extraction 60 °C			Screw press		
		Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
0	PV	0.87±0.06	0.87±0.06	0.87±0.06	0.80±0.00	0.80±0.00	0.80±0.00	0.30±0.00	0.30±0.00	0.30±0.00	0.47±0.06	0.47±0.06	0.47±0.06	0.57±0.06	0.57±0.06	0.57±0.06
	AV	1.50±0.03	1.50±0.03	1.50±0.03	1.44±0.04	1.44±0.04	1.44±0.04	1.12±0.01	1.12±0.01	1.12±0.01	1.30±0.00	1.30±0.00	1.30±0.00	1.37±0.07	1.37±0.07	1.37±0.07
7	PV	1.00±0.00	1.30±0.00	1.67±0.06	0.90±0.00	1.17±0.06	1.57±0.15	0.37±0.06	0.63±0.06	1.00±0.00	0.57±0.06	0.77±0.06	1.23±0.06	0.63±0.06	0.97±0.06	1.40±0.10
	AV	1.76±0.01	1.93±0.03	2.16±0.05	1.69±0.05	1.76±0.04	2.10±0.08	1.30±0.01	1.37±0.10	1.54±0.01	1.52±0.02	1.57±0.01	1.74±0.00	1.61±0.01	1.70±0.02	1.96±0.05
14	PV	1.07±0.06	1.62±0.08	2.33±0.06	1.00±0.10	1.47±0.06	2.20±0.10	0.43±0.06	0.90±0.00	1.63±0.06	0.60±0.10	1.03±0.06	1.80±0.00	0.73±0.06	1.27±0.06	2.03±0.06
	AV	2.06±0.03	2.26±0.06	2.69±0.13	1.90±0.00	2.04±0.02	2.56±0.05	1.46±0.00	1.55±0.03	1.83±0.02	1.68±0.06	1.77±0.01	2.08±0.06	1.84±0.04	1.94±0.05	2.41±0.01
21	PV	1.17±0.15	2.10±0.10	3.20±0.00	1.10±0.00	1.90±0.00	3.03±0.06	0.50±0.00	1.30±0.00	2.37±0.12	0.70±0.00	1.47±0.06	2.53±0.15	0.80±000	1.70±0.10	2.83±0.06
	AV	2.32±0.03	2.71±0.01	3.34±0.00	2.10±0.10	2.44±0.05	3.18±0.25	1.60±0.02	1.80±0.03	2.22±0.11	1.85±0.04	2.06±0.09	2.49±0.05	2.04±0.03	2.30±0.20	2.97±0.07
28	PV	1.23±0.06	2.50±0.10	3.93±0.06	1.17±0.06	2.27±0.06	3.77±0.06	0.53±0.06	1.63±0.06	3.00±0.00	0.73±0.06	1.83±0.06	3.27±0.15	0.87±0.06	2.03±0.06	3.60±0.10
	AV	2.60±0.08	3.10±0.03	3.90±0.15	2.32±0.00	2.74±0.02	3.74±0.02	1.76±0.25	2.02±0.08	2.56±0.09	2.03±0.01	2.30±0.04	2.88±0.14	2.23±0.01	2.58±0.08	3.50±0.08
35	PV	1.37±0.06	2.90±0.00	4.67±0.06	1.27±0.06	2.63±0.06	4.50±0.00	0.60±0.00	2.00±0.00	3.67±0.06	0.83±0.06	2.17±0.06	3.93±0.21	0.97±0.06	2.40±0.00	4.27±0.06
	AV	2.84±0.04	3.50±0.02	4.44±0.04	2.52±0.01	3.06±0.04	4.23±0.02	1.91±0.02	2.24±0.02	2.90±0.01	2.20±0.10	2.55±0.01	3.25±0.06	2.45±0.01	2.85±0.00	3.96±0.00

Mean values of triplicate readings ± SD in the same column and row for PV and AV, respectively are all significantly different ($P < 0.05$) for the different extraction methods. But at day 0 for each extraction methods there is no significant difference ($P>0.05$), respectively. The numbers (0, 7, 14, 21, 28, and 35) represent the day at which the sample was taken and analysed; day 0 being the start of experiment. At ambient temperature for PV soxhlet extraction at day 7 and 14 showed $P>0.05$ and is there is no statistical difference. Similarly day 21, and 28; shaker extraction day 21,and 28; Aq. Extraction 37 °C day 7 and 14, 14 and 21, 21 and 28 and 28 and 35 showed $P>0.05$. Aq. Extraction 60 °C day 7 and 14, and 21 and 28 showed $P>0.05$. Screw press day 0 and 7; day 14 and 21, and day 21 and 28 showed $P>0.05$. Day 0 for soxhlet and shaker at ambient, 45 °C and 65 °C showed $P>0.05$. At day7, Aq. Extraction 60 °C and screw press at ambient temperature, showed $P>0.05$. Day 14 soxhlet and shaker at ambient temperature; day 21 soxhlet and shaker at ambient temperature and day 28 for soxhlet and shaker at ambient temperature showed $P>0.05$. Anisidine value day 0 for soxhlet and shaker at ambient temperature, 45 °C, and 65 °C, showed $P>0.05$. Day 0 Aq. Extraction 60 °C and screw press at ambient temperature, 45 °C and 65 °C showed $P>0.05$. Day 7 soxhlet and shaker extraction method at ambient temperature and at 65 °C showed $P>0.05$, day 21 at ambient temperature shaker extraction and screw press showed $P>0.05$.

Table 4.12: Oxidative stability (shelf life) of moringa seed oil

	PR	Soxhlet Extraction			Shaker Extraction			Aq. Extraction 37 °C			Aq. Extraction 60 °C			Screw press		
		Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
0	PV	0.80±0.00	0.80±0.00	0.80±0.00	0.77±0.06	0.77±0.06	0.77±0.06	0.23±0.06	0.23±0.06	0.23±0.06	0.37±0.06	0.37±0.06	0.37±0.06	0.47±0.06	0.47±0.06	0.47±0.06
	AV	1.44±0.01	1.44±0.01	1.44±0.01	1.38±0.05	1.38±0.05	1.38±0.05	1.00±0.03	1.00±0.03	1.00±0.03	1.20±0.00	1.20±0.00	1.20±0.00	1.32±0.03	1.32±0.03	1.32±0.03
7	PV	0.90±0.00	1.13±0.06	1.57±0.06	0.83±0.06	1.10±0.00	1.53±0.06	0.27±0.06	0.60±0.00	0.90±0.10	0.43±0.06	0.65±0.05	1.10±0.10	0.53±0.06	0.93±0.06	1.30±0.00
	AV	1.70±0.00	1.86±0.06	2.17±0.01	1.54±0.05	1.71±0.02	1.87±0.09	1.12±0.02	1.20±0.01	1.34±0.02	1.38±0.08	1.41±0.01	1.58±0.07	1.47±0.03	1.57±0.01	1.78±0.00
14	PV	0.97±0.06	1.50±0.10	2.20±0.10	0.90±0.00	1.40±0.00	2.17±0.06	0.33±0.06	0.83±0.06	1.47±0.06	0.50±0.00	0.93±0.06	1.70±0.10	0.63±0.06	1.17±0.06	1.97±0.06
	AV	1.96±0.02	2.14±0.02	2.63±0.02	1.75±0.07	1.94±0.02	2.23±0.12	1.21±0.01	1.34±0.00	1.61±0.01	1.53±0.01	1.57±0.00	1.86±0.01	1.66±0.03	1.79±0.06	2.09±0.02
21	PV	1.07±0.06	1.93±0.06	3.07±0.06	1.00±0.00	1.83±0.06	2.97±0.06	0.37±0.06	1.23±0.06	2.20±0.10	0.57±0.06	1.30±0.10	2.47±0.06	0.70±0.00	1.60±0.00	2.73±0.06
	AV	2.23±0.02	2.54±0.02	3.28±0.00	1.97±0.02	2.26±0.06	2.70±0.02	1.31±0.03	1.52±0.02	1.95±0.02	1.68±0.09	1.85±0.02	2.21±0.14	1.85±0.08	2.07±0.06	2.48±0.02
28	PV	1.13±0.06	2.33±0.06	3.77±0.06	1.07±0.06	2.23±0.06	3.70±0.00	0.43±0.06	1.57±0.06	2.83±0.10	0.60±0.00	1.63±0.06	3.10±0.10	0.77±0.06	1.97±0.06	3.43±0.06
	AV	2.49±0.03	2.89±0.13	3.82±0.05	2.18±0.02	2.58±0.09	3.10±0.19	1.40±0.03	1.69±0.00	2.24±0.02	1.85±0.00	2.03±0.02	2.54±0.01	2.01±0.00	2.34±0.06	2.83±0.02
35	PV	1.27±0.06	2.70±0.17	4.43±0.11	1.13±0.06	2.57±0.15	4.40±0.00	0.47±0.06	1.80±0.10	3.50±0.00	0.67±0.06	1.97±0.06	3.73±0.06	0.83±0.06	2.27±0.06	4.10±0.00
	AV	2.78±0.03	3.22±0.02	4.34±0.00	2.40±0.00	2.85±0.00	3.49±0.01	1.50±0.00	1.86±0.00	2.53±0.00	1.99±0.00	2.24±0.01	2.86±0.01	2.20±0.03	2.60±0.04	3.20±0.01

Mean values of triplicate readings ± SD in the same column and row for PV and AV, respectively are all significantly different ($P< 0.05$) for the different extraction methods. But at day 0 for each extraction methods there is no significant difference ($P>0.05$), respectively. The numbers (0, 7, 14, 21, 28, 35) represents the day at which the sample was taken and analysed; day 0 being the start of experiment. At ambient temperature for PV soxhlet extraction at day 7 and 14 showed $P>0.05$ and was therefore not significantly different. Similarly, day 21 and 28 for soxhlet, shaker extraction day 0 and 7; day7 and 14; day 21 and 28, and day 28 and 35 showed $P>0.05$. Aq. Extraction 37 °C day 0 and 7; day 7 and 14; day 14 and 21; day 21 and 28, and day 28 and 35 showed $P>0.05$. Aq. Extraction 60 °C day 0 and 7; day 7 and 14; day 14 and 21; day 21 and 28, and day 28 and 35 showed $P>0.05$. Screw press day 0 and 7; day 14 and 21; day 21 and 28; 28 and 35 showed $P>0.05$. Day 0 for soxhlet and shaker at ambient temperature, 45 °C and 65 °C showed $P>0.05$. Day 7 for soxhlet and shaker at ambient temperature, 45 °C and 65 °C showed $P>0.05$. Day 7 at 45 °C for Aq. Extraction 37 °C and Aq. Extraction 60 °C showed $P>0.05$. Day 14 for soxhlet and shaker at ambient temperature and 65 °C showed $P>0.05$. Day 21 for soxhlet and shaker at ambient temperature and 45 °C, and for Aq. Extraction 37 °C and Aq. Extraction 60 °C showed $P>0.05$. Day 28 for soxhlet and shaker at 65 °C showed $P>0.05$; at 45 °C for Aq. Extraction 37 °C and Aq. Extraction 60 °C showed $P>0.05$. Day 35 for soxhlet and shaker at 65 °C showed $P>0.05$. Anisidine value at day 0 soxhlet and shaker at ambient temperature, 45 °C and 65 °C showed $P>0.05$.

Table 4.13: Comparison of peroxide values of marula and moringa seed oil for aqueous 37 °C at ambient temperature

	Marula seed oil	Moringa seed oil
Day	Peroxide value (meq/kg of oil)	Peroxide value (meq/kg of oil)
0	0.30 ± 0.00^{a}	0.23 ± 0.06^{a}
7	0.37 ± 0.06^{c}	0.26 ± 0.06^{d}
14	0.43 ± 0.06^{e}	0.34 ± 0.06^{f}
21	0.50 ± 0.00^{g}	0.37 ± 0.06^{h}
28	0.53 ± 0.06^{i}	0.43 ± 0.06^{j}
35	0.60 ± 0.00^{k}	0.47 ± 0.06^{l}

Values are mean ± SD of marula and moringa seed oils from different extraction methods analyzed in triplicate. Values in different column followed by different superscript letters are significantly different ($P<0.05$).

Table 4.14: Comparison of anisidine values of marula and moringa seed oil for aqueous extraction at 37 °C at ambient temperature

	Marula seed oil	Moringa seed oil
Day	Anisidine value	Anisidine value
0	1.12 ± 0.01^{a}	1.00 ± 0.03^{b}
7	1.30 ± 0.05^{c}	1.11 ± 0.02^{d}
14	1.46 ± 0.06^{e}	1.21 ± 0.04^{f}
21	1.60 ± 0.02^{g}	1.31 ± 0.03^{h}
28	1.76 ± 0.25^{i}	1.40 ± 0.02^{j}
35	1.91 ± 0.05^{k}	1.50 ± 0.00^{l}

Values are mean ± SD of marula and moringa seed oils from different extraction methods analyzed in triplicate. Mean values in different column followed by different superscript letters are significantly different ($P<0.05$).

A critical look at the peroxide value results of the accelerated storage temperature at 45 °C and 65 °C for the different extraction methods statistically revealed that there was a significant difference between them for the different extraction methods for both oils.

Figures 4.4 to 4.23 show the straight line graphs for peroxide and anisidine values plotted against time (in days) for the different storage temperatures for all extraction methods for marula and moringa seed oils. The straight-line graphs all show R^2 values close to 1 which confirms the linear relationship of peroxide and anisidine values with time. The y-intercept for each method of extraction was shown as the same value for all three storage temperatures (i.e. was forced through the same value) because the peroxide or anisidine values were the same on day zero and therefore had to have the same y-intercept.

Considering the rate of reaction from Fig 4.4 to 4.23, a plot of the peroxide or anisidine values, which is in fact the concentration of the oxidation products, against time shows a straight-line graph and therefore the rate of reaction is a zero-order reaction. This means that the rate of formation of the oxidation products (primary and secondary products) will remain constant with time and is independent of the concentration of the starting reactant. The primary and secondary oxidation products form from oxidation reactions of the fatty acids. Thus the zero order result implies that no matter what concentration of fatty acid is present in the oil, the rate of forming the oxidation products will be the same. The result obtained in this study is in line with that reported by Piedrahita et al. (2015)

The results in Tables 4.15 and 4.17 and Figures 4.4a to 4.8 showed that the rate of change of peroxide values at the degradation temperature of 45 °C and 65 °C was higher compared to that at ambient temperature which is as expected.

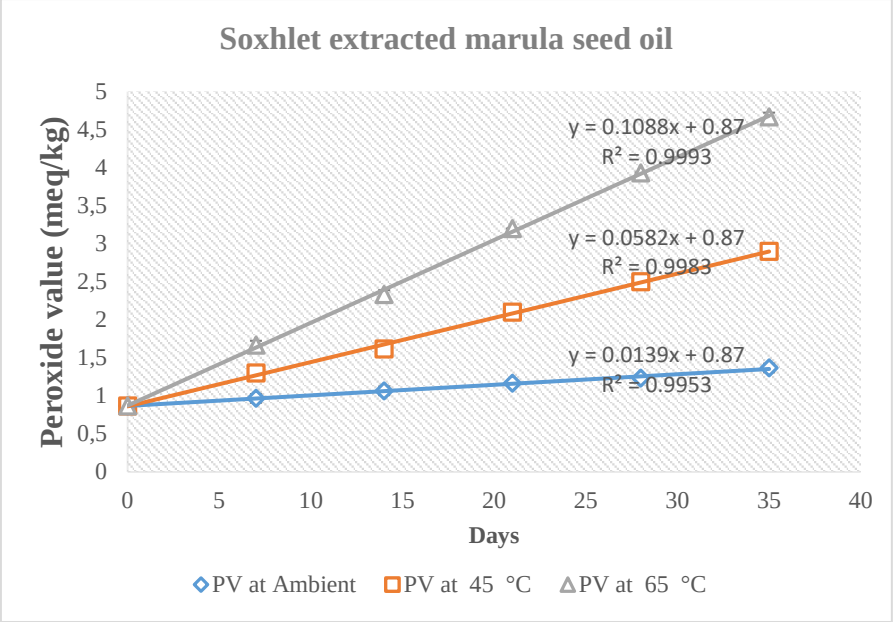


Figure 4.4: Graph showing the rate of increase of peroxide value at ambient temperature, 45 °C and 65 °C for Soxhlet extracted marula seed oil.

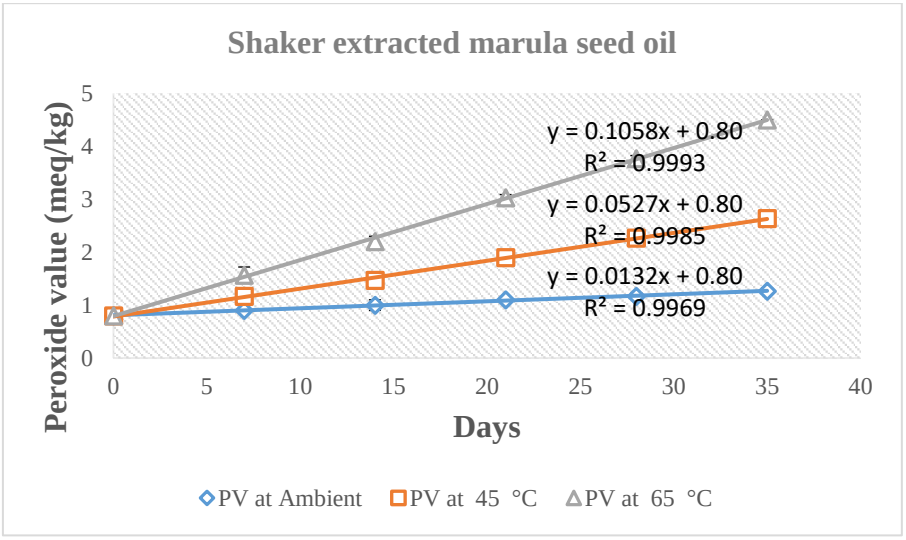


Figure 4.5: Graph showing the rate of increase of peroxide value at ambient temperature, 45 °C and 65 °C for shaker extracted marula seed oil.

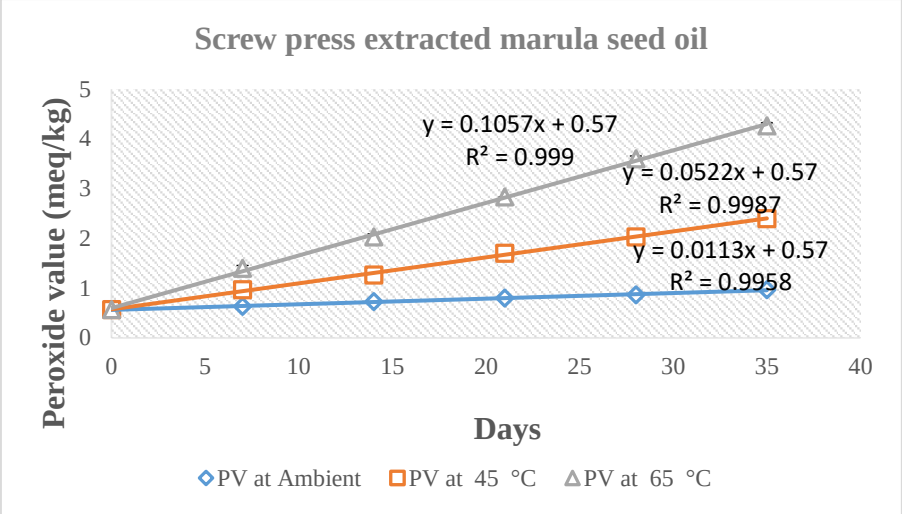


Figure 4.6: Graph showing the rate of increase of peroxide value at ambient temperature, 45 °C and 65 °C for screw press extracted marula seed oil.

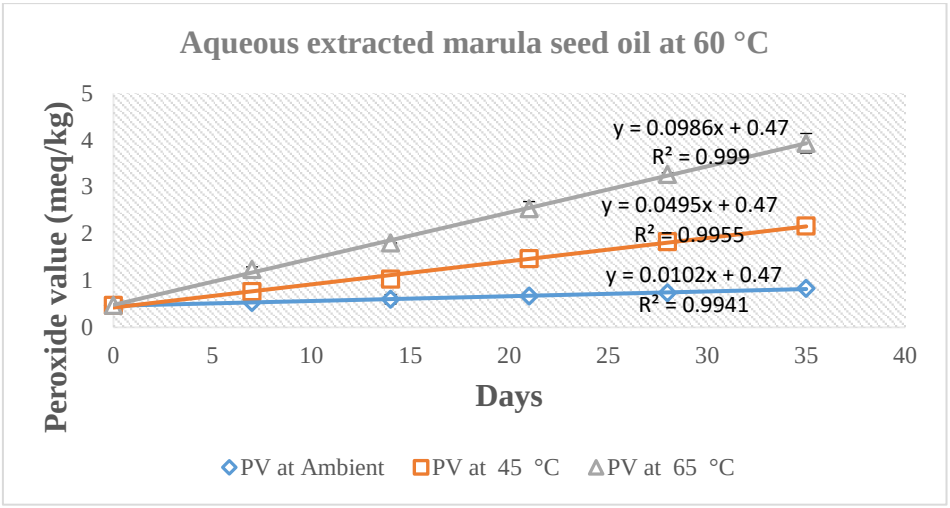


Figure 4.7: Graph showing the rate of increase of peroxide value at ambient temperature, 45 °C and 65 °C for aqueous extracted marula seed oil at 60 °C.

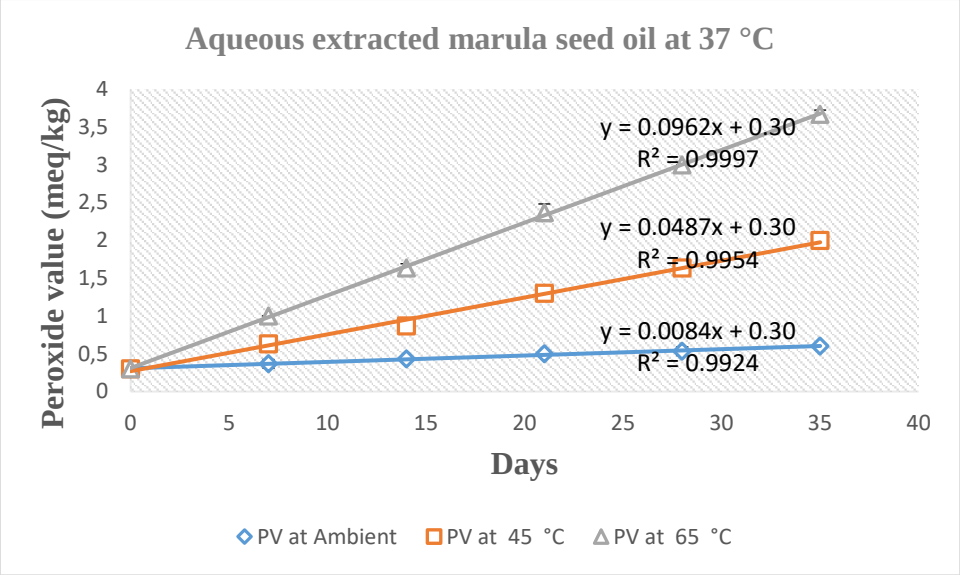


Figure 4.8: Graph showing the rate of increase of peroxide value at ambient temperature, 45 °C and 65 °C for aqueous extracted marula seed oil at 37 °C.

The rate of increase of peroxide value for Soxhlet extracted marula seed oil was the highest at all storage temperatures (ambient, 45 and 65 °C). At ambient temperature, the rate of change was 0.0139 (from the equation of the straight line), and at 45 °C and 65 °C it was 0.0582 and 0.1088, respectively. This could possibly be due to the use of high temperatures and a solvent in the extraction process, which produces more oxidation products resulting in an increased peroxide value for the extracted oil. The high extraction temperature also destroys the natural antioxidants present in the oil resulting in lower antioxidant activity and eventually the formation of more oxidation products (Bajpai and Agrawal, 2015). Further storage of this oil at accelerated temperatures encourages even more oxidation products to form within the short space of time (35 days) resulting in a high peroxide value and an increased rate of oxidation. Aqueous extracted marula at 37 °C stored at the various shelf life conditions had the lowest rate of change compared to the other extraction methods. At ambient temperature, the rate was 0.0084, and at 45 °C and 65 °C it was 0.0487 and 0.0962, respectively. The low rate of oxidation observed for aqueous extracted marula oil could be due to the mild extraction process where lower temperatures and water was used as the extracting solvent. The mild extraction process produces less oxidation products and even though it was stored at accelerated temperatures (45 °C and 65 °C), the presence of initial low oxidation products produces much lesser oxidation products as compared to Soxhlet extracted marula seed oil. Thus, the initial

amounts of oxidation products produced from the extraction method plays an important role in the rate of formation of oxidation products and eventually affects the stability of the oil.

Moringa seed oil showed the same trend with the rate of increase of peroxide value for Soxhlet extraction as the highest for the different shelf life storage conditions (Figure 4.9 to 4.13).

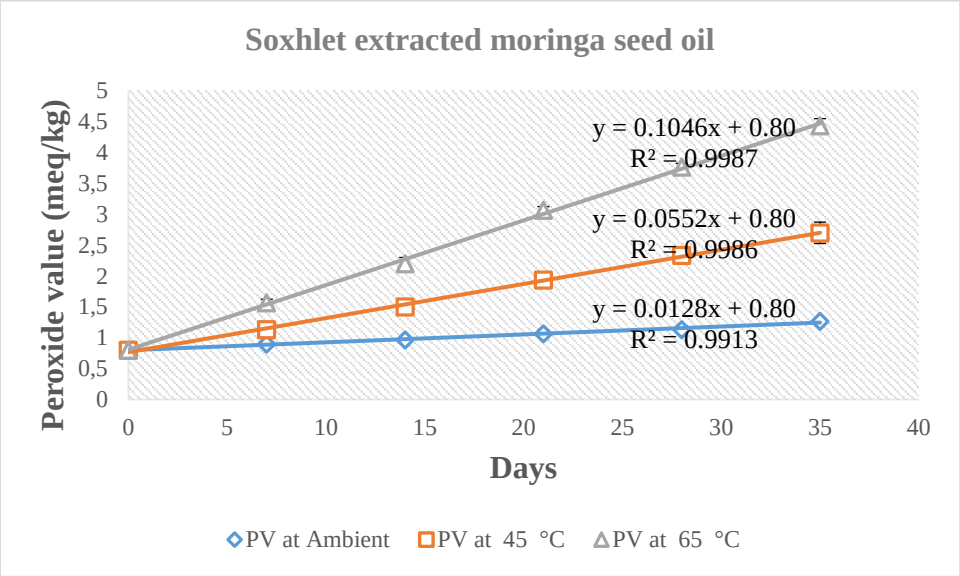


Figure 4.9: Graph showing the rate of increase of peroxide value at ambient temperature 45 °C and 65 °C for Soxhlet extracted moringa seed oil

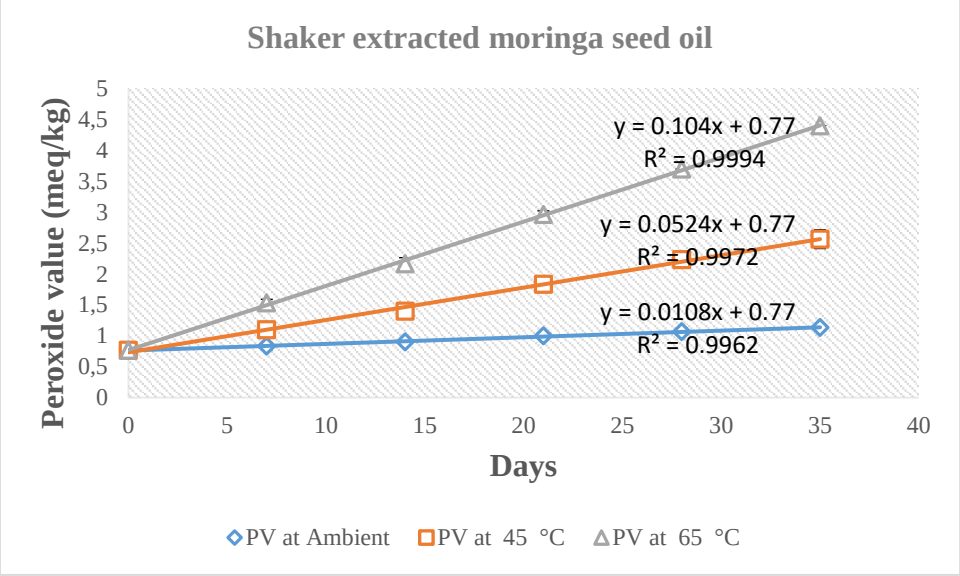


Figure 4.10: Graph showing the rate of increase of peroxide value at ambient temperature 45 °C and 65 °C for shaker extracted moringa seed oil

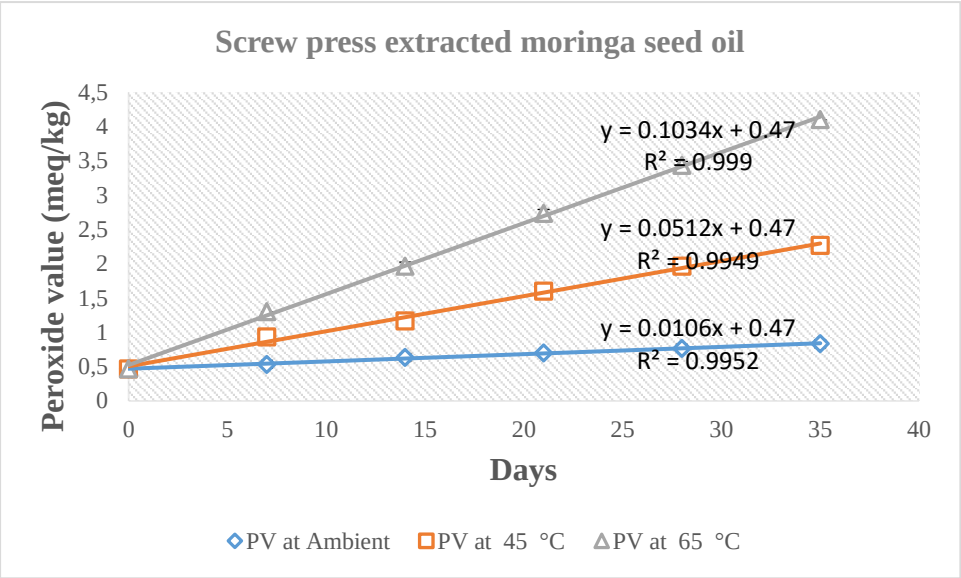


Figure 4.11: Graph showing the rate of increase of peroxide value at ambient temperature 45 °C and 65 °C for screw press extracted moringa seed oil

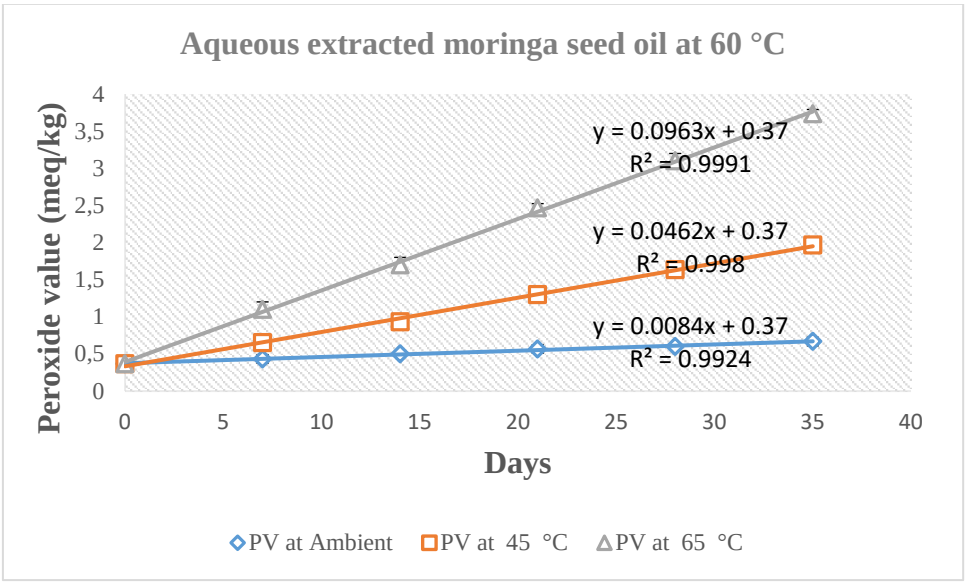


Figure 4.12: Graph showing the rate of increase of peroxide value at ambient temperature 45 °C and 65 °C for aqueous extracted moringa seed oil at 60 °C

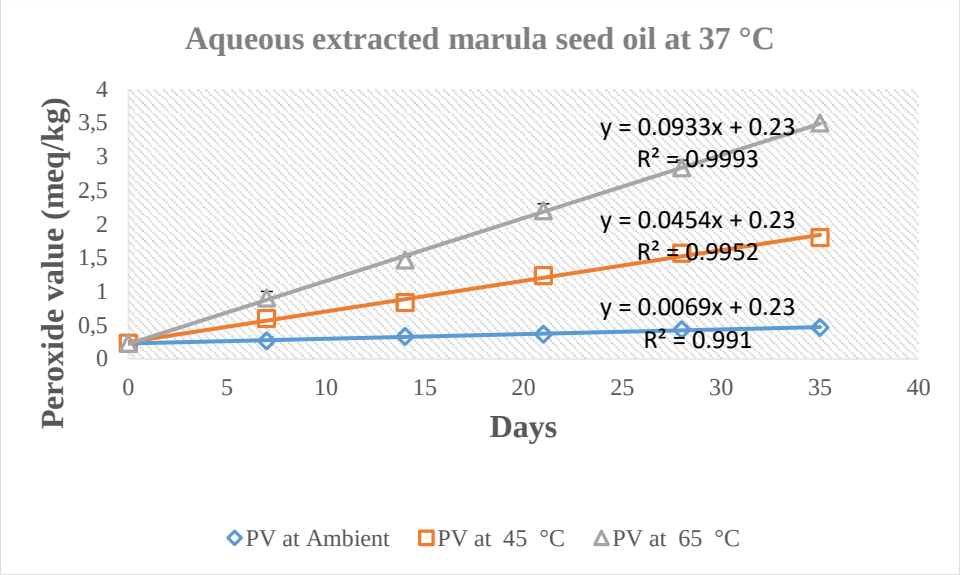


Figure 4.13: Graph showing the rate of increase of peroxide value at ambient temperature 45 °C and 65 °C for aqueous extracted moringa seed oil at 37 °C

Aqueous extracted moringa at 37 °C had the lowest rate of oxidation for all the shelf life storage temperatures. This suggests that aqueous extracted oil takes longer to produce primary oxidation products even at accelerated temperatures and the Soxhlet extracted oil forms primary oxidation products most quickly compared to the other extraction methods used in this study. In the Soxhlet extraction, the rapid oxidation process at accelerated temperatures is due to the initial high amount of the polyunsaturated acid present as well as the low quantities of natural antioxidants present. This leads to the formation of higher amounts of oxidation products and at accelerated storage temperatures, it leads to even more oxidation products being formed. Thus, this extracted oil is highly susceptible to oxidation at accelerated temperatures and will be less stable over long periods. This shows that the fatty acid and the antioxidant activities plays an important role in the oxidation process.

The rate of change of anisidine value is shown in Tables 4.16 and 4.18, and Figures 4.14 to 4.18.

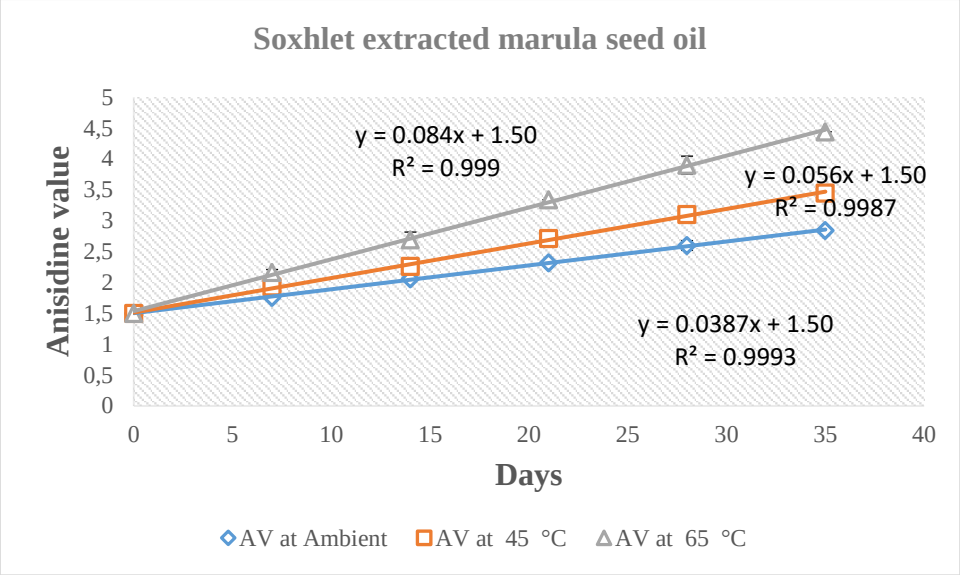


Figure 4.14: Graph showing the rate of increase of anisidine value value at ambient temperature, 45 °C and 65 °C for Soxhlet extracted marula seed oil.

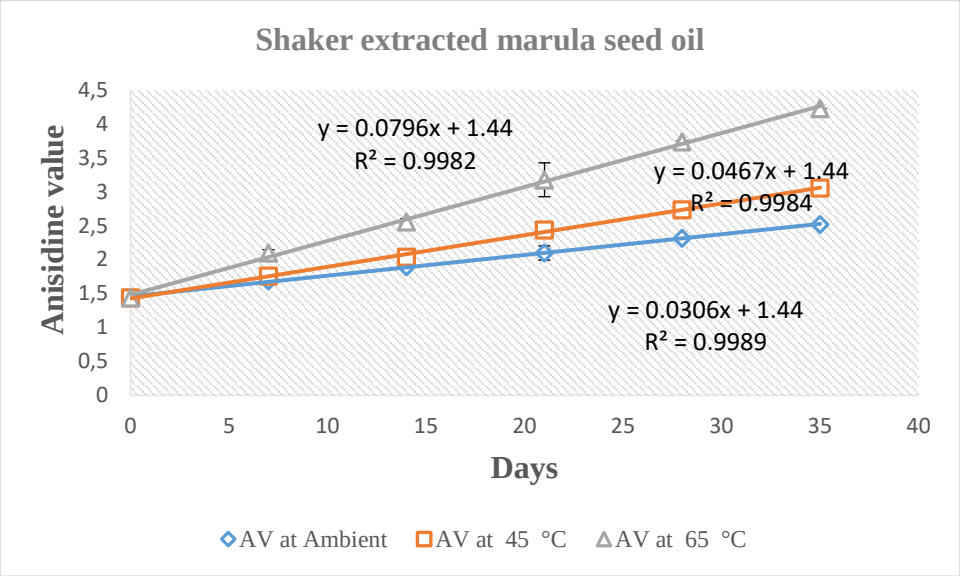


Figure 4.15: Graph showing the rate of increase of anisidine value at ambient temperature, 45 °C and 65 °C for shaker extracted marula seed oil.

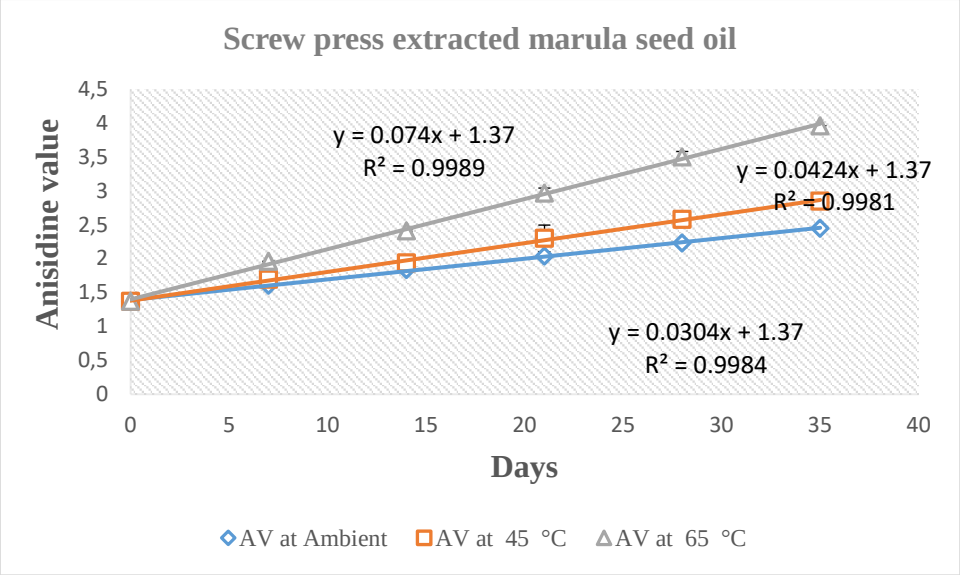


Figure 4.16: Graph showing the rate of increase of anisidine value value at ambient temperature, 45 °C and 65 °C for screw press extracted marula seed oil

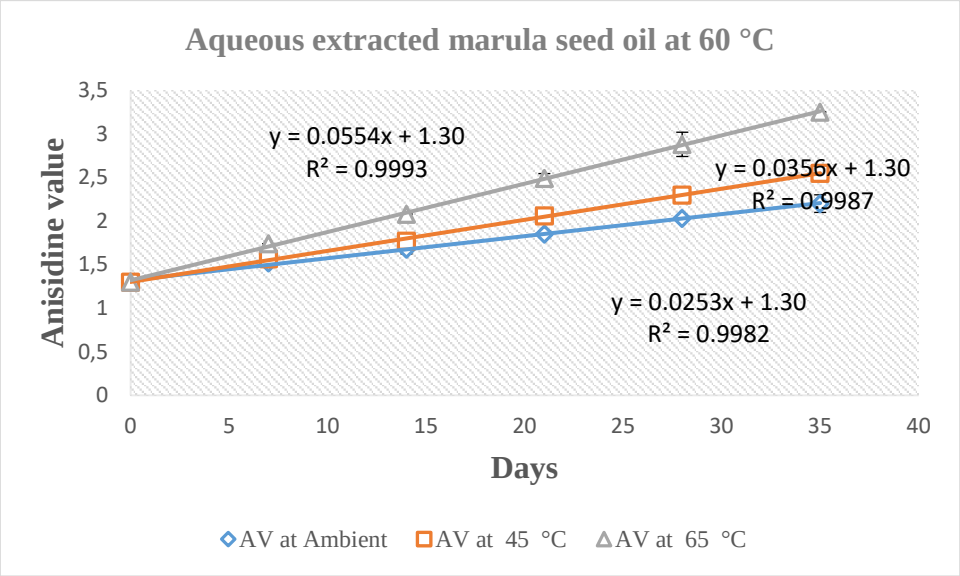


Figure 4.17: Graph showing the rate of increase of anisidine value value at ambient temperature, 45 °C and 65 °C for aqueous extracted marula seed oil at 60 °C

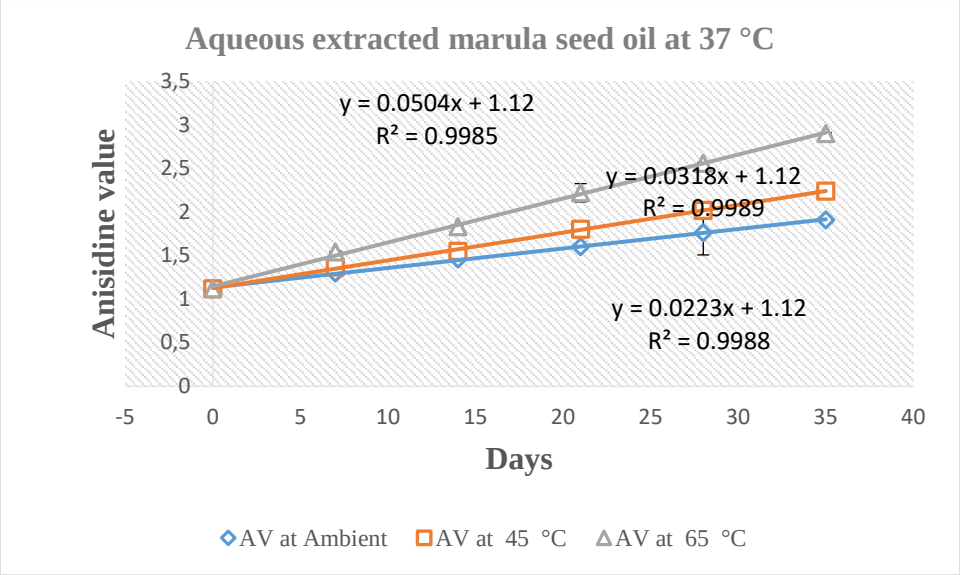


Figure 4.18: Graph showing the rate of increase of anisidine value value at ambient temperature, 45 °C and 65 °C for aqueous extracted marula seed oil at 37 °C

The rate of increase of anisidine value for Soxhlet extracted marula seed oil was the highest for all the storage conditions. Aqueous extracted seed oil at 37 °C had the lowest rate of change stored at ambient temperature. The rate of change of anisidine value for Soxhlet extracted moringa seed oil had the highest value as shown in (Figures 4.19 to 4.23)

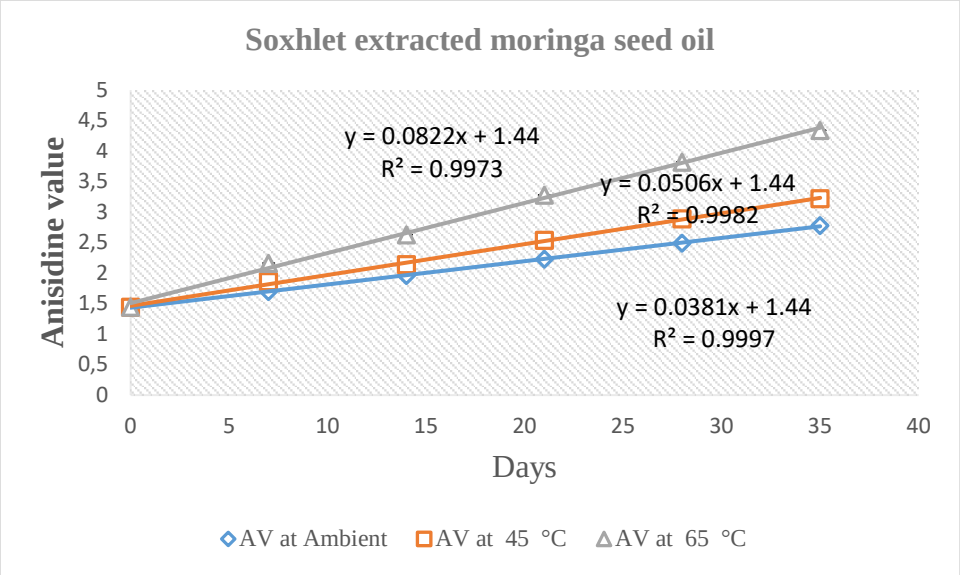


Figure 4.19: Graph showing the rate of increase of anisidine value at ambient temperature 45 °C and 65 °C for Soxhlet extracted moringa seed oil

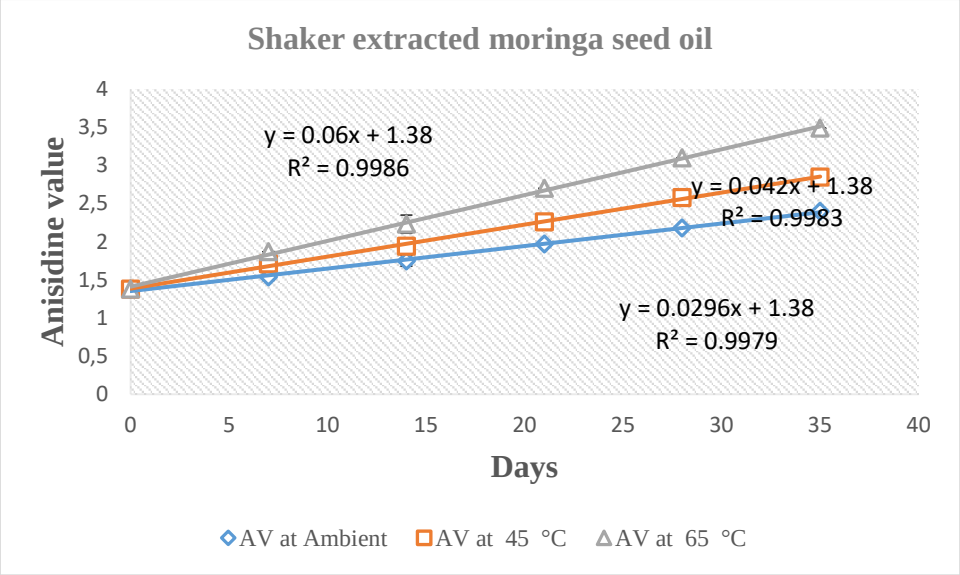


Figure 4.20: Graph showing the rate of increase of anisidine value at ambient temperature 45 °C and 65 °C for shaker extracted moringa seed oil

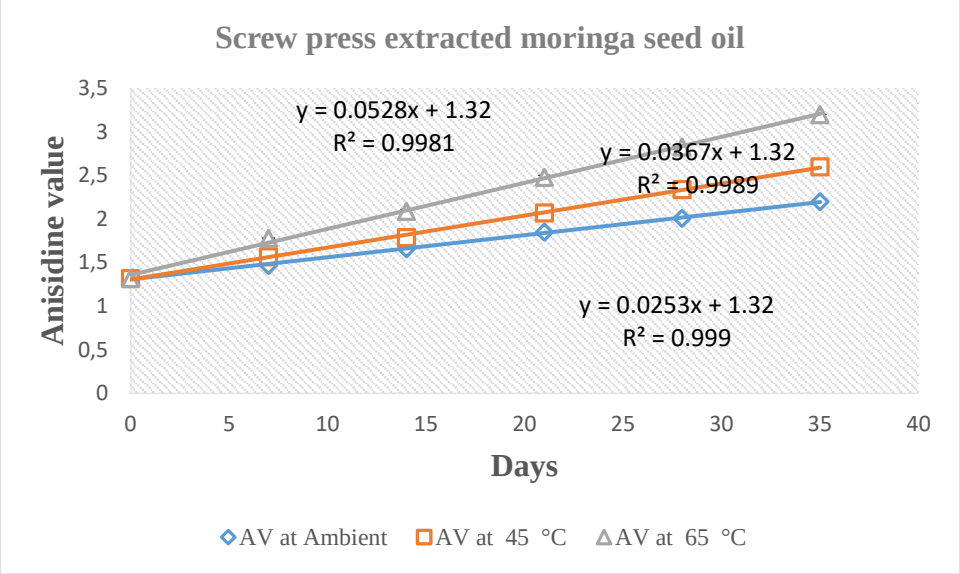


Figure 4.21: Graph showing the rate of increase of anisidine value at ambient temperature 45 °C and 65 °C for screw press extracted moringa seed oil

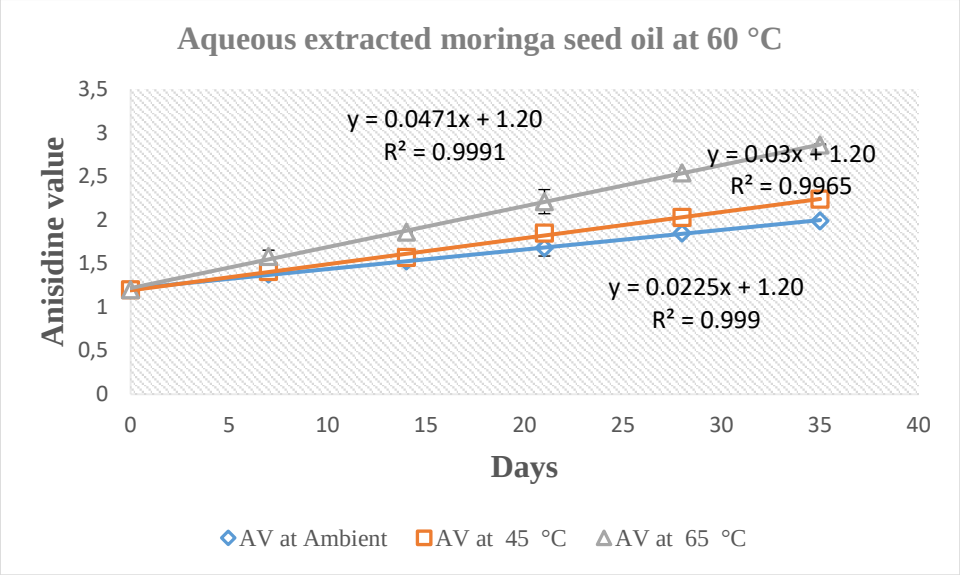


Figure 4.22: Graph showing the rate of increase of anisidine value at ambient temperature 45 °C and 65 °C for aqueous extracted moringa seed oil at 60 °C

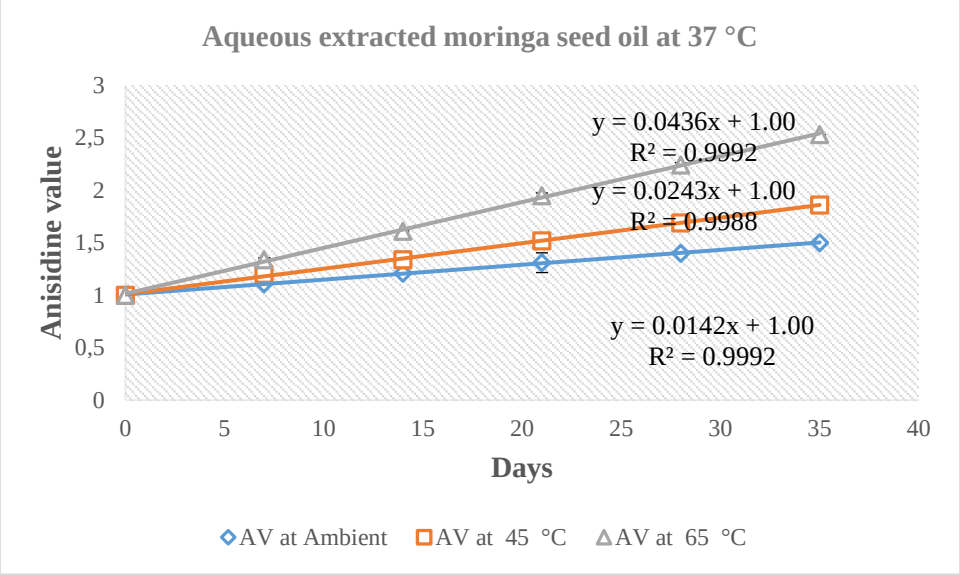


Figure 4.23: Graph showing the rate of increase of anisidine value at ambient temperature 45 °C and 65 °C for aqueous extracted moringa seed oil at 37 °C

The results showed that aqueous extracted seed oil at 37 °C had the lowest rate of change of anisidine value, which implies that the seed oils extracted using the aqueous method will tend to take longer to produce secondary oxidation products, which then results in rancidity. This low rate of change of secondary oxidation could be due to the low temperatures used in this

extraction method as well as the use of water instead of an organic solvent. These mild extraction conditions results in low amounts of primary oxidation products, which will result in fewer secondary oxidation products forming. In addition, the mild extraction temperatures and the use of water as the extracting solvent produces low amounts of polyunsaturated fatty acid (linoleic). Thus, this will lead to less primary oxidation products forming, eventually fewer secondary oxidation products, and a more stable oil. Hence, the aqueous method of extraction is the preferred method of extraction for producing oils with a good stability and good shelf life due to the longer time it takes to produce both primary and secondary oxidation products.

In addition, secondary oxidation (*p*-AV) progresses faster than primary oxidation (PV) at ambient temperature; the contrary is observed at elevated temperature whereby secondary oxidation progresses slower than primary oxidation. This is observed in all samples independent of extraction method or, seed type. Understanding the rationale for this observation requires further investigation in future projects.

Table 4.15: Rate of change of peroxide value for marula seed oil. (PR- parameter, GR- gradient)

PR	Soxhlet Extraction			Shaker Extraction			Screw press			Aq. Extraction 60 °C			Aq. Extraction 37°C		
	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
GR	0.0139	0.0582	0.1088	0.0132	0.0527	0.1058	0.0113	0.0522	0.1057	0.0102	0.0495	0.0986	0.0084	0.0487	0.0962
R^2	0.9953	0.9983	0.9993	0.9969	0.9985	0.9993	0.9958	0.9987	0.9990	0.9941	0.9955	0.9990	0.9924	0.9954	0.9997

Table 4.16: Rate of change of anisidine value for marula seed oil. (PR- parameter, GR- gradient)

PR	Soxhlet Extraction			Shaker Extraction			Screw press			Aq. Extraction 60 °C			Aq. Extraction 37°C		
	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
GR	0.0387	0.0560	0.0840	0.0306	0.0467	0.0796	0.0304	0.0424	0.0740	0.0253	0.0356	0.0554	0.0223	0.0318	0.0504
R^2	0.9993	0.9987	0.9990	0.9989	0.9984	0.9982	0.9984	0.9981	0.9989	0.9982	0.9987	0.9993	0.9988	0.9989	0.9985

Table 4.17: Rate of change of peroxide value for moringa seed oil. (PR- parameter, GR- gradient)

PR	Soxhlet Extraction			Shaker Extraction			Screw press			Aq. Extraction 60 °C			Aq. Extraction 37°C		
	Ambient	45 °C	65°C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
GR	0.0128	0.0552	0.1046	0.0108	0.0524	0.1040	0.0106	0.0512	0.1034	0.0084	0.0462	0.0963	0.0069	0.0454	0.0933
R^2	0.9913	0.9986	0.9987	0.9962	0.9972	0.9994	0.9952	0.9949	0.9990	0.9924	0.9980	0.0963	0.9910	0.9952	0.9993

Table 4.18: Rate of change of anisidine value for moringa seed oil. (PR- parameter, GR- gradient)

PR	Soxhlet Extraction			Shaker Extraction			Screw press			Aq. Extraction 60 °C			Aq. Extraction 37°C		
	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C	Ambient	45 °C	65 °C
GR	0.0381	0.0506	0.0822	0.0296	0.0420	0.0600	0.0253	0.0367	0.0528	0.0225	0.0300	0.0471	0.0142	0.0240	0.0436
R_2	0.9997	0.9982	0.9970	0.9979	0.9983	0.9986	0.9990	0.9989	0.9981	0.9990	0.9965	0.9991	0.9992	0.9988	0.9992

4.7 Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The results in Table 4.19 showed that there was a significant difference (P<0.05) in the radical scavenging abilities of the oils extracted from the different extraction methods. The statistical analysis is determined from the results of the analysis of variance test (ANOVA).

Table 4.19: Radical scavenging ability of marula and moringa seed oil with different extraction methods

	% Inhibition	
Extraction methods	Marula seed oil	Moringa seed oil
BHA	99.09 ± 0.00^{a}	99.09 ± 0.00^{a}
Hexane extraction (Soxhlet)	50.30 ± 0.53^{b}	51.21 ± 0.53^{g}
Hexane extraction (shaker)	52.12 ± 0.53^{c}	53.33 ± 0.53^{h}
Aqueous extraction at 37 °C	59.39 ± 0.53^{d}	62.73 ± 0.00^{i}
Aqueous extraction at 60 °C	56.36 ± 0.00^{e}	59.70 ± 1.05^{j}
Screw press	54.24 ± 0.53^{f}	56.06 ± 0.53^{k}

Values are mean ± SD of marula and moringa seed oils from different extraction methods analyzed in triplicate. Mean values in same column followed by different superscript letters are significantly different (P < 0.05) for marula and moringa seed oil respectively.

Aqueous extracted marula and moringa seed oil at 37 °C had the highest scavenging ability compared to the other extraction methods. This showed that the different extraction methods did affect the effectiveness of the antioxidant compounds present in the oils. Solvent and temperature used in the solvent extraction could be the factor, which affected the natural antioxidant present thus leading to a lower scavenging ability.

The result is also confirmed from the oxidative stability tests (PV, *p*-AV, CD and CT) which showed that the aqueous extracted seed oils had the lowest values, which could be as a result of the natural antioxidant present in the oil being able to scavenge the free radicals. The BHA

standard was used, in this study, as a positive control and to validate the test. The scavenging ability of BHA was 99.09 %, which was higher than that of both marula and moringa seed oil extracted with different extraction methods. This implies that the antioxidant in the standard has a strong resistance against oxidation because it scavenges almost all the free radicals and inhibits lipid oxidation as compared to the antioxidant in the samples, which could scavenge just about 50.30 to 62.73 %.

The Pearson correlation coefficient test carried out between the radical scavenging ability with the oxidative stability tests showed a strong negative correlation. The correlation coefficient between the radical scavenging ability and conjugated diene for marula and moringa seed oil was -0.920 and -0.886, respectively which means that the higher the radical scavenging activity the lower the amount of conjugated diene present in the oil. In addition, a strong negative correlation coefficient of -0.967 and –0.890 for marula and moringa seed oil was shown between the radical scavenging ability and conjugated trienes which means the higher the radical scavenging activity the lower the amount of conjugated trienes present in the oil. A strong negative correlation of –0.980 and –0.946 was shown between radical scavenging ability and peroxide value for marula and moringa seed oil which means that the higher the radical scavenging activity the lower the amount of hydroperoxides (primary oxidation) produced. From the correlation test, we may conclude that the higher the radical scavenging ability the lower the oxidation process (Prescha et al., 2014).

The free radical scavenging results obtained for moringa was higher while that of marula seed oil was lower than that obtained for cranberry seed oil (55.02 to 55.62 %) obtained from screw pressing (Mandal and Lee, 2013). Yu, X., et al. (2013) reported a radical scavenging activity of solvent extracted (Soxhlet method with hexane) moringa seed oil to be approximately 56.00 % which is higher that reported in this study which could be as a result of different harvesting periods, storage conditions, geographical area and climatic conditions of where the seeds were obtained.

Overall, moringa seed oil had a higher radical scavenging ability compared to marula seed oil, which means that moringa has a higher natural antioxidant content. This can be related back to the acid value in section 4.4 where moringa had a higher moisture content but showed a lower acid value. This is probably due to the high natural antioxidant content of moringa oil, which scavenged the free fatty acids resulting in a low acid value.

The results from the radical scavenging ability showed aqueous extracted seed oils at 37 °C to have the highest ability to fight free radicals, which also corresponded to the lowest values for the oxidative stability tests (PV, *p*-AV, CD and CT). This shows that the aqueous extracted seed oils were thus most stable.

5.0 CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

This study was carried out in order to evaluate the impact of extraction methods on the oxidative stability of marula and moringa seed oils rich in oleic acid. The shelf life study is important because it provides information on the oil stability during storage.

The results of this study showed that the aqueous extraction method had a lower oil yield compared to the screw press and the solvent extraction method for marula and moringa seed oil. The results from the GC-MS analysis showed the presence of the following major fatty acids; palmitic, linoleic, oleic and stearic acid. Oleic acid (monounsaturated fatty acid) was the dominant fatty acid in both seed oils and its presence in oil is important in seed oil stability, because it is a poor substrate for oxidation in comparison to polyunsaturated fatty acid. The results from the acid value showed that solvent extracted marula and moringa seed oil had the highest acid value. The aqueous extraction method had the lowest acid value content, which suggested it is an extraction method that produces a better quality of oil.

Other results for oxidative stability tests, such as, peroxide value, *p*-anisidine value and conjugated diene and triene values also showed lower values for the aqueous extraction method indicating that the aqueous extraction method produced a better stability and quality of oil compared to the screw press and solvent extraction methods. Further investigation was done on the oxidative stability of the seed oils by carrying out the peroxide value and the *p*-anisidine test which are the major tests for detecting the stability of seed oils for a period of 35 days and at accelerated shelf life storage temperatures. The results showed that, for the different extraction methods, there was an increase in the peroxide value and the *p*-anisidine value over time but the aqueous extracted seed oil had the lowest increase in peroxide value and *p*-anisidine value throughout the period of 35 days compared to the screw press, shaker and Soxhlet extraction methods. The rate of change of the peroxide value and anisidine value showed that aqueous extracted marula and moringa seed oil at 37 °C had the lowest rate of increase of primary and secondary oxidation products as compared to the other extraction methods. This implies that oils extracted using the aqueous method at 37 °C have better stability and shelf life compared to oils produced from the other extraction methods.

Further analysis was also carried out to determine if the different extraction methods affected the antioxidant activity of the seed oil. This was done by carrying out a radical scavenging activity test using the DPPH stable radical. The results showed that there was a significant

difference between the scavenging ability of the solvent extracted, aqueous extracted and screw pressed extracted marula and moringa seed oils. Aqueous extracted marula and moringa seed oil at 37 °C had the highest scavenging ability and Soxhlet extracted oil has the lowest ability. The radical scavenging ability test also had a good correlation with the oxidative stability tests (PV, CD, CT and *p*-AV).

Finally, the results reported in this study proved the hypothesis that the aqueous extraction method, resulting in a lower oil yield, produced an oil of better quality and stability, which had a good radical scavenging ability as well.

Recommendations and future work

Further studies should consider the following:

1) The oxidative stability based on accelerated shelf life studies should be investigated in order to determine the factors that lead to increased and decreased primary and secondary oxidation at different temperatures.
2) A comparison of the results obtained for marula and moringa seed oils with that of other seed oils such as jacket plum, mongongo nut and Natal mahogany using the same extraction methods used in this study.
3) A comparison of solvent extraction, aqueous extraction at higher temperatures and screw press extraction with supercritical CO_2 extraction and aqueous enzymatic extraction at higher temperatures for marula and moringa seed oils.
4) The effect of frying/cooking temperatures on the oxidative stability of the studied seed oils.
5) The effect of a natural antioxidant such as *Carum copticum* (common name Ajwain) extract and a synthetic antioxidant such as butylated hydroxytoluene on the oxidative stability of marula and moringa seed oil at high temperatures.

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APPENDICES

Appendix A: Representative calculation of percentage oil yield and extraction efficiency in aqueous extracted marula and moringa seed oil at 37 ℃

Example: The percentage oil yield and oil yield was calculated using the formula:

$$Oil\ yield\ = \frac{mo}{ms} \times 100\ \%$$

Where mo is the weight of oil (g) recovered after extraction and ms is the weight of seed samples (g). If the mass of the aqueous extracted marula oil at 37 ℃ was 28.00 g and the mass of the seed sample was 150.01g, and mass of aqueous extracted moringa seed oil at 37 ℃ was 18.0069 g and mass of seed sample was 150.11g, then:

$\frac{28.00}{150.01} \times 100\ = 18.67\ \%$ for aqueous extracted marula seed oil at 37 ℃

$\frac{18.01}{150.11} \times 100 = 12.00\ \%$ for aqueous extracted moringa seed oil at 37 ℃

Calculation for percentage oil yield for Soxhlet extracted marula and moringa seed oil. If the mass of Soxhlet extracted marula seed oil is 27.00 g and the mass of the seed sample was 50.01 g, and the mass of aqueous extracted moringa seed oil was 17.66 g and mass of seed sample was 50.01 g

Marula: $\frac{27.00}{50.01} \times 100 = 53.99\ \%$

Moringa: $\frac{17.66}{50.01} \times 100 = 35.31\ \%$

The extraction efficiency was calculated using the formula:

$$Extraction\ efficiency = \frac{my}{mm} \times 100\ \%$$

Where my is the percentage oil yield for the other extraction methods and mm is the percentage oil yield for Soxhlet extraction which is assumed to have a 100 % extraction efficiency because it had the highest yield.

$\frac{18.67}{53.99} \times 100\ \% = 34.58\ \%$ for aqueous extracted marula seed oil at ℃

$\frac{12.00}{35.31} \times 100\ \% = 33.98\ \%$ for aqueous extracted moringa seed oil at ℃

Appendix B: Representative calculation for relative fatty acids present in moringa seed oils

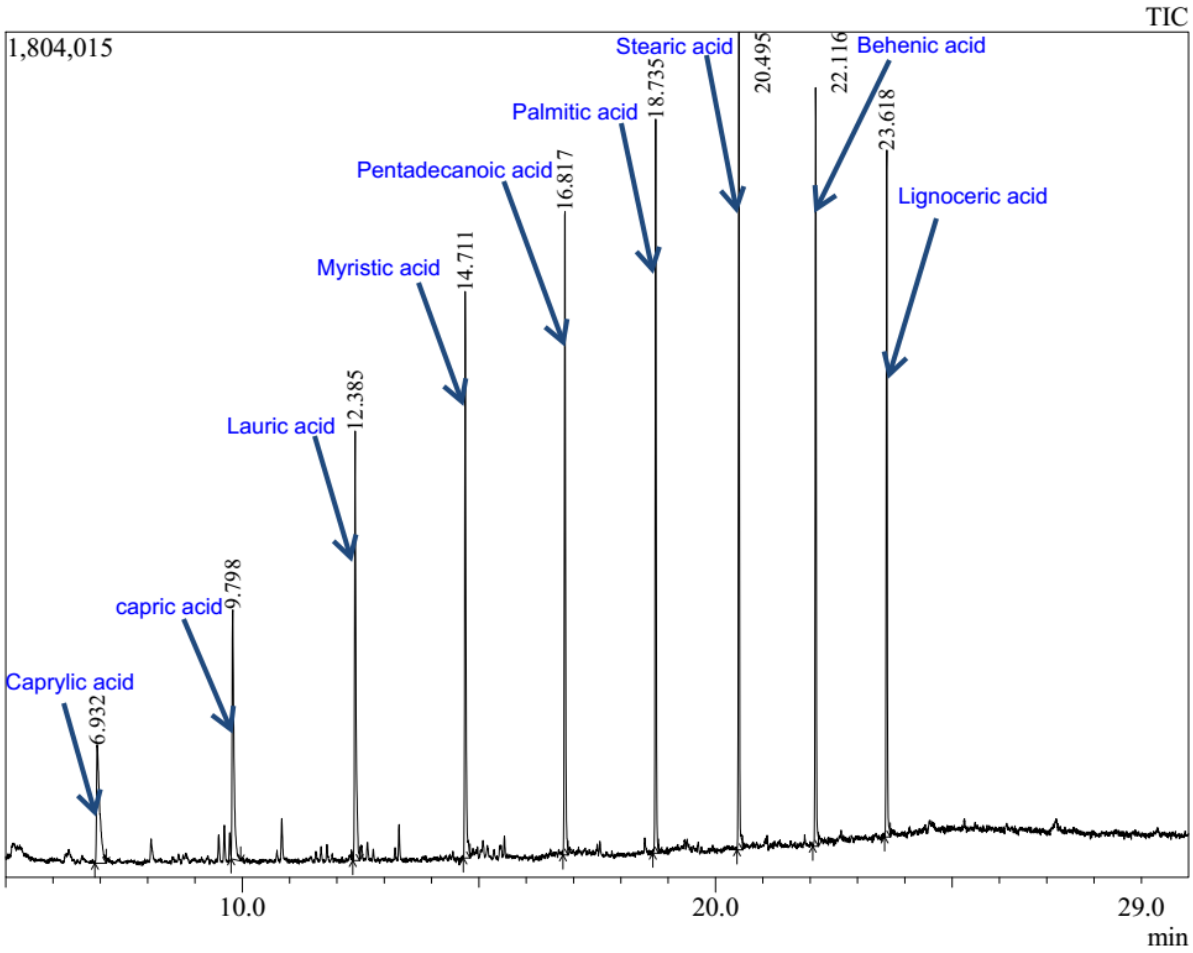
The percentage analyte was calculated using the formula:

$$\%\ Analyte = \frac{A_a}{\sum A_1} \times 100$$

A_a is the peak area of the analyte while $\sum A_1$ is the sum of all peak areas in the chromatogram. For oleic acid the peak area is 5187735 for marula and the total peak area is 7169515, and the peak area is 3376659 for moringa and the total peak area is 4224233, then:

$\frac{5187735}{7169515} \times 100 = 72.36\ \%$ oleic acid for marula seed oil

$\frac{3376659}{4224233} \times 100 = 79.94\ \%$ oleic acid for moringa seed oil



Appendix B.1 - GC-MS chromatogram of 9 fatty acids out of the 36 standard mix that came off the column

Appendix C: Representative example of the oxidative stability test calculation (peroxide value) for aqueous extracted marula and moringa seed oil at 37 °C

The peroxide value was calculated using the formula:

$$Peroxide\ value = \frac{(S-B)\times M\times 1000}{Sample\ weight\ (g)}$$

Where S is the sample titer value, B is the blank titer value and M is the molarity of $Na_2S_2O_3$. If the sample titer value is 0.03 L, the blank 0.00 L, the molarity of $Na_2S_2O_3$ is 0.0100 M and sample weight 2.00 g, then:

$\frac{(0.03-0.00)\times 0.0100\times 1000}{2.00} = 0.15 meq/kg$ of marula oil

$\frac{(0.02-0.00)\times 0.0100\times 1000}{2.00} = 0.10\ meq/kg$ of moringa oil

Appendix D: Representative example of the oxidative stability test calculation (Conjugated diene and triene value) for aqueous extracted marula and moringa seed oil at 37 ℃

$$Conjugated\ diene\ \% = 0.84\left[\left(\frac{A_1}{bc} - 0.07\right)\right]$$

$$Conjugated\ triene\ \% = 0.84\left[\left(\frac{A_2}{bc} - 0.07\right)\right]$$

where A_1 = absorbance of the sample at 232 nm for conjugated diene and A_2 = absorbance at 270 nm for conjugated triene, b = cell length (1 cm), c = concentration of sample used for absorbance measurement in g/L, 0.07 = absorptivity constant (ε) of the ester group 0.84 = not defined.

If the sample concentration 0.98 g, the blank 0.00 L, the absorbance of sample is 1.45 and then:

$$Conjugated\ diene\% = \left(\frac{1.45}{0.98} - 0.07\right) \times 0.84 = 1.18$$

$$Conjugated\ triene\% = \left(\frac{0.99}{0.98} - 0.07\right) \times 0.84 = 0.79$$

Appendix E: Radical scavenging ability of marula and moringa seed oil with different extraction methods

The radical scavenging activity was calculated using the formula:

$$\%\ Radical\ scavenging\ activity = \frac{A\ control - A\ sample}{A\ control} \times 100$$

A control is the absorbance of the control while A sample is the absorbance of the sample. If the absorbance of marula oil was 0.45 and that of the control was 1.10, then:

$\frac{1.10-0.45}{1.10} \times 100 = 59.39\ \%$ for aqueous extracted marula seed oil at 37 °C

$\frac{1.10-0.41}{1.10} \times 100\ \% = 62.73\ \%$ for aqueous extracted moringa seed oil at 37 °C